Cloning and Characterization of a Novel RING-B-box-Coiled-coil Protein with Apoptotic Function*

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We have identified a novel RING-B-box-coiled-coil (RBCC) protein (MAIR for macrophage-derived apoptosis-inducing RBCC protein) that consists of an N-terminal RING finger, followed by a B-box zinc finger, a coiled-coil domain, and a B30.2 domain. MAIR mRNA was expressed widely in mouse tissues and was induced by macrophage colony-stimulating factor in murine peritoneal and bone marrow macrophages. MAIR protein initially showed a granular distribution predominantly in the cytoplasm. The addition of zinc to transfected cells containing MAIR cDNA as part of a heavy metal-inducible vector caused apoptosis of the cells characterized by cell fragmentation; a reduction in mitochondrial membrane potential; activation of caspase-7, -8, and -9, but not caspase-3; and DNA degradation. We also found that the RING finger and coiled-coil domains were required for MAIR activity by analysis with deletion mutants.

The RING-B-box-coiled-coil (RBCC) proteins are a subgroup of the RING finger family characterized by an N-terminal RING finger, followed by one or two additional cysteine-rich zinc fingers (B-box) and a leucine coiled-coil domain forming the RBCC or tripartite motif (1, 2). The core members of the RBCC family possess a B30.2 domain at their C terminus in addition to the RBCC motif (3). However, despite their structural similarity, RBCC proteins show varied subcellular localization and diverse cellular function (4).

Some members are known to be putative transcription factors and are developmentally regulated or expressed in a tissue-specific manner (5). Xnf7 was first detected in the Xenopus oocyte nucleus and is released to the cytoplasm during oocyte maturation (6). At the mid-blastula stage, Xnf7 re-enters the nuclei and is involved in regulating the expression of genes required for axial patterning (7). PwA33 was cloned as a nuclear protein on the loops of amphibian lambrush chromosomes and is suggested to have a role in the synthesis or processing of pre-mRNA during oogenesis (8). Transcriptional intermediary factor-1α and -1β (KAP-1/KRIP-1) bind to the KRAB (Krüppel-associated box) domain of human zinc finger proteins and enhance transcriptional repression exerted by the KRAB domain (9, 10).

Some RBCC proteins show localization in the cytoplasm. SS-A/Ro is an autoantigen in Sjögren’s syndrome and binds to a specific small RNA (11, 12). FYXMID1, the gene responsible for X-linked Opitz syndrome (13), is confined to the cytoplasm and is associated with microtubules (14). Its mutations in the C terminus in patients with Opitz syndrome completely abolish microtubule association (14). The Brain-expressed RING finger protein BERP is associated with myosin V and α-actin-4 (15, 16). The Estrogen-responsive finger protein EFP is induced in response to 17β-estradiol (17) and is essential in estrogen-induced cell proliferation (18). HERF1 was cloned as a downstream target of the acute myeloid leukemia 1/core-binding factor-β transcription factor and is required for the terminal differentiation of erythroid cells, although the precise localization of HERF1 remains to be elucidated (19). In contrast to the RBCC proteins localized in the nucleus, the exact function of most cytoplasmic RBCC proteins remains unknown. The RBCC domain was found to be involved in protein-protein interaction, and some RBCC proteins were discovered in macromolecular complexes, suggesting a role of the RBCC domain in connecting other proteins to form large multiprotein complexes. Borden (4) reported that RING proteins have a common characteristic in that they mediate protein-protein interactions involved in forming large molecular scaffolds.

Apoptosis is a physiological cell suicide process that is indispensable in development, and its malfunction is involved in tumorigenesis. Two RBCC proteins, the proglycelynctic leukemia protein PML and the ret finger protein RFP, form PML nuclear bodies, which play crucial roles in apoptosis (20–22). Several reports indicate that PML is a growth suppressor and that the disturbance of PML functions provides a growth advantage to the leukemic cells (23, 24). Indeed, the overexpression of PML induces apoptosis, and analysis of PML knockout mice has shown that PML is essential in the induction of apoptosis by various stimuli such as DNA damage, Fas, tumor necrosis factor, ceramide, and interferons (25, 26).

