A Novel Single Amino Acid Deletion Caspase-8 Mutant in Cancer Cells That Lost Proapoptotic Activity*

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Caspase-8 is an important initiation caspase that activates the caspase cascade during death receptor-mediated apoptosis. We here report a novel caspase-8 mutant with a naturally occurring deletion of leucine 62 (ΔLeu62casp-8). ΔLeu62casp-8 has a shorter half-life than its wild-type counterpart. Unlike wild-type caspase-8, ΔLeu62casp-8 failed to interact with wild-type caspase-8 or with the adaptor protein FADD. ΔLeu62casp-8 lost its proapoptotic activity in mammalian cells. The leucine 62 therefore is critical for caspase-8 function, and the mutation may be one of the mechanisms through which some types of cancer cells escape from programmed cell death.

Caspases are a key component of the apoptotic machinery of cells, participating in an enzymatic cascade that results in cellular disassembly. Two major pathways of caspase activation during apoptosis have been described (1–3). The extrinsic pathway is involved in apoptosis mediated primarily by tumor necrosis factor (TNF)† family death receptors, such as TNF receptor-1 (TNFR1), Fas, or receptors for TNF-related apoptosis-inducing ligand (TRAIL). Upon activation of the death receptors (DR) the adaptor molecule Fas-associated death domain (FADD)/Mort-1 is recruited to the receptors (4, 5), allowing subsequent binding of caspase-8 to form the death-inducing signal complex (DISC) (6, 7). After caspase-8 is activated, it can process effector caspases, thereby activating a caspase cascade (8–12). In the intrinsic pathway, diverse proapoptotic signals converge at the mitochondrial level, provoking the translocation of cytochrome c from the mitochondria to the cytoplasm (13, 14). Cytochrome c then binds to the apoptotic protease-activating factor-1 (Apaf-1) (15), which permits recruitment of procaspase-9. The complex of cytochrome c, Apaf-1, and caspase-9 is a critical activator of the effector caspases in the mitochondrial pathway (16).

Recent studies have made substantial progress in delineating the signal transduction pathways that couple the initiation caspases to downstream cellular effects. Increasing evidence suggests that defects in these pathways may cause disease (1). Cancer cells may become resistant to death receptor-induced apoptosis because of the expression of decoy receptors, inactivation of death receptors, or loss of their distal signaling molecules. For example, homozygous deletion of the death receptor DR4 gene may lead to TRAIL resistance (17). Mutation in Fas was found in T-lineage acute leukemia (18, 19). Caspase-8 gene silencing due to DNA methylation was found in childhood rhabdomyosarcoma (20). Activation of survival signaling pathways, such as up-regulation of NF-κB, FLIP protein, or members of the IAP family, could also confer on tumor cells resistance to death receptor-mediated apoptosis (21). In this article, we report a novel, naturally occurring caspase-8-defective mutant with a single amino acid deletion of leucine 62 (ΔLeu62casp-8) that we discovered in A431 human vulvar squamous carcinoma cells. Functional analysis indicates that ΔLeu62casp-8 lost its proapoptotic activity when overexpressed in mammalian cells, suggesting that the leucine 62 is critical for caspase-8 function and that the deletion of the amino acid may play a role in tumorigenesis.

EXPERIMENTAL PROCEDURES

Transfection of Cells with Expression Vectors—Cell transfection was performed with the FuGENE™-6 transfection kit (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer’s instructions.

Apolipoprotein Measurements—An apoptosis ELISA kit (Roche) for quantitative measurement of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) and the Hoechst 33342 (Molecular Probes, Inc., Eugene, OR) for staining apoptotic cell nuclei were previously reported (22, 23).

Immunoprecipitation and Immunoblotting—Cells were scraped off from culture dishes with a rubber scraper and lysed in a Nonident P-40 lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin). Protein concentrations were measured by using the Coomassie Plus protein assay reagent (Pierce Chemical Co.). Equal amounts of cell extracts were used for immunoprecipitation and immunoblotting analysis as we previously described (22, 23).

RNA Extraction, cDNA Synthesis, RT-PCR, and cDNA Subcloning—Total RNA from the cell lines was extracted using a modified chloroform/phenol procedure (TRIZOL™, Invitrogen, Carlsbad, CA). First strand of cDNA was generated using reverse transcriptase (RTase) (Roche) following the manufacturer’s protocol, and subsequently amplified by PCR using the Expand™ High Fidelity PCR System (Roche) and the following primer sets: caspase-8 forward primer, 5′-CGGATCCGCACCATGATCTCAGCAAATC-3′ and the reverse primer, 5′-TCCCGGGGACCATGATTAGGTTACG-3′. The semiquantitative PCR control transcript GAPDH was amplified by the specific primers provided by CLONTECH Laboratories, Inc. (Palo Alto, CA). Amplified fragments were confirmed by DNA sequencing and subcloned into the pcDNA3.1-His C version plasmid (Invitrogen).

