Regulation of Apoptotic Protease Activating Factor-1 Oligomerization and Apoptosis by the WD-40 Repeat Region*

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Apoptotic protease activating factor-1 (Apaf-1) has been identified as a proximal activator of caspase-9 in cell death pathways that trigger mitochondrial damage and cytochrome c release. The mechanism of Apaf-1 action is unclear but has been proposed to involve the clustering of caspase-9 molecules, thereby facilitating autoprocessing of adjacent zymogens. Here we show that Apaf-1 can dimerize via the CED-4 homologous and linker domains of the molecule providing a means by which Apaf-1 can promote the clustering of caspase-9 and facilitate its activation. Apaf-1 dimerization was repressed by the C-terminal half of the molecule, which contains multiple WD-40 repeats, but this repression was overcome in the presence of cytochrome c and dATP. Removal of the WD-40 repeat region resulted in a constitutively active Apaf-1 that exhibited greater cytotoxicity in transient transfection assays when compared with full-length Apaf-1. These data suggest a mechanism for Apaf-1 function and reveal an important regulatory role for the WD-40 repeat region.

Apoptosis is an important homeostatic control mechanism for regulating cell numbers in multicellular organisms (1). The molecular machinery that drives the apoptosis program consists of a family of cysteine proteases, the caspases, that cleave their substrates after aspartic acid residues and are normally present in cells as inactive precursors (2–4). Active caspases can typically process their own precursor forms as well as those of other caspases (5–7).

Because of the potential for explosive amplification of multiple caspases by a small initial pool of active caspase, proximal caspase activation events appear to be tightly regulated. An attractive model for activation of initiator caspases is the induced proximity model where molecules that promote close association of caspases facilitate caspase activation by enabling clustered caspases to process one another in trans (8–11). Bi-partite molecules such as FADD and RAIDD that can become recruited to membrane receptors, in addition to binding caspases, can promote caspase clustering and activation upon oligomerization of their associated receptors (12–14).

Apaf-1, a human homologue of the Caenorhabditis elegans CED-4 protein, has been demonstrated to play a critical role in initiating a cascade of caspase activation events in response to stimuli that provoke the release of cytochrome c from the mitochondrial intermembrane space (15–18). Accumulating evidence suggests that mitochondrial damage, accompanied by release of cytochrome c, plays an important role in many forms of apoptosis (19–24). Targeted inactivation of either Apaf-1 or CASP-9 in the mouse results in many extra cells in several tissues, most notably the brain, and results in embryonic lethality (25, 26). Cytochrome c has been shown to bind to Apaf-1, thereby promoting Apaf-1-mediated activation of caspase-9 by an unknown mechanism (15, 17). By analogy with other proximal caspase activation events it is possible that Apaf-1 promotes caspase-9 activation by promoting clustering of this caspase (17). In support of this model, it has been demonstrated recently that the C. elegans homologue of Apaf-1, CED-4, promotes CED-3 activation through oligomerization (27).

Here we show that Apaf-1 can form homodimers via the CED-4 homologous and adjacent linker domains of this molecule. Significantly, Apaf-1 dimerization was strongly repressed by its own C terminus, a region that contains multiple WD-40 repeats. This repression was overcome in the presence of cytochrome c and dATP, suggesting that cytochrome c and dATP facilitate Apaf-1-mediated caspase-9 activation by regulating Apaf-1 dimerization.

EXPERIMENTAL PROCEDURES

Plasmids—Full-length human Apaf-1 cDNA was kindly provided by Dr. Xiaodong Wang. All other constructs described in this paper were generated by polymerase chain reaction-mediated amplification of the relevant coding sequences using full-length Apaf-1 cDNA as a template followed by the insertion of the digested polymerase chain reaction products into either pACT2 (CLONTECH), pAS2–1 (CLONTECH), pcDNA3 (Invitrogen), or pBluescript II SK– (Stratagene). Constructs were verified for authenticity by automated sequencing on an ABI 310 (Applied Biosystems).

