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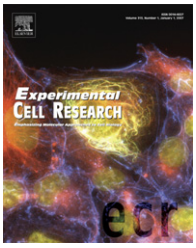

Research Article

Ras-association domain family protein 6 induces apoptosis *via* both caspase-dependent and caspase-independent pathways

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ABSTRACT

The Ras-association domain family (RASSF) comprises six members (RASSF1–6) that each harbors a RalGDS/AF-6 (RA) and Sav/RASSF/Hippo (SARAH) domain. The RASSF proteins are known as putative tumor suppressors but RASSF6 has not yet been studied. We have here characterized human RASSF6. Although RASSF6 has RA domain, it does not bind Ki-Ras, Ha-Ras, N-Ras, M-Ras, or TC21 under the condition that Nore1 (RASSF5) binds these Ras proteins. The message of RASSF6 is detected by RT-PCR in several cell lines including HeLa, MCF-7, U373, A549, and HepG2 cells, but the protein expression is low. The enhanced expression of RASSF6 causes apoptosis in HeLa cells. RASSF6 activates Bax and induces cytochrome C release. Caspase-3 activation is also induced, but the caspase inhibitor, Z-VAD-FMK, does not block RASSF6-mediated apoptosis. Apoptosis-inducing factor and endonuclease G are released from the mitochondria upon expression of RASSF6 and their releases are not blocked by Z-VAD-FMK. The knock down of RASSF6 partially blocks tumor necrosis factor-α-induced cell death in HeLa cells. These findings indicate that RASSF6 is implicated in apoptosis in HeLa cells and that it triggers both caspase-dependent and caspase-independent pathways.

Introduction

A loss of heterozygosity of human chromosome 3p21.3 is frequently observed in lung and other cancers [1]. Studies of candidate tumor suppressor genes that are located within this region have identified a putative Ras effector with a Ras-association (RalGDS/AF-6) (RA) domain [2,3]. This gene was named RA domain family 1 (RASSF1) and because of its significant homology to Nore1, a protein previously demonstrated to bind Ha-Ras in a GTP-dependent manner, was proposed to be a downstream target of the Ras signaling pathways [4]. It is significant that gene silencing by aberrant promoter hypermethylation of a splice variant of RASSF1, RASSF1A, is detectable in many cancers and correlates with tumor stage [5]. Moreover, RASSF1A knockout mice are more susceptible to spontaneous and chemically induced tumors [6]. Four other genes with homology to RASSF1 and Nore1 were subsequently identified, and now comprise the new family of proteins (RASSF1–6; Nore1 has been re-designated RASSF5) [7–9]. Each of the RASSF members harbors a conserved middle RA domain and a C-terminal Sav/RASSF/Hippo (SARAH) domain [10]. The loss of RASSF2, RASSF4, or Nore1 promotes

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tumorigenicity and overexpression of these proteins inhibits cell growth. In addition, RASSF1A inhibits cell cycle progression and the RASSF1, RASSF2, and Nore1 proteins have been implicated in a pro-apoptotic pathway [9,11–13]. These findings suggest that the RASSF proteins play an important role as tumor suppressors *via* the regulation of the cell cycle and the induction of apoptosis.

In our present study, we have analyzed human RASSF6 and show that, unlike Nore1, it does not bind Ki-Ras or any of the other Ras proteins that we examined. In a similar manner to other RASSF family members, however, RASSF6 displays pro-apoptotic properties. RASSF6 expression induces both Bax activation and cytochrome *C* release, and activates caspase-3. Treatment with a caspase inhibitor, Z-VAD-FMK, does not block the RASSF6-mediated apoptotic response. In addition, apoptosis-inducing factor (AIF) and endonuclease G (EndoG) are released from the mitochondria in cells expressing RASSF6. Knock down experiments suggest that RASSF6 is implicated in tumor necrosis factor-α (TNFα)-induced apoptosis in HeLa cells. These observations characterize RASSF6 as a putative tumor suppressor that has the ability to induce apoptosis *via* both caspase-dependent and caspase-independent pathways.