Northern Blot Analysis—Total RNA was prepared as described above. 10 μg of total RNA was loaded into each well, electrophoretically...
substrate PARP at 37°C was added for 15 min followed by incubating with the putative protein. To examine the activities of the products following GrzB processing, the products were analyzed by SDS-PAGE and visualized with autoradiography. The immunoprecipitates were separated by SDS-PAGE and followed by immunoblotting analysis with specific antibodies against caspase-8 or FADD.

In Vitro Translation and Binding Assay and GST Pull-Down Assay—The pcDNA3.1-Casp8, pcDNA3.1-ΔLeu62Casp8, and pcDNA3.1-FADD were in vitro transcribed and translated in the presence of [35S]methionine using the TNT kit from Promega Corp. (Madison, WI). 5 μl of the in vitro translated products were incubated with 5 μg of GST, GST-DED, or GST-ΔDED fusion proteins, respectively, in 500 μl of GST binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Tween 20) at 4°C overnight. The GST and GST fusion proteins were collected by glutathione-Sepharose beads. After extensively washing with 500 μl of buffer, the recovered proteins were resolved by SDS-PAGE and followed by immunoblotting analysis with specific antibodies against caspase-8 and FADD.

In Vitro Processing and Activation of Caspase-8 by Granzyme B—In vitro translated, [35S]-labeled caspase-8 and ΔLeu62Casp8 proteins were incubated with or without recombinant granzyme B (GraB) in a reaction buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 0.1 M NaCl, 10% sucrose, and 10 mM dithiothreitol) at 37°C for 4 h, and the reaction products were analyzed by SDS-PAGE and visualized with autoradiography. To examine the activities of the products following GraB processing, a specific GraB inhibitor (Enzyme System Products, Livermore, CA) was added for 15 min following incubation with the putative substrate PARP at 37°C for another 2 h. The reaction mixture were resolved by SDS-PAGE and followed by immunoblotting analysis with specific antibody against PARP (CHUL Research Center, Laval University, Quebec).

RESULTS AND DISCUSSION

In contrast to many other cell lines, the A431 human vulvar squamous carcinoma cells are insensitive to TRAIL-mediated cytotoxic effect. Fig. 1A shows the result of treating a panel of six tumor cell lines with TRAIL. Exposure of the cell lines to TRAIL induced apoptosis, as measured by an apoptosis ELISA, except in A431 cells. TRAIL is known to induce apoptosis in a caspase-8-dependent manner (25, 26). Western blot analysis indicated that A431 cells had an extremely low level of caspase-8 protein (Fig. 1B). The expression of caspase-8 in A431 cells could not be attributed to caspase-8 gene methylation, which was found in neuroblastomas (20), because treatment of A431 cells with the DNA demethylation agent 5-aza-2′-deoxycytidine failed to activate the expression (data not shown). Northern blot analysis indicated that A431 cells expressed a considerable level of caspase-8 mRNA, compared with the other five cell lines (Fig. 1C). This result further suggested that the low expression level of caspase-8 observed in the A431 cells could not be attributed to changes at the transcription level. We further examined the effects on these cells of two additional caspase-8-dependent apoptosis inducers (the anti-Fas agonistic antibody CH-11 and TNFα), and two caspase-8-independent chemotherapeutic agents (paclitaxel (Bristol-Myers Squibb Company, Princeton, NJ) and cisplatin). A431 cells expressed detectable levels of Fas and TNFR1 (data not shown); however, these cells were resistant to CH-11 or TNFα, but not to paclitaxel or cisplatin (Fig. 1D), indicating the presence of a defective caspase-8-initiated apoptotic pathway in A431 cells.

To explore the underlying mechanism of low expression and low activity level of caspase-8 in A431 cells, we used a semi-
quantitative RT-PCR to amplify the coding sequence of caspase-8 from A431 cells. H460 non-small cell lung carcinoma and MDA468 breast cancer cells served as controls (Fig. 2A).

Consistent with the Northern blot data, the PCR products from all three cell lines each displayed a single band with similar size and intensity. However, sequencing of the PCR products indicated that there was an in-frame deletion of three consecutive base pairs (coding for leucine 62) found in A431 cells, compared with the published sequence of caspase-8 in GenBankTM (Fig. 2B). Caspase-8 is composed of two DEDs at the amino terminus and an ICE-like activity domain at the carboxyl terminus (6, 7). The missing leucine 62 is located in the first DED of caspase-8. This novel caspase-8 mutant is hereafter termed ΔLeu62casp-8. When transiently transfected into MCF7 breast cancer cells, ΔLeu62casp-8 was expressed and even showed a slightly higher expression level than wild-type caspase-8 in our well controlled and repeated experiments (Fig. 2C).

However, a pulse-chase experiment with [35S]methionine metabolically labeled wild-type caspase-8 and ΔLeu62casp-8 indicated that, regardless of its relatively high protein level, ΔLeu62casp-8 had a faster degradation rate, with a half-life of <30 min compared with >1 h for wild-type caspase-8 (Fig. 2D), which may contribute to the low level of caspase-8 (ΔLeu62casp-8) in A431 cells.

