Mouse ING1 Homologue, a Protein Interacting with A1, Enhances Cell Death and Is Inhibited by A1 in Mammary Epithelial Cells

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Abstract
We cloned mouse ING1 homologue (mINGh), an A1/Bfl-1-interacting protein, from mouse mammary glands using a yeast two-hybrid assay and unexpectedly found four splicing variants of mINGh by reverse transcription-PCR assay and sequence analysis. The alternative splicing variants were mINGh-S, mINGh-M, mINGh-L, and mINGh-L2 encoding 171, 248, 166, and 227 amino acids, respectively. Cell death of HC11 cells, induced by serum starvation, was enhanced by mINGhs, and the action of mINGh was inhibited by A1 protein. These results indicate that A1 can inhibit cell death not only via the well known pathway related to the Bcl-2 family but also through direct interaction with mINGh in mammary epithelial cells.

Introduction
Mammary glands repeat proliferation, differentiation, and involution. During the involution phase, the majority of secretory mammary epithelial cells are eliminated by apoptosis [programmed cell death (1, 2)]. Apoptosis plays a major role in the development and maintenance of homeostasis within multicellular organisms. For example, apoptosis is an important process in the elimination of self-reactive lymphocytes, and a failure of such action may lead to the development of neoplasia (3, 4). One of the intercellular mediators for apoptosis is Bcl-2 family proteins, and many of these have been cloned and characterized. The Bcl-2 family, which consists of antiapoptotic members such as Bcl-2 and A1/Bfl-1 and proapoptotic members such as Bak and Bak (5–8), shares four conserved Bcl-2 homology and transmembrane regions. Survival or death of cells is determined by the ratio of proapoptotic:antiapoptotic members. The Bcl-2 homology regions play an important role in the interaction among Bcl-2 family members. A1 is an antiapoptotic Bcl-2 family member and a direct transcriptional target of nuclear factor κB. The expression of A1 can be induced under the influence of inflammatory cytokines, tumor necrosis factor α and interleukin 1 (6, 9). A1 protein showed an ability to prevent cell death induced by tumor necrosis factor α in the presence of actinomycin D in endothelial cells (10).

In this study, we have screened a cDNA library from mouse mammary gland to search for A1-interacting proteins using the yeast two-hybrid assay method. mINGh cDNA was obtained, and the function of A1 and mINGh were examined in HC11 mammary epithelial cells.

Materials and Methods

Screening for A1-interacting Proteins by the Yeast Two-hybrid System. To prepare the bait protein expression plasmid, mouse A1 cDNA containing full coding sequences was cloned into pEG202 expression vector (Origene). pEG202-A1 plasmid was transformed into EGY48 yeast strain and expressed a fusion protein consisting of the LexA DNA binding domain and A1. A mouse mammary cDNA library (Origene) based on pG4–5 plasmid was constructed to express a fusion protein consisting of the B42 transactivation domain and proteins from mouse mammary glands. The library was cloned to screen proteins interacting with A1. Transformed cells were plated on medium containing galactose but lacking tryptophan, histidine, leucine, and uracil. To select potentially positive transformants, colonies were tested for LacZ expression. Positive cDNAs were retransformed individually into Escherichia coli K8 and recovered from transformed cells, which were grown in media lacking tryptophan. Positive clones were characterized by sequence analysis using an ABI Prism 377 DNA Sequencer (Perkin-Elmer).

Cloning of mINGh Splicing Variants Using RT-PCR. Total RNA was prepared from mammary gland of ICR mice using the guanidinium isothiocyanate method (11). First-strand cDNA was synthesized from 1 μg of total RNA by using the SMART PCR cDNA Synthesis Kit (Clontech). Single-stranded cDNA was amplified using 10 pmol of mINGh-specific or splicing variant-specific primers by PCR.

In Vitro Binding Assay. The in vitro binding assay was performed using a GST-A1 fusion protein and mINGhs. Expression plasmid pGEX-A1 for GST-A1 fusion protein was transformed into the BL21 E. coli strain, and the expression of proteins was induced for 4 h after the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. The GST-A1 fusion protein was purified with glutathione-Sepharose beads according to the manufacturer’s procedure (Pharmacia Biotech). 35S-labeled mINGh proteins were produced in vitro using plasmids (pCDNA-mINGhs) and the TNT Quick Coupled Transcription/Translation Systems (Promega) according to the manufacturer’s recommendation. 35S-labeled mINGh proteins were incubated with GST-A1 fusion proteins immobilized on glutathione-Sepharose beads overnight at 4°C. After extensive washing, beads were boiled in SDS sample buffer, and the interaction between the proteins was analyzed by 15% SDS-PAGE.