In this work, we report on the identification of a novel member of the RBCC group of RING finger proteins, referred to as MAIR for macrophage-derived apoptosis-inducing RBCC protein. This gene was identified by cDNA library subtraction in which...
we screened genes up-regulated in bone marrow macrophages by a hematopoietic growth factor, macrophage colony-stimulating factor (M-CSF). We also show the subcellular localization of the novel RBCC protein and its apoptosis-inducing function.

EXPERIMENTAL PROCEDURES

Library Subtraction and Cloning of MAIR—Library subtraction was performed as reported previously (27). Briefly, murine bone marrow macrophages were prepared by culturing femoral bone marrow cells from C57BL/6 mice (Charles River Japan, Yokohama, Japan) with 100 ng/ml M-CSF for 7 days (28). Cells were factor-depleted for 12 h in RPMI 1640 medium containing 10% fetal calf serum and then treated with 100 ng/ml M-CSF for 3 h. Poly(A) RNA from untreated cells or from those treated with M-CSF was prepared using an mRNA separator kit (Clontech, Palo Alto, CA). cDNA library construction and library subtraction were performed with a PCR-Select CDNA subtraction kit (Clontech). The cDNA fragments of the subtracted cDNA library were cloned into the pCR2.1 vector (Invitrogen). Randomly isolated clones were further analyzed by direct sequencing and by Northern hybridization using total RNA from unstimulated and M-CSF-stimulated bone marrow macrophages. A 3.4-kbp cDNA as a cDNA probe for mouse MAIR was isolated by screening a peritoneal macrophage cDNA library with the cDNA fragment obtained by the subtraction approach. 5′-Rapid amplification of cDNA ends (5′-RACE) was performed using a Marathon-ready cDNA amplification kit (Clontech). The 5′-RACE products were cloned into the pCR2.1 vector and sequenced. The following primer was used to amplify the 5′-region: 5′-GCGCTGCTGCTTGTTCGTC-3′ (Northern Blot Analysis—Total RNAs from bone marrow macrophages were isolated using RNAzol B reagent (Tel-Test, Friendswood, TX), electrophoresed on agarose gels, and transferred to a nylon membrane (Hybond N+, Amersham Biosciences). The membrane was hybridized with a radiolabeled MAIR cDNA probe or glyceraldehyde-3-phosphate dehydrogenase cDNA (Clontech) (29). A probe for MAIR was prepared by PCR using primers 5′-CTCTGCGCCCTCCTGCTAAAGGA-3′ and 5′-GGGACACGCCAGGTGGCCAG-3′. Mouse multiple-tissue Northern blots (Ornica, Rocheville, France) were hybridized with the radiolabeled MAIR cDNA or actin cDNA (Clontech).

Expression Constructs—MAIR cDNA was subcloned into an epitope tag expression vector, phM6 or pmH (Roche Applied Science, Mannheim, Germany), to introduce a hemagglutinin (HA) tag at the N or C terminus of MAIR, respectively. The cDNA was also cloned into the pEGFP vector (Clontech) to express a fusion protein of MAIR with the HA-tagged or GFP-fused MAIR cDNA was subsequently inserted into the zinc-inducible expression vector pMEP4 (Invitrogen). The RING finger domain and 5′-end (data not shown). That the designated ATG codon is the true translated initiation codon is supported by its context within a Rokaz consensus sequence (39) and alignment of the MAIR protein with SS-A/Ro autoantigen (11, 12), acid finger protein (AFP) (37), human RFP (38), and HERF1 (19) (Fig. 1B). The MAIR protein shows 24–29% identity and 40–47% similarity to these RBCC proteins. Among these proteins, Xnf7 has a cytoplasmic retention domain that controls its subcellular localization and that precedes the RING finger domain (6). The open reading frame of MAIR cDNA is preceded by a 19-bp upstream sequence, although we were unable to identify an in-frame and upstream stop codon (Fig. 1A). However, analysis of 5′-RACE products suggested that the cDNA has no missing 5′-end (data not shown). That the designated ATG codon is the true translated initiation codon is supported by its context within a Rokaz consensus sequence (39) and alignment of the MAIR protein with SS-A/Ro, RFP, acid finger protein, and HERF1 (Fig. 1B). After our cloning, a cDNA sequence of the putative human homolog of MAIR was submitted to the GenBank™/EBI Data Bank by the Kazusa DNA Research Institute (KIAA1098, accession no. AB029021) as a new cDNA clone (40). These molecules are 78% identical and 85% similar at the amino acid level (Fig. 1B).

