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Molecular Mechanisms Involved in CD43-mediated Apoptosis of TF-1 Cells

ROLES OF TRANSCRIPTION, Daxx EXPRESSION, AND ADHESION MOLECULES*

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CD43 (leukosialin, sialophorin), an abundant leukocyte surface sialoglycoprotein, regulates leukocyte adhesion and transmits activating signals in T cells and dendritic cells. Immobilized anti-CD43 monoclonal antibody (mAb) MEM-59 has been previously shown to induce apoptosis of hematopoietic progenitors. In this study we show that it also triggers apoptosis of the myeloid progenitor-derived cell line TF-1. The kinetics of the MEM-59-induced apoptosis were unusually slow, with the first apoptotic cells appearing 36–48 h after their contact with the immobilized antibody; in 5 days, 90% of the cells were dead. CD43-mediated apoptosis was enhanced by coimmobilized anti-CD45 mAb and partly suppressed by coimmobilized anti-CD50 (ICAM-3) or anti-CD99 mAb. The MEM-59-triggered apoptosis of TF-1 cells was also inhibited by the overexpression of an apoptotic regulator, Daxx. CD43-mediated apoptosis was preceded by the repression of the DNA binding activity of the transcription factor AP-1. DNA array screening revealed that the expression of several genes encoding apoptosis-regulating proteins, including 14-3-3 proteins and the granulocyte macrophage colony-stimulating factor (GM-CSF) receptor β-subunit, was repressed in TF-1 cells bound to immobilized MEM-59. The down-regulation of 14-3-3 proteins and GM-CSF receptor β was accompanied by translocation of the proapoptotic protein Bad to the mitochondria. These results suggest that engagement of CD43 may, presumably through the repressing transcription, initiate a Bad-dependent apoptotic pathway.

CD43, a highly glycosylated and sialylated transmembrane type I protein, is expressed abundantly on the surface of hematopoietic cells, including hematopoietic stem cells and progenitors (1–3). Its extracellular part is modified by an *N*-glycan chain and by 70–85 *O*-linked oligosaccharides and extends from the cell surface much farther than other cell surface molecules (4, 5). This extension, the highly negative charge of the CD43 extracellular domain, and variability in its post-translational modifications suggest that CD43 is responsible for the regulation of the first contacts between cells, either adhesive or repulsive (6). Variability in the glycosylation of the extracellular domain of CD43 was observed during T cell activation. The mobility of CD43 glycosylation isoforms on SDS-polyacrylamide gels ranged from 95 to 130 kDa, and the isoforms were expressed differently on resting *versus* activated T cells (7–9). Human imunodeficiency virus infection of the T cell line CEM led to hypoglycosylation of CD43 and to impaired CD43-mediated homotypic aggregation (10). The intracellular part of CD43 is conserved evolutionarily and contains several protein kinase C phosphorylation sites and a proline-rich sequence resembling SH3 binding consensus (5, 11, 12).

A number of presumed CD43 ligands have been reported including CD54 (ICAM-1),[1] galectin-1, major histocompatibility complex class I glycoproteins, and sialoadhesin (13–16). Cross-linking of CD43 by means of specific antibodies triggers intracellular signals leading to the activation of T cells (17, 18). Molecular events that accompany CD43-induced T cell activation include increased binding of protein-tyrosine kinase Fyn to the intracellular part of CD43, followed by tyrosine phosphorylation of the adaptor protein Shc and the proto-oncogene Vav. CD43-induced signaling then leads to elevated DNA binding of AP-1, NFκB, and NF-AT and to increased expression of IL-2, CD69, and CD40 ligand (12, 19, 20). Furthermore, phosphorylation of the adaptor molecule Cbl has been observed, which may be involved in the negative modulation of T cell activation (21). The cytoplasmic domain of CD43 also mediates connection to the cytoskeleton via its interaction with the ezrin-radixin-moesin proteins (22, 23). Anti-CD43 mAbs also induced the activation and proliferation of NK cells (24, 25) and the maturation of dendritic cells (26).

Activation of the protein-tyrosine kinases Syk and Lyn and phospholipase Cγ by antibody-mediated cross-linking of CD43 in hematopoietic progenitor cells resulted in increased homotypic adhesion, and it also enhanced the integrin-dependent adhesion of human cord blood cells to fibronectin (27, 28). Similar treatment also enhanced the affinity of β_1 and β_2 integrins in T cells and increased homotypic aggregation of the human mast cell line HMC-1 through the activation of protein kinase C and tyrosine kinases (29–31). In contrast, the inter-

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[1] The abbreviations used are: ICAM-1, intercellular adhesion molecule 1; FITC, fluorescein isothiocyanate; GAM, goat anti-mouse IgG antibodies; GM-CSF, granulocyte macrophage colony-stimulating factor; GM-CSF Rβ, β-subunit of GM-CSF receptor; IL, interleukin; mAb(s), monoclonal antibody(ies); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF, nuclear factor; PBS, phosphate-buffered saline.