Cell Culture and Transfection. HC11 cells were cultured in a growth medium containing RPMI 1640 (Life Technologies, Inc.), 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 5 μg/ml insulin (Sigma Chemical Co.), 10 ng/ml epidermal growth factor (Sigma Chemical Co.), and 50 μg/ml gentamicin (Sigma Chemical Co.; Ref. 12). All transfections were performed using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer’s procedure. Cells were transfected with pCDNA3 plasmid (Invitrogen) containing mINGhs (pCDNA-mINGhs) and/or pCDNA3/hygro plasmid (Invitrogen) containing A1 (pCDNA/hygro-A1). Transfectants were selected by growing in media containing G418 (400 μg/ml) and/or hygromycin (200 μg/ml) for 3 weeks. To examine the subcellular localization of mINGh-M, HC11 cells were seeded on a glass slide and grown at 37°C for 24 h. Cells were transiently transfected with pEGFP-C1 (Clontech) containing mINGh-M (pEGFP-mINGh-M) and visualized by confocal laser scanning microscopy.

Viability assay and DNA fragmentation. Stable HC11 cells transfected with vector only, pCDNA-mINGhs, and/or pCDNA/hygro-A1 were seeded on 96-well plates at a density of 20,000 cells/well. On the following day, cells were incubated with serum-free media to induce apoptosis. Viable cells were estimated at 0, 12, 24, and 48 h after serum starvation using Cell Proliferation

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3 The abbreviations used are: mINGh, mouse ING1 homologue; RT-PCR, reverse transcription-PCR; GST, glutathione S-transferase; HL, high ING1 homology region; NLS, nuclear localization signal; GFP, green fluorescence protein.
A mammary gland is an excellent model to investigate factors involving in epithelial growth, differentiation, morphogenesis, and involution. The mammary gland also undergoes repeated modifications in response to hormonal and mechanical stimuli (14, 15). Apoptosis upon the development of mammary gland during the pregnancy cycle is associated with changes in the expression levels of Bel-2 family proteins (16).

To search for A1-interacting proteins, we screened 2 × 10⁶ cDNAs from the mouse mammary gland library using A1 as a bait with the yeast-two hybrid system and obtained 13 cDNAs that contain inserts of different sizes ranging from 0.5 kb to 2.7 kb. Positive cDNA clones were characterized by restriction mapping and nucleotide sequence analysis. One of the positive clones was identified as a mouse candidate tumor suppressor ING1 homologue (mINGh). To confirm the expression of mINGh in mouse mammary gland, RT-PCR was performed using mINGh-specific primers, and unexpectedly, four different sizes of mINGhs were obtained. Splicing variant-specific primers were designed and used for RT-PCR to test artifacts of the four products. The results showed that the mINGhs were not simply PCR artifacts. These four alternative splicing variants of mINGh were expressed not only in mouse mammary gland but also in other organs such as ovary, spleen, and muscle (data not shown). Further nucleotide sequence analysis (n = 3) indicated that the alternative splicing variants of mINGh were mINGh-S, mINGh-M, mINGh-L, and mINGh-L2 encoding 171, 248, 166, and 227 amino acids, respectively (Fig. 1A). The comparison of the deduced amino acid sequence of mINGh-M with ING1 (GenBank accession number AF149820) showed approximately 45% homology (Fig. 1B), and it contains IH1 (76.4%) and IH2 (83.6%) containing the COOH-terminal PHD finger domain. IH1 contains all splicing variants, whereas IH2 contains mINGh-M and a part of mINGh-L2 (Fig. 1C).

ING1 protein, which is known to localize into the nucleus, contains NLS, for instance, amino acids KKKK (17, 18). To examine the subcellular localization of mINGh, mINGh-M was expressed in HC11 cells with GFP as a fusion protein and then visualized using confocal laser scanning microscopy. Unlike the GFP alone, most GFP-mINGh-M fusion proteins localized to the nucleus, suggesting that mINGh is a nuclear protein like ING1 (Fig. 1D). NLS generally consists of high contents of the positively charged amino acids lysine and arginine, and in many nuclear proteins this sequence is split into two blocks of two to four amino acids each. Therefore, we speculate that NLS of mINGh should exist in the lysine-rich region (Fig. 1C).