RESULTS

Cloning of MAIR—To identify M-CSF-inducible genes, we prepared a cDNA library of murine bone marrow-derived macrophages cultured in cytokine-free medium and subtracted this cDNA library from that of cells stimulated with M-CSF (27). A number of genes induced by M-CSF stimulation were obtained. Among them, we found a novel cDNA fragment encoding a RING finger domain. A 3.4-kbp cDNA was isolated by screening a peritoneal macrophage cDNA library with the cDNA fragment obtained by the subtraction approach as a probe (Fig. 1A). The cDNA encodes a 501-amino acid protein, which was designated MAIR for macrophage-derived apoptosis-inducing RBCC protein (see below). The MAIR protein consists of an N-terminal cysteine-rich C3HC4 zinc finger (Fig. 1A, solid line a) (35), followed by a B-box zinc finger (solid line b) (36), a coiled-coil domain (solid line c), and a C-terminal region referred to as the B30.2 domain (dashed lines d–f) (3).

Accordingly, MAIR is a new member of the family of RBCC proteins. There are three putative nuclear localization sequences (Fig. 1A, boxes). Other members of this RBCC family are Xnf7 of Xenopus (6), PwA33 (8), human 52-kDa SS-A/Ro autoantigen (11, 12), acid finger protein (AFP) (37), human RFP (38), and HERF1 (19) (Fig. 1B). The MAIR protein shows 24–29% identity and 40–47% similarity to these RBCC proteins. Among these proteins, Xnf7 has a cytoplasmic retention domain that controls its subcellular localization and that precedes the RING finger domain (6). The open reading frame of MAIR cDNA is preceded by a 19-bp upstream sequence, although we were unable to identify an in-frame and upstream stop codon (Fig. 1A). However, analysis of 5′-RACE products suggested that the cDNA has no missing 5′-end (data not shown). That the designated ATG codon is the true translated initiation codon is supported by its context within a Rokaz consensus sequence (39) and alignment of the MAIR protein with SS-A/Ro, RFP, acid finger protein, and HERF1 (Fig. 1B).

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Tissue Distribution and M-CSF-induced Expression of MAIR—Northern analysis showed that the MAIR mRNA was ubiquitously expressed in the mouse tissues examined (Fig. 1C). The mRNA was 3.4–3.6 kb, suggesting that our clone (3.4 kb) was a nearly full-length cDNA. It was relatively highly expressed in the brain, lung, spleen, thymus, heart, and muscle (Fig. 1C). According to the HUGE Database provided by the Kazusa DNA Research Institute, the human homolog (KIAA1098) is relatively highly expressed in the brain, liver, spleen, ovary, testis, and heart.

MAIR cDNA was originally isolated from M-CSF-stimulated macrophages, but there was a relatively low level of MAIR expression in bone marrow cells. We therefore certified MAIR expression in primary macrophages and several macrophage cell lines. Bone marrow macrophages were factor-depleted and then stimulated with M-CSF for the time periods indicated. Total RNA was blotted onto a membrane and hybridized with the MAIR or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe.

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**Transient MAIR Expression Promotes Cell Death—**To analyze the function of MAIR, we initially attempted to express

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3 Available at www.kazusa.or.jp/huge.
HA-tagged MAIR in mouse NIH3T3 cells. When we transiently transfected NIH3T3 cells with the HA-tagged MAIR expression vector, we could easily detect the HA-tagged protein by immunoblot analysis using anti-HA antibody as a band with a molecular mass of 55 kDa (data not shown). However, none of the G418-resistant stable clones showed a detectable level of HA-tagged MAIR protein (data not shown).