action of proliferating hematopoietic progenitors with plastic-immobilized anti-CD43 mAb MEM-59 led to their apoptosis (32–34). Interestingly, stem cells and nonproliferating progenitors were resistant to MEM-59-induced apoptosis. Another anti-CD43 mAb also triggered apoptosis in the Jurkat T cell line (35). It seems likely that the apoptosis induced in hematopoietic progenitors by the cross-linking of their surface CD43 with a putative ligand in bone marrow may be important in regulating their growth and/or differentiation. Very little is known about the molecular basis of the apoptotic signaling triggered by the cross-linking of CD43. Therefore, in the present study we investigated processes that led to or affected apoptosis induced by immobilized anti-CD43 mAb MEM-59 in a model myeloid progenitor-derived cell line TF-1.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Reagents—TF-1 (ATCC, Manassas, VA) cells were grown in RPMI 1640 medium containing 10% fetal calf serum and 10 ng/ml recombinant GM-CSF (Leukomax, Shering-Plough, Basel, Switzerland) (36). Daxx-overexpressing TF-1 cells (TF-1/Daxx) were grown in the same culture medium containing in addition 0.4 mg/ml G418 (Sigma).

The monoclonal antibodies MEM-59 (CD43), MEM-28 (CD45), MEM-131 (CD99), MEM-171 (CD50), and AFP-01 (human α-fetoprotein), previously prepared and characterized in the Prague laboratory, were purified on a protein A-Sepharose column (Amersham Biosciences, Inc.). Anti-Myc tag mAb 9E10 was purchased from Roche Molecular Biochemicals. Antibodies against 14-3-3 proteins, Bad, and GM-CSF receptor β subunit (GM-CSF Rβ) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). mAb TU-01 (α-tubulin) was kindly provided by Dr. P. Dráber (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague), mAb αF1 (mitochondrial F_1F_0-ATPase) by Dr. J. Houštek (Institute of Physiology, Academy of Sciences of the Czech Republic), and mAb 1C1 recognizing GM-CSF Rβ by Dr. A. F. Lopez (Hanson Center for Cancer Research, Adelaide, Australia). The annexin V-FITC/propidium iodide apoptosis determination kit was purchased from Alexis Biochemicals (San Diego, CA), and GAM was from Sigma.

Plasmids and Oligonucleotides—The intracellular parts of human cDNAs encoding CD43 (amino acids 275–400) and human Fas (CD95) (amino acids 191–335) were amplified from a leukocyte cDNA library (CLONTECH, Palo Alto, CA) by PCR and subcloned into the yeast two-hybrid bait vector pLexA (CLONTECH). Daxx cDNA (provided by Dr. A. Pluta, Johns Hopkins University, Baltimore) was subcloned in-frame into a modified pCDNA3 plasmid containing the Myc epitope upstream of the cloning site. This construct and the parental plasmid were transfected into TF-1 cells, and Myc-Daxx expressing cells (TF-1/Daxx) or mock-transfected cells were selected by limiting dilution in a culture medium containing in addition 1 mg/ml G418. The oligonucleotides (5′ → 3′) used for electrophoretic mobility shift assays were CGCTTGATGACTCAGCCGGAA (AP-1) and AGTTGAGGGGACTTTCCCAGCC (NFκB). Mutations that compromised specific DNA binding were TGACTCA to TGACTTG (AP-1) and GGGGACTTT to GGCGACTTT (NFκB). The expression of GM-CSF Rβ and β-actin mRNAs was determined by reverse transcription-PCR using oligonucleotides GACAGGCCGTGGAAGTGGAGAG, GGCCGGGGAGGAAGCAATAG, and CCTGGGGGCTTCTTGACTTG (reverse transcription primer) (GM-CSF Rβ), and GACGAGGCCCAGAGCAAGAG and GGGCCGGACTCATCGTACTC (β-actin). All PCR-made constructs were sequenced.

Treatment of Hematopoietic Cells with Monoclonal Antibodies, Cell Staining, and Flow Cytometry—For cell treatment with immobilized mAbs, tissue culture plates/dishes were incubated with purified mAbs (100 μg/ml in PBS) at 37 °C for 1 h and then washed three times with PBS. Cells at the indicated cell concentrations were then added and incubated with the immobilized mAbs for the various time periods indicated. Cells in suspension were treated with a mixture of soluble mAb and GAM (both at 10 μg/ml). Cells attached to the MEM-59-coated plastic were released by treating with 0.25 mg/ml *O*-sialoglycoprotein endopeptidase (Cedarlane Laboratories, Hornby, Canada) at 37 °C for 45 min. The enzyme treatment did not affect the viability of the treated cells (32, 33). The released cells were then harvested by gentle pipetting and analyzed by flow cytometry (FACSort, Becton Dickinson, Franklin Lakes, NJ).

For immunostaining, cells were incubated in the staining buffer (PBS containing 0.2% gelatin and 0.1% sodium azide) with 50 μg/ml cell surface marker-specific mAb on ice for 1 h. After washing, cells were incubated in the staining buffer containing FITC-conjugated GAM (Jackson Immunoresearch Laboratories, West Grove, PA). Cells were then washed, resuspended in the staining buffer, and analyzed by flow cytometry. The apoptosis assay using annexin V-FITC/propidium iodide staining was carried out according to the manufacturer's recommendations. The stained cells were analyzed by flow cytometry.