Expression of GST Fusion Proteins—A GST-Apaf-11–601 fusion was constructed by polymerase chain reaction-mediated amplification of the relevant coding sequence from the full-length Apaf-1 cDNA followed by subcloning of the resulting polymerase chain reaction product in-frame with the GST coding region of pGEXTK2 (Amersham Pharmacia Biotech). Plasmids encoding GST and GST fusion proteins were transformed into Escherichia coli DH5α, and bacteria were induced to express recombinant proteins in the presence of 100 μM isopropyl-1-thio-

β-D-galactopyranoside for 2–4 h at 30 °C. Recombinant proteins were subsequently purified using glutathione-Sepharose (Amersham Pharmacia Biotech) according to standard procedures.

Yeast Transformation—Saccharomyces cerevisiae strain Y190 (CLONTECH) was transformed by the lithium acetate method as described by Gietz et al. (28). Briefly, a colony of Y190 was inoculated into

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1 The abbreviations used are: Apaf-1, apoptotic protease activating factor-1; AD, activation domain; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
Yeast transformants were prepared as follows. Yeast transformants expressing the appropriate plasmids were expanded in 50 ml of YPD growth medium to an OD₆₀₀ of 0.4 ± 0.1. Cells were harvested and washed once in ice-cold water followed by pelleting. Pelleted yeast were snap frozen in liquid nitrogen followed by resuspension in ~ 250 μl of cracking buffer prewarmed to 60 °C (35 mM Tris-HCl, pH 6.8, 90 mM EDTA, 7 mM urea, 4.5% SDS, 0.35 mg/ml bromphenol blue, 125 mM 2-mercaptoethanol) supplemented with protease inhibitors (6 μg/ml pepstatin A, 2 μg/ml leupeptin, 9 mM benzamidine, 20 μg/ml aprotinin, 4.5 mM phenylmethylsulfonyl fluoride). Samples were then normalized with respect to cell density by the addition of further cracking buffer. Samples were then brought to 70 °C for 10 min followed by vigorous vortexing for 1 min in the presence of 300 mg of acid-washed glass beads (Sigma). Protein lysates were centrifuged at 22,000 × g for 5 min at 4 °C to pellet beads and intact cells.

Proteins were subjected to standard SDS-polyacrylamide gel electrophoresis at 60–70 V and were transferred onto 0.45 μm polyvinylidene difluoride membranes (Costar, United Kingdom) for 3 h at 50–75 mA. Antihistidine monoclonal, antileucine, and antityrosine were detected using a Gal4-based two-hybrid assay (29), utilizing one of the following independent reporter assays: the ability to grow on medium lacking histidine (Y190 strain). Fusion proteins in yeast (Y190 strain). Fusion proteins were detected by Western blot as described under “Experimental Procedures.” The blot is overexposed to facilitate detection of the Gal4AD fusion protein (lane 6).

**Regulation of Apaf-1 Dimerization**

**A** Apaf-1

**B** Apaf-1 Gal4 activation domain hybrids

**C** Activation domain hybrids

1. Gal4AD
2. Gal4AD-Apaf-1 CED-3
3. Gal4AD-Apaf-1 CED-4
4. Gal4AD-Apaf-1 CED-4 linker
5. Gal4AD-Apaf-1 linker
6. Gal4AD-Apaf-1 ACED-3
7. Gal4AD-Apaf-1 ADW-40

**Fig. 1.** Schematic representation of human Apaf-1 and the various deletion mutants generated in this study. A, structure of human Apaf-1 protein. Caspase recruitment domain, CED-4 homologous, linker, and WD-40 repeat regions are indicated. Numbers represent amino acid residues. B, schematic representation of Gal4-Apaf-1 fusions that were generated for the purposes of this study. C, expression of Gal4-Apaf-1 AD fusion proteins in yeast (Y190 strain). Fusion proteins were detected by Western blot as described under “Experimental Procedures.” The blot is overexposed to facilitate detection of the Gal4AD fusion protein (lane 6).
Transient Transfections—MCF-7 cells were transiently transfected with pCDNA3-based Apaf-1 or FADD expression plasmids in conjunction with the pCMV
gal reporter construct (CLONTECH) using the Fugene-6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Transfected cells were incubated for 24–48 h followed by fixation in paraformaldehyde fixative (2% paraformaldehyde, 0.1% gluteraldehyde in phosphate-buffered saline, pH 7.2) at 4 °C for 5 min. Fixed cells were washed with phosphate-buffered saline, pH 7.2, followed by staining for β-galactosidase reporter gene expression by the addition of β-galactosidase staining buffer (phosphate-buffered saline, pH 7.2, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.02% Nonidet P-40, 0.01% SDS) containing 1 mg/ml X-gal. Plates were incubated at 37 °C for 30 min to 2 h to allow blue color development.