To investigate the fate of MAIR-transfected cells and the subcellular localization of MAIR, we transiently expressed MAIR as a GFP fusion protein in NIH3T3 cells (Fig. 2A). Despite the presence of putative nuclear localization signals (Fig. 1A), fluorescence microscopic analysis revealed a granular distribution of GFP-MAIR predominantly in the cytoplasm at 12 h post-transfection (Fig. 2A, panel a). The fact that MAIR localized in the cytoplasm was confirmed using HeLa cells (data not shown). Confocal microscopic examination showed partial colocalization of MAIR with mitochondria (Fig. 2B). At 24 h post-transfection, GFP-MAIR-expressing NIH3T3 cells shrunk and began to detach from the dish (Fig. 1A, panel b). Some cells appeared to be fragmented (Fig. 1A, panel b, arrowheads). Consistent with the results, flow cytometric analysis showed that propidium iodide-positive dead cells increased in number 72 h after transfection of GFP-MAIR compared with mock transfection (Fig. 2C).

Zinc-inducible Expression of MAIR in GM-CSF-dependent TF-1 Cells—To demonstrate more clearly that the expression of MAIR promotes cell death, we transfected TF-1 cells with a zinc-inducible pMEP4-HA-MAIR construct, in which a metallothionein promoter directs MAIR expression. TF-1 cells are leukemic cells of human origin whose proliferation is dependent on the presence of GM-CSF (30). Three independent clones (clones 18, 19, and 24) that expressed HA-tagged MAIR after zinc treatment were established (Fig. 3A). As shown in Fig. 3B, the addition of 75 or 100 μM ZnCl₂ to the culture medium induced cell death in the three clones, whereas the treatment did not affect the viability of mock-transfected TF-1 cells. Among MAIR transfectants, clone 24, which expressed more HA-tagged MAIR compared with clones 18 and 19 (Fig. 3A), showed markedly reduced viability after zinc treatment (Fig. 3C). Phase-contrast microscopic examination revealed the presence of particles of fragmented TF-1-HA-MAIR cells after zinc induction (Fig. 3C).

Next, we made a pMEP4-GFP-MAIR construct, in which GFP fused to the N terminus of MAIR could be monitored by flow cytometry, and introduced the construct into TF-1 cells. GFP-MAIR was dose-dependently expressed after exposure to ZnCl₂ in a stable transfectant (clone 12) (Fig. 4A). A low but detectable level of MAIR expression could be found even if the cells were not treated with ZnCl₂ (Fig. 4A, upper panel). ZnCl₂ addition to the medium induced cell death in the transfectants (clones 12 and 25) (Fig. 4B), as shown in experiments in which HA-tagged MAIR was expressed (Fig. 3B).

Using this clone (clone 12), we analyzed the time course relationship between the expression of MAIR and ΔΨₘ. GFP-
MAIR expression (fluorescent intensity of GFP) could be detected within 2 h after zinc treatment (Fig. 5A, lower panels). The amount of MAIR increased until 8 h. The increase in the side scatter could be detected at 4 h, which was followed by a decrease in the forward scatter (Fig. 5A, upper panels). This change is consistent with certain characteristics of early apoptosis (41). Simultaneously, the uptake of MitoTracker CMX-Ros, which indicates mitochondrial membrane potential, decreased in response to zinc treatment (Fig. 5A, lower panels). Dye exclusion was conserved at 12 h, but membrane-damaged cells increased over 24 h (data not shown). We then analyzed the DNA content of TF-1 cells expressing GFP-MAIR by flow cytometry after culturing for 24 h in the presence of zinc. A large proportion of TF-1-GFP-MAIR cells (67%) contained degraded DNA, a characteristic of apoptosis (Fig. 5B). There was no increase in hypodiploid DNA in the control cell line and untreated TF-1-GFP-MAIR cells (Fig. 5B). These features in inducible MAIR overexpression suggested that the cell death observed was apoptosis.