Yeast Two-hybrid Screening for Proteins Interacting with the Intracellular Part of CD43—pLexA-CD43(ICP) was used for screening a Jurkat cDNA library cloned in the prey vector pB42AD (CLONTECH) for interacting proteins. The control plasmid, pLexA-lamin, was also supplied by CLONTECH. Postscreening analysis, including β-galactosidase assays, was done according to the manufacturer's protocols (CLONTECH).

MTT Cell Proliferation Assay—MTT solution (5 mg/ml in PBS, Sigma) was added to cells cultured in 96-well plates to a final concentration of 0.5 mg/ml. After 3 h of incubation at 37 °C, an equal volume of acid isopropyl alcohol (0.04 M HCl in isopropyl alcohol) was added, and the plates were incubated at room temperature for 10 min. The liquid in the wells was resuspended by pipetting, and the absorbance at 570 nm was determined using a Victor microplate reader (PerkinElmer Life Sciences).

Preparation of Nuclear Extracts, Electrophoretic Mobility Shift Assays, and Protein Gel Electrophoresis—Nuclear extracts from TF-1 cells, both untreated and treated with mAbs, were prepared by hypotonic lysis and nuclei extraction. Cells in suspension were harvested by centrifugation, washed with PBS and hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (Sigma), 0.5 mM dithiothreitol, pH 7.9 at 4 °C), resuspended in 3 packed cell volumes of hypotonic buffer and, after a 15-min incubation on ice, lysed by repeatedly passing the cell suspension through a 30-gauge needle. The nuclei were centrifuged and washed with hypotonic buffer, and nuclear proteins were extracted with hypotonic buffer containing in addition 0.35 M NaCl. The nuclear extract was centrifuged (13,000 × *g* at 2 °C for 30 min), and the supernatant was stored in aliquots at −80 °C. The protein concentration in the nuclear extracts was determined by Bio-Rad protein assay reagent. Cells attached to the immobilized MEM-59 were washed on the culture dishes and scraped in hypotonic buffer; nuclear proteins were purified as above.

Equal amounts of the nuclear extracts (3 μg, usually 1–2 μl) were incubated in binding buffer (10 mM HEPES, 100 mM NaCl, 100 μg/ml bovine serum albumin, 4% glycerol, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.5 mM dithiothreitol, pH 7.9, at 20 °C) with the ^{32}P end-labeled oligonucleotide probe for 20 min at room temperature. The protein-DNA complexes were resolved by electrophoresis in native 6% polyacrylamide gel, visualized by autoradiography, and quantified by a PhosphorImager BAS-5000 (FUJIFILM Medical Systems, Stamford, CT).

For the analysis of protein expression, cells were lysed in SDS sample buffer, separated by SDS-PAGE, transferred to nitrocellulose or polyvinylidene difluoride membranes, and immunodetected by specific antibodies. Heavy membrane and cytoplasmic fractions of TF-1 cells were prepared as described by Rice and Lindsay (37). The cytoplasmic fraction was clarified by ultracentrifugation (100,000 × *g* at 4 °C for 2 h) and contained no membranes (*i.e.* it was F_1F_0-ATPase-negative). Gel loadings were normalized either to α-tubulin (whole cell lysates and cytoplasmic fractions) or to F_1F_0-ATPase (heavy membrane fractions) signals.

Isolation of Total RNA from TF-1 Cells, Reverse Transcription-PCR, and DNA Arrays—Total RNA from TF-1 cells was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) with additional acid phenol and two chloroform extractions. Its purity was confirmed by spectrophotometry ($A_{260}/A_{280} > 1.8$) and by agarose gel electrophoresis. The purified RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and specific primers. Synthesized cDNA was used either for semiquantitative PCR with gene-specific primer pairs or for the hybridization of nylon membranes with the DNA arrays of apoptosis-related genes (R&D Systems, Minneapolis, MN). In the latter case, [α-^{32}P]dCTP was included into the reverse transcription reaction. The PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. ^{32}P-Labeled cDNAs were hybridized according to the manufacturer's protocols (R&D Systems) to DNA array membranes containing 198 cloned, apoptosis-related cDNAs, including cytokines, their receptors, caspases, signal transduction, and other factors. Radioactive signals on the membranes were detected by autoradiography and quantified by a PhosphorImager BAS-5000. Relative gene expression was calculated as the ratio of averaged signals of the

