The casp9 protein plays a critical role in apoptosis induced by a variety of death stimuli. A regulator of apoptosis, Apaf-1, binds to and activates pro-casp9 in the presence of cytochrome c and dATP, a requirement that is bypassed by deletion of the WD-40 repeats located in the C-terminal half of Apaf-1. In this report, we used constitutively active Apaf-1 mutant lacking the WD-40 repeat region to study the mechanism and regulation of pro-casp9 activation. Mutational analysis revealed that only a small portion of the CED-4 homologous region (residues 456–559) could be deleted without destroying the ability of Apaf-1-(1–559) to activate pro-casp9. Apaf-1 can self-associate to form oligomers. Disruption of Apaf-1 self-association by deletion (∆109–559) or mutation of the P-loop region (K149R) abrogated Apaf-1-mediated pro-casp9 activation. Forced oligomerization of the caspase recruitment domain of Apaf-1 was sufficient for pro-casp9 activation. Dimerization of chimeric Fpk-pro-casp9 protein with the dimerizer drug FK1012 induced pro-casp9 processing and apoptosis in cells. Significantly, the C-terminal region containing WD-40 repeats interacted with its N-terminal CED-4 homologous region, as determined by immunoprecipitation experiments. Importantly, expression of the WD-40 repeat region inhibited Apaf-1 self-association and proteolytic activation of pro-casp9. These studies provide a mechanism by which Apaf-1 promotes autoactivation of pro-casp9 through Apaf-1 self-association, a process that is negatively regulated by the WD-40 repeats.

Programmed cell death, or apoptosis, an evolutionarily conserved and genetically regulated biological process, plays a critical role in development and tissue homeostasis of multicellular organisms (1–4). Dysfunction of this process contributes to the pathogenesis of several diseases in humans and mice (4–6). A family of cysteine proteases (designated caspases) related to Caenorhabditis elegans CED-3 appears to represent the executionary arm of the apoptotic program. Each caspase contains conserved residues important for specific proteolytic activity cleaving substrates after aspartate residues (7–9). Caspases are synthesized in cells as inactive zymogens and, upon stimulation with apoptotic signals, are processed into mature forms composed of a tetramer of two large and two small subunits (10, 11). The apoptotic process is characterized by sequential activation of multiple caspsases. Depending on their order of activation in the proteolytic cascade, caspsases can be divided into upstream and downstream caspsases. The casp9 protein is an upstream caspase that contains a caspase recruit domain (CARD) in its N terminus, a region that is also present in the prodomain of several death proteases including CED-3, casp1, and casp2 (12). Activation of casp9 initiates a protease cascade with subsequent activation of downstream casp3 and casp7, leading to cleavage of target proteins and execution of the apoptotic program (13).

Our understanding of how caspsases are activated in mammalian cells has been greatly enhanced by recent biochemical studies of the nematode proteins CED-3, CED-4, and CED-9 (14–19). CED-4 physically interacts with both CED-3 and CED-9, forming a multimeric protein complex (14–17). CED-4 promotes the activation of CED-3, and this activation process is inhibited by CED-9 (17–19). Apaf-1, a mammalian homolog of CED-4, has been recently identified (20). The N-terminal half of Apaf-1 shares extensive homology with CED-4 and contains a CARD followed by a putative ATPase domain with conserved Walker’s A and B motifs (20, 21). The C-terminal region of Apaf-1 lacks homology with CED-4 and is composed of 12 WD-40 repeats (20). In the presence of cytochrome c and dATP, Apaf-1 adopts a conformation that can bind to pro-casp9, an event that leads to its proteolytic activation (13). However, little is known about the mechanism by which Apaf-1 regulates the proteolysis of pro-casp9.