Materials and methods

Construction of expression vectors and recombinant proteins

Human RASSF6, rat RASSF6, and mouse Nore1 cDNAs were obtained from human, rat, and mouse lung libraries (BioChain Institute Inc.) by PCR using the primer sets (5′-caattgactatgatggctcaccagt-3′ and 5′-gtcgactaaactgttgtctctgtttt-3′), (5′-caattgacaacgatggatcaccgg-3′ and 5′-gtcgactagactgtggtctccgtttt-3′), and (5′-gaattcgcttccccggccatcgggca-3′ and 5′-gtcgactaccccggcttccctggga-3′), respectively. The sequence of the obtained RASSF6 was confirmed to correspond to that of the gene deposited as AY217664.1 (RASSF6a) in the GenBank. The sequence was extended to the 5′ side through PCR using synthetic oligonucleotides as sense primers (5′-tcctcagaccatctcaaaaaggaggaaaagatgactatgatggctcacca-3′ for the first PCR and 5′-ctcgagcccagctgagccatgctctgggaggagacaggcgccgcccctgcgcccgcgcgggcctcggacctcccctacaggatatcctcagaccatctcaaaaaggag-3′ for the second PCR) to obtain the gene AK126346.1 (RASSF6b). The pMal C2 vector (New England Biolabs) was used to produce maltose-binding protein (MBP). The pCIneoFLAG-His6 vector was generated by ligation of a linker (5′-ctagccggggccatggactacaaagacgatgacgacaagg-3′ and 5′-aattccttgtcgtcatcgtctttgtagtccatggcccgg-3′) into the *Nhe*I/*Eco*RI sites of pCIneo (Promega) followed by insertion of an additional linker (5′-aattgcaccatcatcatcatcatcatgaattcacgcgt-3′ and 5′-ctagacgcgtgaattcatgatgatgatgatggtgc-3′) into the *Eco*RI/*Xba*I sites. The pCIneoFLAG-His6-FLAG vector was generated by ligation of a linker (5′-aattgactacaaagacgatgacgacaaggaattcacgcgt-3′ and 5′-ctagacgcgtgaattccttgtcgtcatcgtctttgtagtc-3′) into the *Eco*RI/*Xba*I sites of pCIneoFLAG-His6. pCIneoMyc, pBudCE4.2, and pBudC green fluorescent protein (GFP) vectors were described previously [14,15]. pLGFPC2 vector, which was previously described, was digested by *Nhe*I and *Mlu*I and ligated into the same sites of pCIneo to generate pClneoGFP vector [16]. Expression vectors harboring the Ras proteins Ki-Ras, Ha-Ras, N-Ras, M-Ras, and TC21 were donated by Y. Takai and M. Matsuda of Osaka University. pBudCGFP Ligand of Numb-protein X 1 (LNX1) was described [15]. The proteins generated from the RASSF6 and Nore1 expression constructs contain the following amino acid regions of their respective RASSF products; pCIneoFLAG-His6, pCIneoMyc, pBudCGFP, and pMal RASSF6, 2-337 (AY217664.1); pCIneoFLAG-His6-FLAG and pCIneoMyc RASSF6b, 2-369 (AK126346.1); pCIneoMyc rat RASSF6, 2-341 (NP_001020842); pCIneoGFP, pClneoFLAG-His6, and pMal Nore1, 2-413 (AAH89605.1). *Nhe*I/*Sal*I fragment from pCIneoFLAG-His6-RASSF6 was ligated into *Xba*I/*Sal*I sites of pBudCGFP to generate pBudCGFP-FLAG-RASSF6. *Nco*I/*Not*I fragment from pCIneoFLAG-His6 RASSF6 was ligated into the same sites of pET32a (Novagen) to generate pET32a-His6-FLAG-His6 RASSF6. MBP-RASSF6 and Nore1 and His6-FLAG-tagged RASSF6 were isolated from *Escherichia coli* transformed by pMal RASSF6, pMal Nore1, and pET32a-His6-FLAG-His6 RASSF6 using amylose resin (New England BioLabs) and Ni-NTA column (Qiagen). pLN-FLAG JAM4 used in the previous study was digested with *Bgl*II/*Not*I and the resulting fragment was ligated into *Bgl*II/*Not*I sites of pBudCE2 to generate pBudCE-FLAG JAM4 [17]. Human neuroepithelial cell transforming gene 1 (NET1) (BC010285.1) was purchased from Open Biosystems and used to generate pCIneoFLAG NET1, which encodes the amino acids 2-542. pCIneoFLAG MAGI-1 covers the amino acids 2-1256 (AB010894.1) of human membrane-associated guanylate kinase inverted-1.

Antibodies and chemical reagents

Rabbits were immunized by MBP-RASSF6. The mouse monoclonal anti-Myc 9E10 antibody was obtained from the American Type Culture Collection. Additional antibodies and reagents used in this study were obtained from commercial sources: mouse monoclonal and rabbit polyclonal anti-FLAG, mouse monoclonal anti-Bax (clone 6A7) and rabbit polyclonal anti-EndoG (Sigma-Aldrich); rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling Technology); goat polyclonal anti-AIF (D-20) and mouse monoclonal anti-GFP (Santa Cruz Biotechnology); mouse monoclonal anti-Ras (BD Transduction); fluorescein-isothiocyanate (FITC)- and rhodamine-conjugated secondary antibodies (Chemicon International Inc.); cycloheximide (Sigma-Aldrich); TNFα (Peprotech); and Z-VAD-FMK (Calbiochem).

RT-PCR

Messenger RNA was purified from various human cells using QuickPrep™ micro mRNA purification kit (GE HealthCare Bio-sciences). Reverse transcription was performed using ImProm-II reverse transcription system (Promega) with oligo dT_{15} primer. PCR primers for RASSF6, RASSF1, and Nore1 are; RASSF6, 5′-acgtcttctccagcaaagga-3′ and 5′-cagagctgcttcactcatgg-3′; RASSF1, 5′-acaagggcacgtgaagtcat-3′ and 5′-ccttcaggacaaagctcagg-3′; and Nore1, 5′-gacagctacaacacgcgaga-3′ and 5′-aggggcaggtagaaggatgt-3′. The primers for RASSF1 and Nore1 are designed in the RA domains, using the software named Primer 3, so that all splicing variants with the RA domain should be detected [18]. Cycling parameters involved