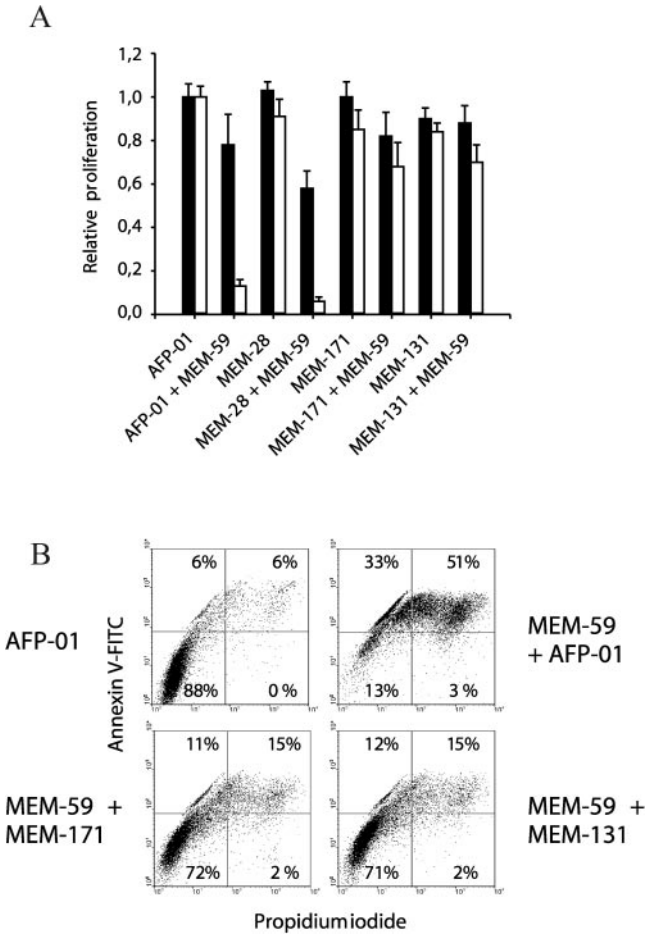

FIG. 1. **Immobilized mAb MEM-59 inhibits the proliferation of TF-1 cells through induction of apoptosis; pro- and antiapoptotic effects of CD45, CD99, and CD50 mAbs.** *Panel A*, cells were cultured in triplicate in a 96-well tissue culture plate (5×10^3 cells/well) coated with the indicated plastic-immobilized mAbs for 3 days (*black bars*) or 5 days (*white bars*). Cultures treated with plastic-immobilized AFP-01 (control), MEM-59 (anti-CD43), MEM-28 (anti-CD45), MEM-131 (anti-CD99), MEM-171 (anti-CD50) mAbs or their indicated combinations were analyzed by MTT assays, and the percent inhibition of cell proliferation was calculated. The means ± S.D. of three independent experiments are shown. *Panel B*, cells were seeded in a 24-well plate (5×10^4 cells/well) with the indicated immobilized mAbs and cultured for 4 days. Cells were released by treatment with *O*-sialoglycoprotein endopeptidase, stained with annexin V-FITC/propidium iodide, and analyzed by flow cytometry.

duplicate gene spots on the membrane hybridized with ^{32}P-labeled cDNA isolated from TF-1 cells treated with the immobilized MEM-59 to the signals of corresponding spots on the membrane hybridized with the ^{32}P-labeled cDNA from cells treated either with the control mAb or with a mixture of soluble MEM-59 and GAM (10 μg/ml of both). The results were normalized by the corresponding ratios of the signals of the housekeeping genes *L19* and *HLA-A*.

RESULTS

Growth of TF-1 Cells Is Suppressed by Immobilized Anti-CD43 mAb MEM-59 Because of Apoptosis Induction; Antiapoptotic Effect of CD50 or CD99 Antibody—Immobilized, but not soluble, anti-CD43 mAb MEM-59 specifically induced apoptosis of human hematopoietic progenitors (32, 33). However, these cells are available only in limited amounts, and therefore we looked for a suitable model cell line. The hematopoietic progenitor-derived cell line TF-1 was such a sensitive model. In the presence of plastic-immobilized CD43 mAb MEM-43, the relative proliferation of these cells in a 5-day assay was only 14 ± 2% of the control (Fig. 1*A*), and as expected, soluble MEM-59 did not affect their growth.[2] Suppression of TF-1 cell proliferation by the immobilized MEM-59 was clearly caused by the induction of apoptosis, as shown by annexin V-FITC/propidium iodide staining (Fig. 1*B*). TF-1 cells strongly expressed the early hematopoietic markers CD34 and CD38 as well as other surface markers such as CD5, CD7, CD29, CD45, CD50, CD99, and CD147.[2] Coimmobilization of mAbs to most of these molecules (in addition to the immobilized MEM-59) had no further effect, but coimmobilized mAb to CD45 (MEM-28) further enhanced the growth inhibitory effect, although coimmobilized mAb MEM-131 (CD99) or MEM-171 (CD50) partially counteracted it (Fig. 1*A*), obviously because of reduced apoptosis (Fig. 1*B*).

TABLE I
Intracellular parts (ICP) of CD43 and Fas (CD95) interact with the C-terminal part of Daxx in the yeast two-hybrid system

Liquid culture β-galactosidase assays were performed, and the β-galactosidase activities of yeast cell extracts were calculated as described in the *Yeast Protocol Handbook* (Clontech). The numbers in parentheses stand for the positions of amino acids in the Daxx protein sequence. Full-length Daxx contains 740 amino acids. pLexA-lamin was used as a negative control.