The emerging view of apoptosis is that this complex biochemical event is carried out by a family of cysteine proteases called caspases (42). To address the role of caspases, we used several kinds of caspase inhibitors. The addition of Z-Asp-CH2-DCB, but not Z-VAD-fmk, completely prevented a reduction in MitoTracker CMX-Ros in TF-1-GFP-MAIR cells (Fig. 6A). However, the percentage of membrane-damaged cells with Z-Asp-CH2-DCB was not greater than that without inhibitors even after 24 h of ZnCl2 induction (data not shown). These observations suggest that the inhibitor Z-Asp-CH2-DCB did not induce necrotic cell death in addition to preventing apoptotic cell death. The specific inhibitors Ac-YVAD-CHO (for caspase-1 and -4), Ac-DEVD-CHO (for caspase-3 and -7), Ac-IETD-CHO (for caspase-8 and -6), and Ac-LEHD-CHO (for caspase-9) could not effectively impede the decrease in $\Delta \Psi_m$ when used alone (data not shown). We then performed immunoblot analysis for caspases to detect their activation. When TF-1-GFP-MAIR cells were treated with zinc (Fig. 6B), it was obvious that caspase-7, -8, and -9 were cleaved, whereas no processing of caspase-2 or -3 was observed (Fig. 6B) (data not shown for caspase-2). Caspase-9 is activated by binding to Apaf1 in the presence of cytochrome c released from the mitochondria (43). Consistent with the decrease in $\Delta \Psi_m$ as mentioned above, this pattern of caspase activation suggests that the mitochondria are involved in MAIR-induced apoptosis. We also detected a cleaved form of poly(ADP-ribose) polymerase, a substrate of executioner caspases (Fig. 6B).

The RING Finger and Coiled-coil Domains Are Important for the Apoptosis-inducing Activity of MAIR—To examine which domain of MAIR is responsible for its apoptosis-inducing activity, we generated a series of MAIR truncation mutants (Fig. 7A), performed transient transfections into NIH3T3 cells, and screened for cell death by determining the mitochondrial membrane potential (Fig. 7B). The expression of the mutant with the B-box domain deleted ($\Delta BB$) or with the B30.2 domain deleted ($\Delta B30.2$) was comparable to that of the wild type (Fig. 7B, left panels). However, for unknown reasons, the expression
of the mutant with the RING finger domain deleted (ΔRF) or with the C-terminal region containing the coiled-coil and B30.2 domains deleted (ΔCC-B30.2) was somewhat higher than that of the wild type (Fig. 7B, left panels). The B30.2 domain-deleted and B-box-deleted mutants induced a reduction in mitochondrial membrane potential at a comparable level to the wild type (Fig. 7B, right panels). Thus, these domains might not be involved in the apoptosis-inducing function of MAIR. In contrast, the reduction in mitochondrial membrane potential by the RING finger domain-deleted MAIR mutant or the mutant in which the C-terminal region containing the coiled-coil domain was deleted was less severe than that of the wild type (Fig. 7B, right panels), despite the higher expression of these mutants in transfected cells (see left panels). These data indicate that the RING finger and coiled-coil domains are required for the apoptosis-inducing activity of MAIR.

Finally, we investigated how the difference in the apoptotic activity of the mutants corresponds to their subcellular localization (Fig. 7C). The B-box-deleted (ΔBB panel) and B30.2 domain-deleted (ΔB30.2 panel) mutants showed a similar cellular distribution to the wild type (MAIR panel). In contrast, the less potent mutants, i.e. the RING finger domain-deleted MAIR mutant (ΔRF panel) and the mutant in which the C-terminal region containing the coiled-coil domain was deleted (ΔCC-B30.2 panel), showed a distinct distribution pattern from the wild type. The RING finger domain-deleted mutant showed a filamentous appearance in the cytoplasm, and the C-terminal region-deleted mutant spread throughout the cytoplasm. Thus, the change in the subcellular distribution appears to correlate with apoptotic activity.