In the present studies, we sought to understand how Apaf-1 promotes the maturation of pro-casp9 into an active protease, and to establish a role for the WD-40 repeat region (WDR) in the regulation of Apaf-1. We show that Apaf-1 can self-associate and its self-association can promote the proteolytic maturation of pro-casp9. Similarly, we show that forced dimerization of pro-casp9 was sufficient for induction of pro-casp9 activation, suggesting that self-association of Apaf-1 promotes casp9 activation by bringing two or more pro-casp9 molecules into close proximity. We also show that WDR disrupts Apaf-1 self-association, providing a mechanism by which WDR negatively regulates Apaf-1-mediated casp9 activation.

MATERIALS AND METHODS

Plasmid Construction—The plasmids pcDNA3-Apaf-1-Myc, pcDNA3-Apaf-1-(1–559)-Myc, and pcDNA3-Apaf-1-(1–455)-Myc and pcDNA3-β-galactosidase were described previously (22). Apaf-1-(1–455)-Myc and Apaf-1-(1–328)-Myc were obtained by truncating Apaf-1 at EcoRV and ScaI, respectively. A PCR fragment containing amino acids 1–108 of Apaf-1 was cloned into pcDNA3-Myc to produce pcDNA3-Apaf-1-(1–

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108)-Myc. The P-loop mutants (K149R, K149T, or K149G) were generated by PCR mutagenesis and cloned into pcDNA3-Myc. pcDNA3-Apaf-1-(1–559)-FLAG and pcDNA3-Apaf-1-(468–1194)-HA were constructed by cloning the corresponding fragments into pcDNA3-FLAG or pcDNA3-HA, respectively. Fragments containing Apaf-1 mutants in pcDNA3 were excised and cloned into similarly digested pGKT2 or pACT2 to produce pGKT8-Apaf-1-(1–559), pGKT8-Apaf-1-(1–455), pGKT8-Apaf-1-(1–328), pGKT8-Apaf-1-(1–108), pGKT8-Apaf-1-(1–559KR), and pACT2-Apaf-1-(1–559). The coding region of casp9 was inserted into SalI-digested pSH/S-Fpk1-E (23) to generate pSH/S-Fpk1-casp9-E and pSH/S-Fpk1-casp9(Δ1–101)-E. To construct pcDNA3-Apaf-1-(1–101)-E, BamHI/SalI fragment from pSH/S.E.-Fpk3-E (23) was first inserted into pcDNA3-Myc, and then the BamHI/XhoI fragment from pcDNA3-Apaf-1-(1–108)-Myc was cloned into the resulting pcDNA3-Fpk3. Authenticity of all constructs made by PCR amplification was confirmed by sequencing.

Transfection, Immunoprecipitation, and Western blot analysis—Human embryonic kidney 293 or 293T cells (2–5 × 10⁶) were transfected with 5 μg each of the indicated plasmid DNA by the calcium phosphate method. Protein immunoprecipitation and Western blot analysis with relevant antibodies were performed as described (14). The proteins were detected by using an enhanced chemiluminescence system (Amersham Pharma Biotech).

Apoptosis assay—Mouse embryonic fibroblast (MEF) cells (1 × 10⁶) were seeded in each well of 12-well plates. After 24 h, cells were transiently transfected with 0.25 μg of the reporter pcDNA3-β-galactosidase plasmid plus the following plasmids: 0.2 μg each of pSH/S-Fpk1-E, pSH/S-Fpk1-casp9-E, or pSH/S-Fpk1-casp9(Δ1–101)-E. pcDNA3 was used to adjust total plasmid DNA to an equal amount. PK1012 (200 μt) was added for 4 h after transfection. Percentage of apoptotic cells was determined 16–18 h after transfection in triplicate cultures as described (24).

In vitro caspase-9 assay—293T cells were transfected with plasmid constructs producing Apaf-1 mutants and cytosolic extracts prepared as described (20). Caspase-9 assay was performed as described previously (22).

Yeast two-hybrid assay—Yeast transformation, growth assay, colony lift assay, and Western blotting analysis were performed as reported previously (25).