Bait	Prey	β-Galactosidase activity
		units
pLexA-lamin	pB42AD-Daxx(558–740)	3 ± 0.5
pLexA-CD43(ICP)	pB42AD-Daxx(558–740)	59 ± 4.6
pLexA-Fas(ICP)	pB42AD-Daxx(558–740)	54 ± 5.2
pLexA-lamin	pB42AD-Daxx(1–740)	2.5 ± 0.8
pLexA-CD43(ICP)	pB42AD-Daxx(1–740)	15 ± 2.3
pLexA-Fas(ICP)	pB42AD-Daxx(1–740)	13 ± 2.7

Expression of Daxx Together with Engagement of CD50 or CD99 Inhibits MEM-59-induced Apoptosis of TF-1 Cells—Proteins interacting with the intracellular part of CD43 should mediate or regulate proximal steps in the apoptotic signaling induced by mAb-cross-linked CD43. Thus, we employed yeast two-hybrid screening of a Jurkat cDNA library and searched for proteins interacting with the intracellular part of CD43. The screening revealed that the C-terminal part of the protein Daxx specifically interacted with the intracellular part of CD43 (Table I.). Under the same conditions, Daxx also interacted with the intracellular part of Fas (CD95) used as a positive control (see "Discussion"). To examine whether Daxx could affect the MEM-59-induced apoptosis of TF-1 cells, human Daxx with the N-terminal Myc tag was overexpressed in TF-1 cells (Fig. 2*A*). TF-1/Daxx cells were markedly more resistant to MEM-59-induced growth suppression/induction of apoptosis than the parental or mock-transfected cells (Fig. 2, *B* and *C*). Overexpression of Daxx also significantly reduced the growth inhibitory effect of coimmobilized CD43 (MEM-59) and CD45 (MEM-28) mAbs, and in combination with coimmobilized CD50 (MEM-171) or CD99 (MEM-131) mAbs almost eliminated MEM-59-induced growth suppression and apoptosis (Fig. 2, *B* and *C*).

Immobilized and Soluble mAb MEM-59 Have Different Effects on the DNA Binding Activities of Transcription Factors AP-1 and NFκB in TF-1 Cells—The relatively slow kinetics of the immobilized MEM-59-induced apoptosis of TF-1 cells suggested a possible requirement of *de novo* transcription/proteosynthesis. However, inhibition of proteosynthesis even by very low doses of cycloheximide led to relatively rapid (within 24–36 h) apoptosis of TF-1 cells.[3] In agreement with previous data on Jurkat T cells (20), the cross-linking of CD43 with soluble MEM-59 significantly enhanced the DNA binding activities of the transcription factors AP-1 and NFκB in TF-1 cells as well

[2] L. Andera, unpublished data.
[3] Š. Šímová, unpublished data.

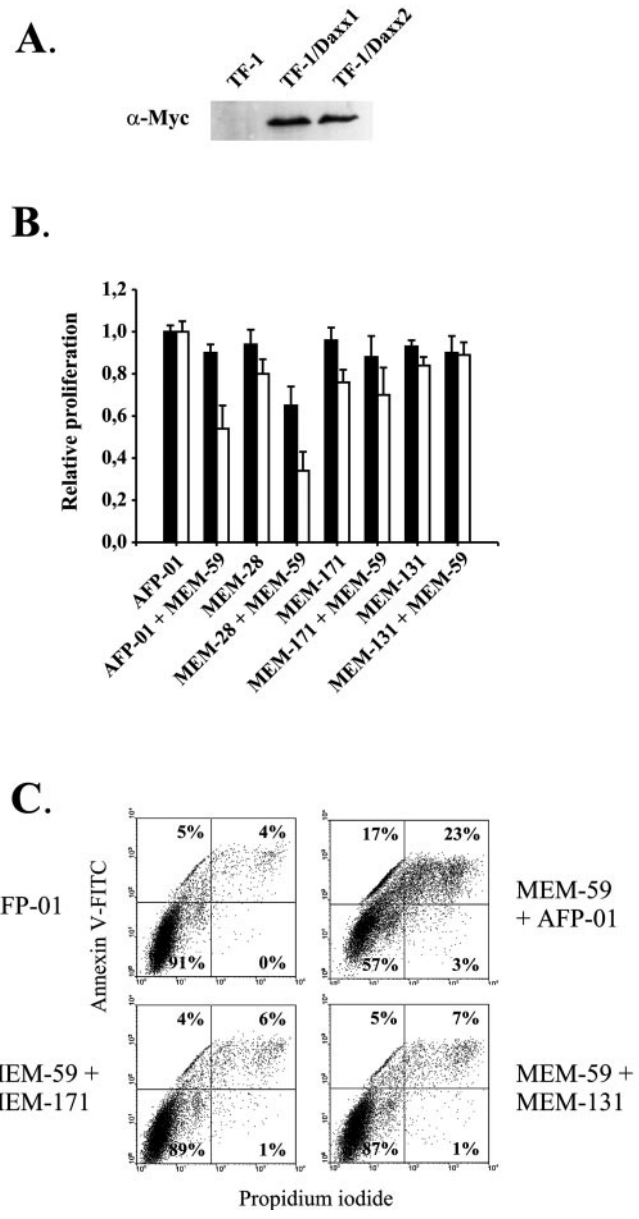

FIG. 2. **Overexpression of Daxx, in cooperation with anti-CD99 and anti-CD50 mAbs, inhibits MEM-59-induced apoptosis of TF-1 cells.** *Panel A*, Myc-tagged human Daxx expression in mock-transfected cells (TF-1) and in two TF-1 clones transfected with pCDNA3-MycDaxx (TF-1/Daxx1 and TF-1/Daxx2) analyzed by anti-Myc mAb 9E10 on a Western blot of whole cell lysates. *Panel B*, TF-1/Daxx cells (5×10^3/well, mixture of equal fractions of TF-1/Daxx1 and TF-1/Daxx2 cells) were cultured in triplicates in a 96-well tissue culture plate coated with the indicated plastic-immobilized mAbs for 3 days (*black bars*) or 5 days (*white bars*). Their proliferation was analyzed by the MTT assay as described in the Fig. 1*A* legend. *Panel C*, TF-1/Daxx cells were seeded in a 24-well plate (5×10^4 cells/well) with the indicated immobilized mAbs, cultured for 4 days, and analyzed by flow cytometry as described in the Fig. 1*B* legend.