Caspase-8 is proteolytically activated by oligomerization following its recruitment to the FADD molecule upon death receptor activation (4–7). Overexpression of caspase-8 alone was sufficient to induce caspase-8 oligomerization and autoactivation, triggering the caspase-8-initiated cell death pathway (7). We therefore hypothesized that the relative lower level of transfected wild-type caspase-8 could be due to an autoactivation-related autocleavage, which might not be the case for transfected ΔLeu62casp-8. To address this hypothesis, we examined the capability of ΔLeu62casp-8 to undergo oligomerization and interact with FADD. GST-fused wild-type caspase-8 DED domain (GST-DED) and ΔLeu62casp-8 DED domain (GST-ΔDED) (Fig. 3A) were used, respectively, for in vitro binding assays with in vitro translated FADD, wild-type caspase-8, or ΔLeu62casp-8.
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caspase-8, or ΔLeu62casp-8 (Fig. 3B). An interaction was found between FADD and the wild-type caspase-8 DED but not between FADD and the ΔLeu62casp-8 DED (Fig. 3C). Furthermore, wild-type caspase-8 interacted with the caspase-8 DED but not with the ΔLeu62casp-8 DED, and the ΔLeu62casp-8 DED failed to interact with either wild-type caspase-8 or itself (Fig. 3D). To further confirm these in vitro results, the protein-protein interaction assays were repeated with the caspase-8 or ΔLeu62casp-8 protein were mixed with 30 μM of GrB-specific inhibitor (Enzyme System Products) for 15 min prior to a 2-h incubation with 200 ng of recombinant PARP. The reaction products were resolved by SDS-PAGE, followed by autoradiography.

To determine whether ΔLeu62casp-8 retained its enzymatic activity, in vitro translated wild-type caspase-8 and ΔLeu62casp-8 were incubated with recombinant granzyme B, a serine protease that activates caspase-8 by proteolytic processing (7). Both wild-type caspase-8 and ΔLeu62casp-8 were efficiently processed by granzyme B (Fig. 3E). The granzyme B-processed wild-type caspase-8 and ΔLeu62casp-8 showed equal activities in cleaving PARP (Fig. 3F). To block any potential direct effect of granzyme B on PARP, in this assay, a selective inhibitor of granzyme B was used before PARP was incubated with granzyme B-processed caspase-8 or ΔLeu62casp-8. The results showed that although ΔLeu62casp-8 was defective in protein-protein interaction, it could still be processed by granzyme B and was enzymatically active once processed.

Caspase-8 can trigger apoptosis when overexpressed, presumably through proximity-induced autoactivation (6, 7, 27). To further investigate whether ΔLeu62casp-8 remained proapoptotic, wild-type caspase-8 and ΔLeu62casp-8 were tran-
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A431 and MCF7 cells were individually transiently transfected with pcDNA3.1 control vector, or the vectors containing wild-type caspase-8, or ΔLeu62casp-8 using the FuGENE™-6 reagent for 20 h, followed by cell lysis for the detection of wild-type caspase-8, Δcaspase-8 and β-actin expressions by Western blot analysis (A), and for quantification of apoptosis by ELISA (B). C, induction of nuclear condensation by wild-type caspase-8 but not by ΔLeu62casp-8. MCF7 cells were transiently transfected with vectors described in A and B, the cells were then stained with 1 μg/ml Hoechst 33342 in phosphate-buffered saline for 5 min at 37 °C and then observed and photographed under a fluorescent microscope (arrow, condensed nuclei).

Fig. 4. Loss of proapoptotic activity of ΔLeu62casp-8. A and B, induction of apoptosis by wild-type caspase-8 but not by ΔLeu62casp-8. A431 and MCF7 cells were individually transiently transfected with pcDNA3.1 control vector, or the vectors containing wild-type caspase-8, or ΔLeu62casp-8 using the FuGENE™-6 reagent for 20 h, followed by cell lysis for the detection of wild-type caspase-8, Δcaspase-8 and β-actin expressions by Western blot analysis (A), and for quantification of apoptosis by ELISA (B). C, induction of nuclear condensation by wild-type caspase-8 but not by ΔLeu62casp-8. MCF7 cells were transiently transfected with vectors described in A and B, the cells were then stained with 1 μg/ml Hoechst 33342 in phosphate-buffered saline for 5 min at 37 °C and then observed and photographed under a fluorescent microscope (arrow, condensed nuclei).
here that deletion of the leucine 62 in caspase-8 (ΔLeu62casp-8) dramatically altered the proapoptotic function of caspase-8. Our findings are significant. From the mechanistic point of view, the results indicate that leucine 62 located in the first DED of caspase-8 plays a critical role for caspase-8 oligomerization and for its interaction with FADD for triggering the caspase cascade. From the cancer biology point of view, our observation, along with several other types of reported caspase-8 genetic changes in cancer cells, including gene deletion, methylation, and point mutation, suggests a possible mechanism by which some cancer cells may escape this genetically programmed, caspase-8-initiated cell death. Restoration of the caspase-8-mediated cell death pathway may be therefore a rational strategy for cancer therapeutics.

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