RESULTS

Ability of Apaf-1 Mutants to Promote Proteolytic Processing of Pro-casp9—We previously reported that Apaf-1-(1–559), an N-terminal mutant form of Apaf-1 lacking WDR, can activate pro-casp9 independently of cytochrome c and dATP (22). We took advantage of this previous observation to further assess the function of Apaf-1-(1–559) in mammalian cells, as the endogenous Apaf-1 protein requires cytochrome c and dATP for pro-casp9 activation. We constructed a series of deletion mutants that span residues 1–559 of Apaf-1, prepared 293T cytosolic extracts producing these mutants and determined their ability to promote processing of pro-casp9 in a cell-free system (Fig. 1A). A mutant with deletion of residues 456–559, Apaf-1-(1–455), was still capable of promoting pro-casp9 processing, although its activity was markedly reduced compared with Apaf-1-(1–559) (Fig. 1B). Two additional mutants, Apaf-1-(1–328) and Apaf-1-(1–108), failed to promote pro-casp9 processing in the cell-free assay, suggesting that the CED-4 homologous region of Apaf-1 is critical for promoting pro-casp9 activation (Fig. 1B). The region with homology to CED-4 (residues 97–412) includes a putative ATPase domain with conserved Walker A (P-loop) and B sequences. The diminished activity of mutant Apaf-1-(1–455) indicates that residues 456–559 are important but not required for proteolytic processing of pro-casp9. Another mutant, Apaf-1-(109–559), in which the CARD was deleted, failed to activate pro-casp9,2 indicating that the most N-terminal 1–108 amino acids of Apaf-1 are also critical for pro-casp9 activation. Protein blots confirmed that equivalent protein expression was obtained with each of the Apaf-1 constructs (Fig. 1C). To assess the requirement for the P-loop region (residues 139–157), we constructed a mutant in which the conserved Lys149 was substituted with Arg (1–559K149R). Mutation of Lys149 to Arg drastically reduced the ability of Apaf-1-(1–559) to promote proteolytic processing of pro-casp9 (Fig. 1B). These results were verified by analysis of two additional mutants in which Lys149 was substituted with Thr or Gly.3 These results indicate that the P-loop motif is critical for Apaf-1 to activate pro-casp9.

Dimerization of Pro-casp9 Is Sufficient for Proteolytic Activation and Apoptosis—To begin to determine the mechanism by which Apaf-1 promotes the proteolytic activation of pro-casp9, we determined whether Apaf-1-(1–559)-mediated activation of pro-casp9 requires an intrinsic protease activity of casp9. Both endogenous Apaf-1 and constitutively active Apaf-1-(1–559) mutant required casp9 activity for processing pro-casp9, as a catalytically inactive pro-casp9 mutant (C287S) did not produce the intermediate p35 proteolytic fragment (Fig. 2A). These results suggest that Apaf-1-dependent pro-casp9 processing is mediated by autocatalysis. Because proteolytic activation of casp9 has been suggested to occur through self-association (28–30), we tested whether pro-casp9 can be activated by a similar mechanism. We used anti-Myc antibody to induce dimerization of N-terminally Myc-tagged pro-casp9 in the cell-free system. Incubation with rabbit anti-Myc antibody induced the proteolytic processing of pro-casp9, whereas control rabbit anti-HA antibody did not (Fig. 2B), indicating that dimerization is sufficient for pro-casp9 processing. To determine if dimerization of pro-casp9 can induce apoptosis in cells,
WD-40 Repeats Regulate Apaf-1 Self-association