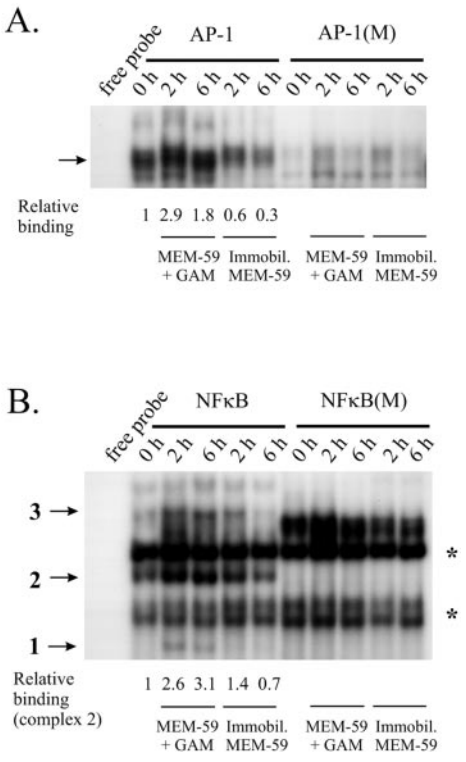

FIG. 3. **Changes in the DNA binding activities of transcription factors AP-1 and NFκB induced by cross-linking of CD43.** TF-1 cells (10^6 cells/ml) were incubated either with a mixture of MEM-59 and GAM (10 μg/ml of both) or with immobilized MEM-59 for 2 or 6 h. Nuclear extracts (see "Experimental Procedures") from these cells were incubated with double-stranded oligonucleotide DNA probes containing either wild type or mutated (*M*) AP-1 (*panel A*) or NFκB (*panel B*) DNA binding sites. Protein-DNA-protein complexes were separated electrophoretically in polyacrylamide gels, visualized by autoradiography, and scanned by a PhosphorImager. *Arrows* on the *left* point to specific complexes. *Asterisks* on the *right* indicate nonspecific complexes.

TABLE II
Changes in the expression of some genes in TF-1 cells treated with either immobilized or soluble MEM-59

Gene	Relative change in gene expression in TF-1 cells treated with	
	Immobilized MEM-59 *versus* control (AFP-01) mAb	Immobilized MEM-59 *versus* soluble MEM-59
IL-4 Rα	2.43	0.92
14–3-3 proteins	0.36	0.46
IL-2 Rγ	0.57	1.04
GM-CSF Rβ	0.45	0.41
ref-1	0.53	0.43

Relative changes in gene expression were determined from a PhosphorImager analysis of apoptotic cDNA array membranes (see "Experimental Procedures").

(Fig. 3). In contrast, the cross-linking of CD43 with immobilized MEM-59 resulted in a substantial inhibition of the DNA binding activity of AP-1 (3-fold after 6 h of treatment), whereas the DNA binding activity of NFκB remained almost unchanged with only a slight increase after 2 h of treatment (Fig. 3).

Immobilized MEM-59 Changes the Expression Patterns of Several Apoptosis-related Genes in TF-1 Cells and Induces Translocation of Bad to Mitochondria—The next set of experiments was aimed at the characterization of the MEM-59-induced changes in the expression of apoptosis-related genes. Hybridization of ^{32}P-labeled cDNA from control and TF-1 cells treated with immobilized MEM-59 mAb to a nylon membrane with a cDNA array of apoptosis-related genes revealed that the immobilized MEM-59 suppressed the expression of genes encoding IL-2 Rα, *ref-1*, GM-CSF Rβ, and 14-3-3s, whereas the expression of IL-4 Rα was up-regulated (Table II, center column). The down-regulation of *ref-1*, GM-CSF Rβ, and 14-3-3 gene expression was specifically elicited by immobilized mAb, whereas changes in the expression of the IL-2 Rγ and IL-4 Rα genes were also induced by soluble mAb (Table II, right column).

Immunostaining of Western blots with pan-specific anti-14-3-3 mAb confirmed that a 24-h treatment of TF-1 cells with immobilized MEM-59 already caused a significant down-regu-