RESULTS

Apaf-1 Dimerization in Yeast Is Regulated by the WD-40 Repeat Region—Preliminary investigations using co-immuno-precipitation assays have suggested that Apaf-1 can self-associate either directly or via putative adaptor proteins (17, 27). To discriminate between these possibilities, we used a Gal4-based yeast two-hybrid interaction trap assay to ask whether Apaf-1 can dimerize and to map the region(s) involved. We generated a series of Apaf-1 deletion mutants fused to the Gal4 AD and co-transformed these into yeast in combination with similar Gal4 DNA-binding domain Apaf-1 deletion mutants (Fig. 1, A and B). Expression of Apaf-1 deletion mutants in yeast was confirmed by Western blotting (Fig. 1C).

Using two different reporter assays (the ability to grow in the absence of histidine and β-galactosidase reporter gene expression) Apaf-1 dimerization, mediated via the CED-4 and linker domains (amino acid residues 92–601), was readily detected (Fig. 2). Both the CED-4 homologous and linker domains were required for maximal interaction, although the CED-4 homologous domain alone (residues 92–412) appeared to be capable

Fig. 2. Apaf-1 dimerization in yeast requires the CED-4 homologous and linker domains and is regulated by the WD-40 repeat region. A, Y190 cells were co-transformed with a pAS2–1 bait plasmid encoding the CED-4 and linker regions of Apaf-1 (amino acid residues 92–412) along with either empty pACT2 vector (None) or pACT2 encoding the indicated Gal4AD-Apaf-1 fusions. Co-transformants were grown on Leu- and Trp-selective medium for 5–7 days in the presence (His+) or absence (His−) of histidine as described under “Experimental Procedures.” Growth in the absence of histidine indicates a positive interaction. B, plasmids encoding the indicated binding domain (BD) and AD fusions were co-transformed into Y190 cells and selected as described above. Co-transformants were assessed for their ability to grow in the absence of histidine or their ability to activate transcription of the β-galactosidase reporter gene, as indicated. The strength of interaction was assessed by colony size and abundance (in His reporter assay) or intensity and kinetics of blue color development (in β-galactosidase reporter assay). (+++, strong; ++, medium; +/−, weak; −, no interaction. ND, not determined. Results are representative of at least three independent experiments.

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Fig. 3. Apaf-1 dimerization in vitro. A, schematic representation of the 35S-labeled Apaf-1 deletion mutants that were used in the in vitro capture assay. Proteins were produced by in vitro transcription and translation as described under “Experimental Procedures.” B, left panel, purified GST or GST-Apaf-1ΔWD-40 fusion protein (amino acid residues 1–601) was assessed for their ability to capture the 35S-labeled Apaf-1 deletion mutants depicted in A. 35S-labeled caspase-9, a known binding partner of Apaf-1, was used as a positive control in these assays. Right panel, input amounts of all proteins were normalized by signal intensity, as indicated. Captured proteins were detected by SDS-polyacrylamide gel electrophoresis followed by fluorography. Results are representative of three independent experiments.
of mediating weak but significant association with the CED-4/ linker regions in both the growth and the β-galactosidase reporter assays (Fig. 2, A and B). Strikingly, Apaf-1 dimerization in yeast was strongly inhibited by the C-terminal portion of the molecule, which contains multiple WD-40 repeats (Fig. 2, A and B), suggesting that this region regulates Apaf-1 self-association.

Dimerization of Apaf-1 in Vitro—To confirm these observations, in vitro protein-protein interaction assays were performed using a GST-Apaf-1 ΔWD-40 fusion protein (amino acid residues 1–601) in conjunction with various 35S-labeled in vitro transcribed and translated Apaf-1 deletion mutants (Fig. 3A). These assays confirmed that Apaf-1 can self-associate via the CED-4 homologous and linker domains of the molecule and that the CED-4 homologous domain alone, in agreement with the two-hybrid data, was far less efficient in promoting dimerization (Fig. 3A). Interestingly, caspase-9 was found to be more efficiently captured in the pulldown assays relative to the Apaf-1 deletion mutants, suggesting that the caspase-9/Apaf-1 interaction is of a higher affinity than the Apaf-1/Apaf-1 interaction (Fig. 3B).