Self-association of Apaf-1 Can Induce Proteolytic Activation of Pro-casp9—Since Apaf-1-(1–559) can self-associate, it is possible that Apaf-1 induces autoactivation of pro-casp9 by bringing into close proximity two or more molecules of pro-casp9. Apaf-1-(1–108) is sufficient to interact with pro-casp9 (Ref. 13 and data not shown) but is unable to mediate activation of pro-casp9 (Fig. 1B). We therefore used Apaf-1-(1–108) to determine whether dimerization or self-association of its CARD can promote activation of pro-casp9 in the cell-free system. Dimerization of C-terminally Myc-tagged Apaf-1-(108) with rabbit anti-Myc antibody induced the processing of pro-casp9, which was slightly enhanced by goat anti-rabbit IgG (Fig. 4A). In control experiments, mock treatment or incubation with anti-HA failed to induce activation of pro-casp9 (Fig. 4A). We verified these results by engineering a chimeric Fpk-Apaf-1 expression construct to express Apaf-1-(1–108) fused to three tandem repeats of Fpk. Incubation of the lysates with the dimerization agent AP1510 induced proteolytic activation of pro-casp9 in the cell-free system (Fig. 4B). In control experiments, pro-casp9 did not undergo proteolytic activation in the absence of AP1510 or when AP1510 was added to cell extracts that express control Fpk protein (Fig. 4B). Therefore, self-association of Apaf-1-(1–108) can induce activation of pro-casp9, suggesting that proteolytic processing of pro-casp9 might be mediated by Apaf-1 self-association. The latter process could bring into close juxtaposition two or more pro-casp9 molecules and result in autoactivation. A similar mechanism has been proposed for the activation of casp1, casp3, and casp8 (23, 28–30).

WDR Interacts with the N-terminal CED-4 homologous Domain—Apaf-1 contains an extra C-terminal domain with 12 WD-40 repeats when compared with its C. elegans homolog CED-4 (20). Apaf-1 mutants with deletion of WDR are constitutively active and capable of processing pro-casp9 independently of cytochrome c and dATP (Ref. 22, Fig. 1A). These observations suggest WDR negatively regulates the activity of Apaf-1. We hypothesized that WDR might inhibit the activity of N-terminal Apaf-1 through physical association. To test this model, we performed in vivo binding experiments to determine whether WDR can associate with N-terminal Apaf-1-(1–559). We transfected 293T cells with HA-tagged Apaf-1-(1–559), as determined by immunoblotting analysis shown in Fig. 5A revealed that WDR co-immunoprecipitated with Apaf-1-(1–559). Similar immunoprecipitation experiments with Apaf-1 mutants showed that a
region between amino acids 109 and 328 of Apaf-1 is critical for association with WDR (Fig. 5A). This region partially overlaps with the putative self-association domain and contains conserved Walker A and B motifs. Therefore, we determined next whether the P-loop mutant Apaf-1-(1–559K149R) can bind to WDR. Significantly, Apaf-1-(1–559K149R) retained its ability to bind to WDR, as determined by immunoprecipitation experiments (Fig. 5A). These latter results suggest that WDR interacts with Apaf-1-(1–559) through a region similar but not identical to that which mediates self-association, as the Apaf-1-(1–559K149R) mutant had reduced ability to interact with Apaf-1-(1–559) (Fig. 3C).

**Fig. 3. Apaf-1-(1–559) self-associates.** A and B, Apaf-1 interacts via its CED-4 homologous region. 293 cells were transfected with plasmids producing Apaf-1-(1–559)-FLAG- and Myc-tagged Apaf-1 or indicated Apaf-1 mutants shown at top of panels. 24 h after transfection, cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG (upper panel). Immunoblotting of lysates with anti-FLAG (middle panel) or anti-Myc (lower panel) antibody is shown. C, mutation of lysine 149 in the P-loop region disrupts Apaf-1 self-association. Asterisks indicate nonspecific bands. WB, Western blotting. D, interaction of Apaf-1-(1–559) with Apaf-1 mutants in yeast. HF7c yeast cells were transformed with pACT2-Apaf-1-(1–559) and indicated Apaf-1 mutants in pGBT8 and streaked on solid growth media with (+His) or without (−His) histidine. Growth of colonies on His-free plates is an indication that two proteins interact in yeast. 1–559KR indicates 1–559K149R. Rare colonies observed with Apaf-1-(1–108) are due to reversion.