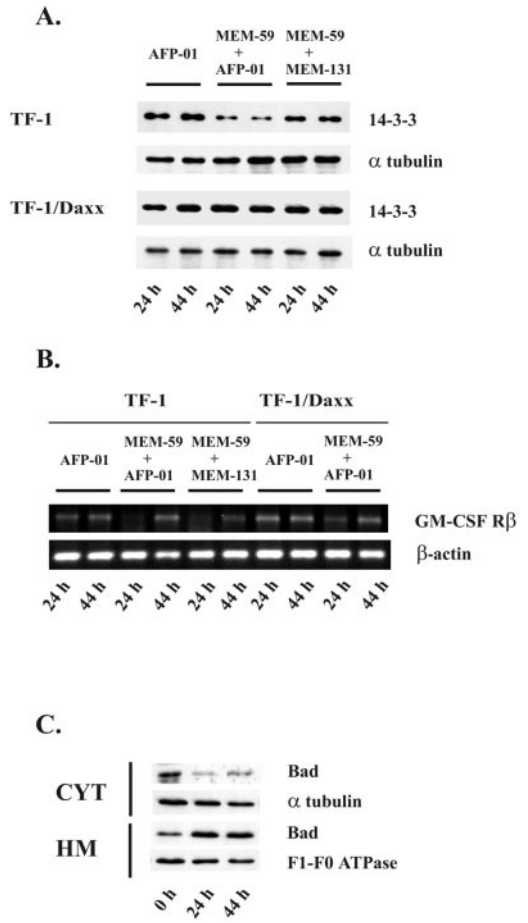

FIG. 4. **Immobilized MEM-59 induces changes in the expression of 14-3-3 proteins, and GM-CSF Rβ, and in the cellular localization of Bad.** *Panel A*, immunodetection of 14-3-3 proteins in TF-1 and TF-1/Daxx cells. Western blots of TF-1 cell extracts that were harvested 24 or 44 h after their binding to immobilized MEM-59 or MEM-59 + MEM-131 (CD99) mAbs were immunostained with antibodies to 14-3-3 proteins or α-tubulin (loading control). *Panel B*, analysis of MEM-59-induced changes in GM-CSF Rβ mRNA expression in TF-1 and TF-1/Daxx cells by semiquantitative reverse transcription-PCR. Equal amounts (5 μg) of total RNA from TF-1 or TF-1/Daxx cells were reverse transcribed by Superscript II using either a gene-specific primer (GM-CSF Rβ) or oligo(dT) primer (β-actin) and amplified by pairs of specific primers (see "Experimental Procedures"). PCR products were analyzed electrophoretically in agarose gels stained with ethidium bromide. *Panel C*, MEM-59-induced translocation of Bad from the cytoplasm to the mitochondria was analyzed by Western blotting of SDS lysates of cytoplasmic (*CYT*) and mitochondria-containing, heavy membrane (*HM*) fractions of TF-1 cells treated with immobilized MEM-59. Anti-α-tubulin and anti-mitochondrial F_1F_0-ATPase mAbs TU-1 and αF1, respectively, were used as loading controls.

lation of 14-3-3 proteins (Fig. 4*A*). This decrease in the 14-3-3 protein level was less pronounced when MEM-59 was coimmobilized with CD99 mAb and was not observed in TF-1 cells overexpressing Daxx (Fig. 4*A*). Because none of the tested anti-GM-CSF Rβ mAbs recognized the GM-CSF receptor on the surface of TF-1 cells with sufficient sensitivity or under the conditions of Western blotting, the expression of GM-CSF Rβ was analyzed by reverse transcription-PCR. As expected, a 24-h treatment of cells with immobilized MEM-59 suppressed the expression of GM-CSF Rβ mRNA in TF-1 cells and, to lesser extent, also in TF-1/Daxx cells (Fig. 4*B*). Coimmobilized antiapoptotic CD99 mAb did not modulate this suppression. However, after 44 h of treatment, the expression of GM-CSF Rβ mRNA was restored in both TF-1 and TF-1/Daxx cells (Fig. 4*B*).

Both 14-3-3 proteins (by sequestering of serine-phosphorylated proteins) and the GM-CSF receptor (through activation of Akt kinase) cooperate in inhibiting the apoptosis-inducing activity of the proapoptotic protein Bad (38, 39). Thus, downregulation of both of these effectors should lead to the translocation of Bad from the cytoplasm to the mitochondria. Indeed, a 24-h treatment of TF-1 cells with immobilized MEM-59 already induced a significant mobilization of Bad to the mitochondrial (heavy membrane) fraction (Fig. 4*C*).

DISCUSSION

Receptor-mediated apoptotic signaling in hematopoietic cells is carried out primarily by the death receptors of the tumor necrosis factor receptor family. However, the cross-linking of other cell surface receptors such as major histocompatibility complex class I glycoproteins, the T cell receptor, or the B cell receptor either with a ligand or with an agonistic mAb could also induce apoptosis (40–42). Cross-linking of sialoglycoprotein CD43, an abundant cell surface protein of most hematopoietic cells, with mAbs was reported to induce apoptosis of proliferating human hematopoietic progenitors (32–34). Although the natural ligands inducing apoptosis via CD43 are currently unknown, possible candidates might be galectins (43–45) or the sialoglycoprotein-binding soluble or membrane-bound lectins such as the recently described sialoadhesin (16).