DISCUSSION

We have identified a novel RBCC protein involved in the apoptotic pathway. The subcellular localization of proteins provides valuable information on their functions. Many RBCC proteins are localized in the nucleus and are involved in transcriptional regulation (5). For example, Xnf7, a maternally expressed cytoplasmic protein, moves to the nucleus during mid-blastula transition (7). The intracellular distribution of some RBCC proteins was reported to be dependent on the cell types. Endogenous RFP is located predominantly in the cytoplasm with low levels in the nucleus in NIH3T3 and mouse A9 cells (44). In contrast, the protein was shown to be a nuclear protein in HepG2, HeLa, and mouse fetal heart cells (44). When HeLa cells were transfected with wild-type RFP, the exogenous protein showed the same distribution pattern as the endogenous protein (44). MAIR possesses three putative sites of nuclear localization signals (Fig. 1A). However, upon examining the subcellular localization of exogenous MAIR using NIH3T3 cells (Fig. 2A) and HeLa and TF-1 cells (data not shown), we found MAIR to be predominantly localized in the cytoplasm. We also found that MAIR was distributed as granules in the cytoplasm (Fig. 2A). This is in good accordance with the hypothesis that the RBCC domain mediates protein oligomerization and the fact that RBCC proteins are often found in large complexes, possibly acting as scaffolding elements (4). How-
MAIR is an RBCC family member that has a B30.2 domain in the C-terminal portion in addition to the RBCC motif. The apoptotic activity of PML appears to be mediated through its RING domain (24). In addition, deletion of the coiled-coil motif in PML yields a diffuse nuclear localization pattern and results in no growth suppression (50). Although MAIR is a cytoplasmic protein rather than a nuclear protein, corresponding regions of MAIR appear to be required for the apoptosis-inducing activity. The deletion of the RING finger domain and the coiled-coil region of MAIR, but not the B-box or B30.2 domain, partially abolished the MAIR activity (Fig. 7B). Of importance, the less potent mutants showed a distinct cytoplasmic distribution pattern from the wild type (Fig. 7C). Therefore, the RING finger domain and the coiled-coil region of MAIR might be important in forming a molecular scaffold with cytoplasmic proteins. Interestingly, stable transformants obtained by transfection with the RING finger domain-deleted MAIR mutant showed detectable MAIR expression upon immunoblotting even when the cells were not treated with ZnCl₂,² raising the possibility that the RING finger domain is involved in the stability of MAIR itself. There is recent evidence that the RING finger domain can act as a ubiquitin-protein isopeptide ligase (E3) to target proteins for degradation (51). Ubiquitin-protein isopeptide ligase (E3) proteins are responsible for providing specificity to ubiquitin conjugation by acting as links or bridges between ubiquitin-conjugating enzymes and substrate. Therefore, ubiquitin conjugation may control the stability or degradation of MAIR through its RING finger.

MAIR was originally isolated by cDNA subtraction, in which genes that were up-regulated in bone marrow macrophages by M-CSF stimulation were screened (Fig. 1D). Thus, the finding that MAIR possesses apoptosis-inducing activity seems to be contradictory to the fact that M-CSF is a growth factor for macrophages. Differentiated macrophages are known to be resistant to various death stimuli such as death receptor ligation, ever, we cannot rule out the possibility that MAIR moves to the nucleus during the apoptotic process (Fig. 2A, panel b).