**Fig. 4. Self-association of Apaf-1 is sufficient for proteolytic activation of pro-casp9.** A and B, forced self-association is sufficient for Apaf-1-(1–108) to activate pro-casp9 processing. In A, Apaf-1-(1–108) extract was incubated in the absence (−) or presence of control (anti-HA, 0.2 μg) or anti-Myc (0.2 μg) purified rabbit antibody. 2° indicates addition of 0.4 μg of goat anti-rabbit secondary antibody. In B, Fpk3-Apaf-1-(1–108) extract was incubated with or without 20 nM AP1510. Immunoblotting of Fpk-Apaf-1 chimeric proteins is indicated (lower panel).

region between amino acids 109 and 328 of Apaf-1 is critical for association with WDR (Fig. 5A). This region partially overlaps with the putative self-association domain and contains conserved Walker A and B motifs. Therefore, we determined next whether the P-loop mutant Apaf-1-(1–559K149R) can bind to WDR. Significantly, Apaf-1-(1–559K149R) retained its ability to bind to WDR, as determined by immunoprecipitation experiments (Fig. 5A). These latter results suggest that WDR interacts with Apaf-1-(1–559) through a region similar but not identical to that which mediates self-association, as the Apaf-1-(1–559K149R) mutant had reduced ability to interact with Apaf-1-(1–559) (Fig. 3C).

**Fig. 5.** A and B, forced self-association is sufficient for Apaf-1-(1–108) to activate pro-casp9 processing. In A, Apaf-1-(1–108) extract was incubated in the absence (−) or presence of control (anti-HA, 0.2 μg) or anti-Myc (0.2 μg) purified rabbit antibody. 2° indicates addition of 0.4 μg of goat anti-rabbit secondary antibody. In B, Fpk3-Apaf-1-(1–108) extract was incubated with or without 20 nM AP1510. Immunoblotting of Fpk-Apaf-1 chimeric proteins is indicated (lower panel).
559) plus Apaf-1-(468–1194) and compared their ability to induce pro-casp9 activation. Significantly, activation of pro-casp9 induced by Apaf-1-(1–559) was inhibited by WDR of Apaf-1 (Fig. 5A) (13). However, the N-terminal region of Apaf-1 containing the CARD and CED-4 homologous region can promote pro-casp9 processing in the absence of dATP (22, 27). In addition, we have found that a nonhydrolyzable analogue of ATP fails to inhibit Apaf-1-(1–559)-mediated pro-casp9 processing, providing further evidence that ATP or dATP hydrolysis is not required for Apaf-1-(1–559) to activate pro-casp9. However, an Apaf-1 P-loop mutant, Apaf-1-(1–530K149R), has been shown to promote pro-casp9 processing (27). Although the reason for this discrepant result is unclear, it could be explained by the use of different systems. Whereas the other group employed purified recombinant Apaf-1 proteins expressed in *Escherichia coli*, we expressed Apaf-1 mutants in mammalian cells and used their extracts to assess the activation of pro-caspase-9. A possible explanation is that aggregation of *E. coli* Apaf-1 mutant protein might have bypassed the requirement for self-association, a process that we have shown is P-loop-dependent. An important role for the P-loop in Apaf-1 function is also suggested by the observation that similar mutations in CED-4 disrupt its ability to induce chromatin condensation in yeast and CED-3 processing in both insect and mammalian cells (18, 19, 26). Here, we provide evidence that mutants with substitutions of the conserved Lys residue of the P-loop, which is critical for binding to γ-phosphate, fail to bind Apaf-1-(1–559) and to activate pro-casp9. These seemingly conflicting results suggest that, in addition to contributing to dATP binding or hydrolysis, the P-loop region might be involved in the formation of the self-association surface. Such a role for the P-loop is consistent with our observation that substitution of the conserved Lys of the CED-4 region with several amino acids disrupts Apaf-1-(1–559) self-association. Alternatively, mutation of the P-loop could subtly change the conformation of Apaf-1 and therefore affect its ability to self-associate and to activate pro-casp9. Molecular modeling analysis has suggested that the dATP-binding pocket of Apaf-1 is formed from sequences across the CED-4 homologous region including the Walker A and B motifs (31). The proposed structural role for the P-loop is in agreement with observations that nucleotide-binding pockets of many proteins are precisely formed from