To investigate the molecular mechanisms of MEM-59-induced apoptosis, we used a model hematopoietic cell line TF-1 (36), which responded to immobilized MEM-59 with extensive apoptosis. The co-cross-linking of both CD43 and CD45 enhanced the MEM-59-induced apoptosis of TF-1 cells, implying that a ligand recognizing both CD43 and CD45 might be more efficient in inducing apoptosis of hematopoietic progenitors *in vivo*. In contrast, the co-ligation of the adhesion-related molecules CD50 (ICAM-3) (46, 47) or CD99 (48) partially inhibited MEM-59-induced apoptosis, suggesting a possible way of suppressing CD43-induced apoptosis through adhesion-connected antiapoptotic signaling. Although the cross-linking of CD50 or CD99 inhibited MEM-59-mediated apoptosis of TF-1 cells, anti-CD50 or anti-CD99 mAbs were reported to induce apoptosis of thymocytes, Jurkat T cells, and Ewing's sarcoma cells (49–52). Interestingly, other reports show that mAb-mediated cross-linking of CD43 on thymocytes, peripheral blood mononuclear cells, or even on TF-1 cells did not lead to apoptosis but rather stimulated their proliferation (17, 20, 53). Apparently, the cell-specific environment, the nature of the CD43 epitope involved, or cell type-dependent changes in the glycosylation of CD43 could affect the final outcome of CD43-mediated signaling.

In addition to anti-CD50 and CD99 mAbs, the overexpression of the apoptotic regulator Daxx, which interacts with the intracellular part of CD43 in a yeast two-hybrid system, also inhibited MEM-59-induced apoptosis in TF-1 cells. Although Daxx is predominantly a nuclear protein, it was reported to associate with the cytoplasmic domains of Fas (CD95) (54–56) or type II transforming growth factor-β receptor (57) and to enhance apoptosis induced by these receptors. Recent reports suggest that protein kinase ASK1 regulates the cellular localization of Daxx (58, 59) as well as its interaction with Fas (and therefore possibly also with CD43). Deletion of Daxx in mice results in extensive apoptosis and embryonic mortality, also implicating its antiapoptotic function (60). Our results support an antiapoptotic role for Daxx, at least in the CD43 cross-linking-induced apoptosis of TF-1 cells. Moreover, overexpression of Daxx enhanced the antiapoptotic effect of anti-CD50 and anti-CD99 mAbs, suggesting their nonoverlapping functions in the inhibition of MEM-59-induced apoptosis of TF-1 cells. More detailed studies on the role of ASK1 and Daxx in CD43-mediated apoptosis are presently under way in our laboratory.

The soluble anti-CD43 mAbs MEM-59 and L10 cross-linked

with secondary antibody were reported to increase the DNA binding activities of AP-1, NF-AT, and NFκB transcription factors in Jurkat T cells, resulting in the induction of IL-2, CD69, and CD40L gene expression in peripheral T cells (20). Indeed, we also confirmed that cross-linking of CD43 by soluble, apoptosis-noninducing MEM-59 on TF-1 cells enhanced the DNA binding activities of both AP-1 and NFκB. In contrast, immobilized, apoptosis-inducing MEM-59 significantly suppressed the DNA binding activity of AP-1, suggesting that this suppression could be an important aspect of MEM-59-induced apoptotic signaling. Apoptosis of Jurkat T cells induced by another anti-CD43 mAb, J393, was reported to be accompanied by suppression of NFκB DNA binding activity, but in contrast to our observation, the DNA binding of AP-1 remained unchanged (35).

The differences in the DNA binding activities of AP-1 and NFκB in TF-1 cells treated with soluble *versus* immobilized MEM-59, as well as the slow kinetics of MEM-59-induced apoptosis of TF-1 cells, suggested an important role for either *de novo* proteosynthesis or transcription in the process. Indeed, the expression of several genes implicated in antiapoptotic signaling, including GM-CSF Rβ and 14-3-3 proteins, was suppressed (Table II and Fig. 4). GM-CSF is known to activate the expression of Bcl-2 and Mcl-1 in TF-1 cells and to induce phosphorylation of the proapoptotic protein Bad via activation of protein kinase B/Akt- and mitogen-activated protein kinase/extracellular signal regulated kinase- dependent pathways (38, 39, 61). 14-3-3 proteins mediate essential antiapoptotic signaling through sequestering Bad and other proapoptotic proteins (62). Interestingly, overexpression of Daxx inhibited the MEM-59-induced down-regulation of 14-3-3 proteins. This is in contrast to its proposed function as a transcriptional repressor (63, 64). An intriguing possibility would be that Daxx might act as a repressor of another repressor(s). The down-regulation of GM-CSF Rβ and 14-3-3 proteins, both negative regulators of Bad, suggested that translocation of Bad to the mitochondria could be responsible for the apoptosis of TF-1 cells induced by immobilized MEM-59; this was indeed confirmed in our experiments (Fig. 4).

Thus, the MEM-59-induced suppression of the DNA binding activity of AP-1 could be at least in part responsible for the down-regulation of GM-CSF Rβ and 14-3-3 gene expression, which in turn leads to an accumulation of proapoptotic Bad in the mitochondria, resulting eventually in apoptosis. It seems likely that different ways of ligation of a receptor (in our case CD43) could result in quite opposite outcomes. The cross-linking of CD43 by soluble mAb, imitating an interaction with a hypothetical soluble ligand, led to increased DNA binding activity of AP-1 and NFκB transcription factors in both Jurkat and TF-1 cell and to the expression of activation markers on T cells (20). In contrast, plastic-immobilized mAb, presumably mimicking the contact of hematopoietic cells with a cell surface ligand in bone marrow, induced a drop in the DNA binding activities of AP-1 and subsequent apoptosis.

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