SS-A/Ro, FXY/MID1, and BERP were reported to be cytoplasmic RBCC proteins (14, 15, 45). The gene for BERP is mapped to chromosome 11p15, which is frequently deleted in several human cancers and is speculated to be a tumor suppressor gene (46). However, the exact molecular functions of these cytoplasmic RBCC proteins remain to be elucidated. In this study, we demonstrated that MAIR was involved in programmed cell death. The induction of the MAIR protein in TF-1 cells caused apoptosis of the cells characterized by cell fragmentation (Fig. 3C); a reduction in mitochondrial membrane potential (Fig. 5A); and activation of caspase-7, -8, and -9 (Fig. 6B). The caspase cascade and mitochondria play central roles in the cytoplasmic process of apoptosis (47–49). Some apoptotic stimuli such as growth factor starvation and reactive oxygen species cause mitochondrial membrane permeabilization and release of cytochrome c, apoptosis-inducing factor, and some caspases (48). Released cytochrome c forms complexes with Apaf1 and procaspase-9 and accordingly activates caspase-9, which then processes and activates other caspases (42). Apoptosis induced by MAIR was found to be partially dependent on caspases because the pan-caspase inhibitor Z-Asp-CH₂-DCB prevented a reduction in the mitochondrial membrane potential (Fig. 6A). Dissipation of the mitochondrial membrane potential in the early phase and activation of caspase-9 and -7 imply that MAIR triggers the event upstream or in the vicinity of the mitochondria. In fact, confocal microscopic examination showed partial colocalization of MAIR with the mitochondria (Fig. 2B). Furthermore, KIAA1098, the putative human homolog of MAIR, has been mapped to chromosome 8p21, which is frequently deleted in human cancers (Mitelman Database of Chromosome Aberrations in Cancer).⁴

⁴ Available at cgup.nci.nih.gov/Chromosomes/Mitelman.
antineoplastic agents, and ionizing irradiation, whereas monocytes are susceptible to Fas-mediated apoptosis (52). Flip, Bcl-xL, A1, and cytoplasmic p21Cip1/WAF1 (53–56). From the view point of M-CSF stimulation, M-CSF induces Ets2 expression, and in turn, Ets2 up-regulates Bcl-xL expression in the BAC1.2F5 macrophage cell line (57). In NIH3T3 cells expressing the M-CSF receptor, M-CSF promotes cell survival through Akt-induced inhibition of caspase-9 activation (58). Akt mediates IκB kinase-α phosphorylation, leading to NFκB activation, which is essential for tumor necrosis factor signaling (59). M-CSF up-regulates XIAP (X-linked inhibitor of apoptosis (52) upon activation of NFκB possibly through activated Akt (60). However, at the same time, M-CSF was reported to induce the expression of pro-apoptotic factors procaspase-3 and -9 and Bax in M-CSF-induced bone marrow macrophages (60). An increased expression of procaspase-3 and -8 was also observed in phorbol 12-myristate 13-acetate-stimulated U937 monocytic cells (61). These observations imply that M-CSF is able to up-regulate both pro-apoptotic and anti-apoptotic proteins and tilts the balance to an anti-apoptotic state. We recently demonstrated that p53δak-2, a molecule that negatively regulates signal transduction and cell proliferation mediated by cyto- kinies, is up-regulated in macrophages by M-CSF stimulation (27). Thus, the up-regulation of the gene for MAIR in macro- phages by M-CSF is also likely to be a negative feedback mechanism.

Based on the results of our transient and inducible expression experiments with the cell lines, we conclude that the novel RBCC protein MAIR possesses an apoptosis-inducing function. Our data presented here suggest that the cell death induced by MAIR is at least in part dependent on the mitochondria. However, the precise mechanism of MAIR-induced apoptosis is a subject of future investigation. Many RBCC proteins interact with other proteins and seem to construct macromolecular complexes as molecular blocks. Accordingly, identification of MAIR-associated proteins will reveal the role of MAIR in the apoptotic pathway more precisely as well as the structure of the macromolecular complexes in which MAIR is involved. We are now screening MAIR-associated proteins in a bone marrow cDNA library using a yeast two-hybrid system.

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Note Added in Proof—Following the acceptance of this article, we became aware of an article describing the RBCC protein family. MAIR was not investigated minutely in this study, but the coiled-coil region was essential for both homo-oligomerization and proper subcellular localization of RBCC proteins as a common character (62). The human homologue KIAA1098 was renamed TRIM35, a HUGO (Human Genome Organisation) nomenclature committee-approved gene symbol.

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