FIG. 5. WDR binds to the CED-4 homologous domain and inhibits Apaf-1-(1–559)- mediated pro-casp9 activation. A, WD-40 repeats interact with CED-4 homologous region. 293 cells were transfected with plasmids producing Apaf-1-(468–1194)-HA- and Myc-tagged Apaf-1 mutants as indicated. 24 h after transfection, cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA (upper panel) or anti-Myc antibody (upper panel). Immunoblotting of lysates with anti-HA (lower panel) is shown. Asterisks indicate nonspecific bands. H, IgG heavy chain; WB, Western blotting. B, WDR disrupts Apaf-1-(1–559) self-association. 293 cells were transfected with indicated plasmids and total lysates immunoprecipitated with anti-Myc antibody and immunoblotted with anti-FLAG (upper panel). Immunoblotting of total lysates with anti-FLAG (middle panel) and anti-Myc (lower panel) antibodies is shown. C, Apaf-1-(1–559)-mediated pro-casp9 activation is inhibited by co-expression of WDR. 20 μg of protein from Apaf-1-(1–559) or Apaf-1-(1559/1194) extracts were used in each reaction. Immunoblotting analysis of Apaf-1-(1–559) and WDR (residues 468–1194) with anti-Myc antibody is shown in lower panel.
regions widely scattered throughout their primary sequences and sensitive to mutations (32–34). Then, what is the role of dATP hydrolysis in Apaf-1 function? One possible model is that dATP hydrolysis and/or cytochrome c binding are required to overcome the inhibitory effect of the WD-40 repeats (see below). Alternatively, dATP hydrolysis could provide energy for conformational changes that are required to promote the release of processed casp9. This hypothesis is consistent with a recent report showing that processed casp9 activated by an N-terminal constitutive Apaf-1 was unable to process casp3 (27).

Previous studies suggested that WDR of Apaf-1 plays an inhibitory role in Apaf-1 function, as deletion of these repeats results in a constitutively active Apaf-1 protein (22). However, the basis for such an inhibitory role in the regulation of Apaf-1 remained unknown. We provide evidence that WDR can associate with the N-terminal region of Apaf-1 and that its expression abrogates Apaf-1-(1–559) self-association. Because self-association of Apaf-1-(1–559) can trigger pro-casp9 activation, these studies suggest a mechanism whereby WDR negatively regulates the activation of pro-casp9. Consistent with this hypothesis, we show that expression of WDR (residues 468–1194) inhibits the maturation of pro-casp9 promoted by Apaf-1-(1–559). The precise mechanism by which WDR inhibit Apaf-1-(1–559) self-association needs to be established. WDR might directly compete with N-terminal Apaf-1 for binding to the self-association domain (residues 109–328) and/or alternatively it might inhibit the Apaf-1-(1–559)–Apaf-1-(1–559) interaction by steric hindrance or another mechanism. Based on our studies, it is tempting to speculate that binding of cytochrome c and/or dATP hydrolysis induces a conformational change from a “closed conformation,” in which the N-terminal region of Apaf-1 is in contact with WDR, to an “open conformation,” in which WDR-Apaf-1 interaction is disrupted so that Apaf-1 can oligomerize via its N-terminal interaction domain. This open conformation promoted by cytochrome c and/or dATP hydrolysis might enable Apaf-1 to bind multiple pro-casp9 molecules and to promote pro-casp9 autoprocessing. WD-40 repeats are present in a variety of proteins with diverse functions and have been proposed to play a regulatory role (35). Inhibition of Apaf-1 self-association by WDR of Apaf-1 is consistent with such a role in Apaf-1 function.

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WD-40 Repeat Region Regulates Apaf-1 Self-association and Procaspase-9 Activation

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