As expected from the yeast two-hybrid data, GST-Apaf-1 ΔWD-40 very weakly bound full-length Apaf-1 (data not shown) or a deletion mutant lacking the CED-3 homologous region (Fig. 4), again suggesting that the WD-40 repeat region prevents Apaf-1 self-association. Significantly, the addition of cytochrome c/dATP to the in vitro interaction assay overcame this inhibition and restored dimerization (Fig. 4B). This suggests that cytochrome c activates the intrinsic caspase-activating properties of Apaf-1 by overcoming the inhibition exerted by the WD-40 repeat region, which is likely by binding to this region and inducing a conformational change that permits Apaf-1 aggregation.

Removal of the WD-40 Repeat Region from Apaf-1 Enhances the Pro-apoptotic Properties of this Molecule—Apaf-1 has previously been reported to be a relatively poor promoter of apoptosis when transiently overexpressed in mammalian cells (15, 31), presumably because cytochrome c is required to unlock the caspase-activating properties of this molecule. A logical extension of our observations with regard to Apaf-1 dimerization would predict that the removal of the C-terminal WD-40 repeat region (amino acid residues 602–1194) should generate a constitutively active Apaf-1 that would promote apoptosis in the absence of cytosolic cytochrome c. To test this possibility we transiently transfected MCF-7 cells with either full-length Apaf-1 or an Apaf-1 deletion mutant lacking the WD-40 repeat region.
We have provided evidence that the WD-40 repeat region of Apaf-1 negatively regulates Apaf-1 self-association. Clearly, further studies are required to determine the mechanism of this inhibition. Possibilities include long range effects on the CED-4 homologous and linker domains that alter their configuration and disrupt the dimerization surface; alternatively, the WD-40 repeat region may physically occlude the binding sites. Whatever the mechanism, our data suggest that this block to self-association is overcome in the presence of cytochrome c, suggesting that cytochrome c may bind to the WD-40 repeat region and alter its conformation. It is possible that other WD-40 repeat-binding proteins may be capable of performing a similar role in cytochrome c in certain situations. In this context, it is interesting that Apaf-1 contains a number of caspase consensus cleavage sites close to the junction between the linker and WD-40 repeat regions, raising the possibility that caspase-catalyzed removal of the WD-40 repeat region may also activate Apaf-1.

During the preparation of this report, Nunez and colleagues (35) also reported data to suggest that the WD-40 repeat region regulates Apaf-1 self-association. These authors showed that overexpression of an Apaf-1 deletion mutant spanning part of the linker region in addition to the WD-40 repeat region (amino acid residues 468–1194) could block co-immunoprecipitation of epitope-tagged Apaf-1 deletion mutants lacking the WD-40 repeat region (amino acids I–559). In the present study, we have directly demonstrated the ability of the WD-40 repeat region to regulate Apaf-1 self-association, both in yeast as well as in vitro, and have extended these findings by showing that cytochrome c/dATP can overcome this repression. There is also very good agreement between our studies with respect to the region of Apaf-1 involved in promoting self-association.

Targeted inactivation of Apaf-1 in mice results in profound developmental abnormalities in cell number regulation in the brain as well as in other tissues such as the peripheral nervous system, resulting in embryonic lethality (25, 26). These data strongly implicate Apaf-1 as an important player in pathways that result in neuronal apoptosis during development. Although it is unclear whether Apaf-1 plays a similar role in regulating neuronal apoptosis in nondevelopmental settings, it is likely to be involved in at least a subset of these cell deaths. Thus, strategies aimed at disrupting Apaf-1 dimerization may be capable of attenuating cell loss in neurodegenerative disease. Such strategies may involve exploiting natural regulators of Apaf-1 function (such as Bcl-XL or Diva) or small molecule inhibitors targeted to the domains required for efficient Apaf-1 self-association. Further studies will explore these possibilities.

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