An in vitro binding assay was performed to determine whether all splicing variants of mINGh were able to interact with A1. As shown in Fig. 2, mINGh-S, mINGh-M, and mINGh-L2 specifically interacted with GST-A1 but not with beads or GST alone. We also examined the interaction between mINGh and p53, and we were not able to detect any binding activity between the two proteins (data not shown).

Fig. 1. Nucleotide and deduced amino acid sequence analysis of mINGh splicing variants and subcellular localization of mINGh. A, the open reading frames of four mINGh splicing variants were determined by nucleotide sequence analysis. B, the deduced amino acid sequence of mINGh-M was compared with those of mING1, and identical residues are shaded. C, the number of amino acid residues for mINGh splicing variants is indicated, and the deduced amino acid sequence of mINGh-M is shaded. The boxed portions denote high ING1 homology and a lysine-rich region. The nucleotide sequence data reported in this study are in the European Molecular Biology Laboratory, GenBank, and DNA Data Bank of Japan nucleotide sequence data bases with the following accession numbers: mINGh-S, AY036107; mINGh-L, AY035880; and mINGh-L2, AY035881. D, HC11 cells were transfected with vector only (A and C) or GFP-mINGh-M (B and D) and incubated for 30 h. Cells were visualized by confocal laser scanning microscopy (C and D), and the same field of cells was visualized by green fluorescence (A and B).

Fig. 2. Interaction between mINGh and A1 in vitro. A bead only, GST, and a GST fusion protein purified by glutathione-Sepharose beads were incubated with 35S-labeled mINGhs, and the proteins were separated on 15% SDS-PAGE. Lanes 1–4 represent input, GST, bead only, and GST-A1, respectively.

Kit I [3-(4',5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide assay; Roche] according to the manufacturer’s procedure. Absorbance at 620 nm was subtracted from absorbance at 540 nm for each well. DNA fragmentation was assessed using the method of Smith et al. (13). Equal volumes of lystate were run on a 1.2% agarose gel and stained with ethidium bromide.
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To examine the function of mINGhs and the interaction between A1 and mINGhs, HC11 cells were transfected with A1- and/or mINGh-expressing plasmids. The induction of apoptosis was achieved by growing cells in serum-free media (23). Because mINGh-M was expressed at the highest level compared with other splicing variants in the tissues, stable transfectants expressing mINGh-M were used to examine the ability of mINGh to induce apoptosis in HC11 cells under serum starvation. As shown in Fig. 3A, cells expressing mINGh-M enhanced cell death more rapidly than those expressing vector only, coincident with the expression level. On the other hand, cells expressing antisense mINGh-M showed a level of cell death similar to that of cells expressing the vector only. Indeed, cells expressing mINGh-M showed enhanced the formation of apoptotic bodies and DNA fragmentation compared with cells expressing vector only or antisense mINGh (Fig. 3B).

To confirm whether all of the mINGhs enhanced cell death, the stable cells expressing mINGhs were monitored in serum-free media at various time points. All mINGh forms gradually enhanced cell death up to about 20–30% compared with that of the control at 48 h after serum starvation (Fig. 3C). On the other hand, mammary epithelial cell death was inhibited temporarily by overexpression of A1 for up to 24 h after serum starvation, and the enhancing effect on cell death by overexpression of mINGhs was almost completely inhibited by A1 (Fig. 3D). It is interesting to point out that the enhancing effect of mINGhs was inhibited by A1 because mINGh proteins localize to the nucleus, but A1 proteins localize primarily to the cytoplasmic area (24). Therefore, we propose that the functional activity of mINGh may have been decreased by interaction with A1 in the cytosol because most nuclear proteins are localized from the cytosol to the nucleus by NLS. The mechanism of action of mINGh modulation by A1 remains unknown at the present time, and additional studies are required to reveal the exact mechanism.

In this study, we have cloned mINGhs in the mouse mammary gland. The protein sequence showed 98% homology compared with the human ING1 homologue protein, indicating high sequence conservation through evolution. mINGh also has two high ING1 homology parts and showed a very similar function to ING1 protein. These results indicate that mINGh could be an essential protein for the function of mINGh and ING1 proteins should be categorized as one family based on sequence and function tests. Finally, we demonstrate that A1 can inhibit mammary epithelial cell death not only via the pathway related to the Bcl-2 family but also by inhibition of mINGh function.

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