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Parcs Is a Dual Regulator of Cell Proliferation and Apaf-1 Function*

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Here we identify a novel protein, named Parcs for pro-apoptotic protein required for cell survival, that is involved in both cell cycle progression and apoptosis. Parcs interacted with Apaf-1 by binding to the oligomerization domain of Apaf-1. Apaf-1-mediated activation of caspase-9 and caspase-3 was markedly decreased in a cytosolic fraction isolated from HeLa cells with reduced parcs expression. Interestingly, parcs deficiency blocked cell proliferation in non-tumorigenic cells but not in multiple tumor cell lines. In MCF-10A cells, parcs deficiency led to early G1 arrest. Conditional inactivation of parcs in genetically modified primary mouse embryonic fibroblasts using the Cre-LoxP system also resulted in the inhibition of cell proliferation. We conclude that Parcs may define a molecular checkpoint in the control of cell proliferation for normal cells that is lost in tumor cells.

Apaf-1 (apoptotic protease-activating factor 1), a mammalian homolog of the Caenorhabditis elegans cell death gene product CED-4, functions as an adaptor in an ~700-kDa multiprotein complex (named the apoptosome) to mediate the activation of caspase-9 (1–5). Activation of caspase-9, an initiator caspase, leads to the subsequent cleavage and activation of downstream executioner caspases, caspase-3 and caspase-7, which in turn cleave specific protein substrates, leading to the final destruction of cells.

The formation of the apoptosome during apoptosis is regulated by multiple factors. In an elegant series of biochemical studies, Wang and co-workers (4) demonstrated that cytochrome c, released from mitochondria during apoptosis, is a key factor in the formation of the apoptosome. Mouse embryonic fibroblasts deficient in apaf-1 or expressing a mutant allele of cytochrome c with reduced efficiency in mediating apoptosome formation show resistance to UV light-induced apoptosis (6). In addition, a number of proteins, including Aven (7), Hsp70 (8, 9), Hsp90 (10), NAC (11), and APIP (12), have been shown to have the ability to directly interact with Apaf-1. Among these proteins, only NAC was shown to be a positive regulator of Apaf-1 function (11).

Recently, Kroemer and co-workers (13, 14) demonstrated that apaf-1 deficiency and loss-of-function mutations in ced-4 compromise the arrest of DNA synthesis and sensitize cells to chromosomal instability in response to DNA damage in the absence of apoptosis. These results suggest that Apaf-1 might have a hitherto unsuspected role in the maintenance of genomic stability and cell cycle arrest, independent from its function in the apoptosis machinery.

In this study, we describe a novel protein that we termed Parcs for pro-apoptotic protein required for cell survival, which was isolated based on its ability to interact with the oligomerization domain of Apaf-1. We show that Parcs regulates the competency of Apaf-1 to form the apoptosome, as a cytosolic extract isolated from HeLa cells deficient in parcs expression was functionally defective in mediating caspase-9 activation in response to the addition of cytochrome c and dATP. We show that Parcs also has a role in mediating cell proliferation in a subset of cell lines. Inhibition of parcs expression by short hairpin RNA (shRNA)3 in MCF-10A cells but not in HeLa cells completely blocked cell proliferation by interfering with cell cycle progression. Furthermore, conditional deletion of parcs in mouse embryonic fibroblasts also led to a blockage of cell proliferation. Our results demonstrate that Parcs may serve a dual role in regulating both apoptosis and cell proliferation.

**EXPERIMENTAL PROCEDURES**

Screening of the Small Pool cDNA Expression Library—A mouse spleen cDNA expression library (15) divided into ~1600 “small pools” of 100 cDNA clones each was transcribed and translated using rabbit reticulocyte lysate (Promega) in the presence of a [35S]methionine/cysteine mixture (PerkinElmer Life Sciences).

GST-Apaf-1-(1–412), GST-Apaf-1-(98–412), or GST alone was produced in Escherichia coli BL21. An overnight bacterial culture was diluted 1:100 in fresh LB medium, and bacteria were grown to A600 = 0.8, followed by induction with 0.1 mM isopropyl β-d-thiogalactopyranoside for 2 h at 37 °C. GST or GST-Apaf-1 fusion proteins were purified with glutathione-Sepharose 4B beads from the soluble fraction prepared by sonication of a bacterial pellet in phosphate-buffered saline containing 1% Triton X-100.

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3 The abbreviations used are: shRNA, short hairpin RNA; GST, glutathione S-transferase; HA, hemagglutinin; MEFs, mouse embryonic fibroblasts.
The screening of the small pool library for Apaf-1-binding proteins was performed by incubating 2 μl of the [35S]-labeled protein mixture synthesized as described above with equal amounts of GST or GST-Apaf-1(1–412) fusion protein immobilized on Sepharose 4B beads. After a 2-h incubation at 4 °C in binding buffer (150 mM NaCl, 1% Triton X-100, and 50 mM Tris-HCl, pH 7.4) with rotation, the beads were spun down and washed four times with 1 ml of cold binding buffer. SDS sample buffer was added to the beads, and bound [35S]-labeled proteins were separated by SDS-PAGE and visualized by autoradiography.

Production of Anti-Parcs Antibodies—Recombinant His-Parcs protein encoded in a pET-28a vector (Novagen) was produced in BL21(DE3) bacteria. Induction of the proteins was performed with 1 mM isopropyl β-D-thiogalactopyranoside, and His-Parcs was isolated from the insoluble fraction using 6 M guanidine in Niquel beads (Novagen) and eluted with imidazole. Purified His-Parcs in 6 M guanidine was extensively dialyzed against phosphate-buffered saline before injection into rabbits for the production of polyclonal antibodies (Covance) or into rats for the production of monoclonal antibodies.

shRNA-mediated Depletion of Parcs—To stably suppress parcs expression, we employed pSRP, a self-inactivating retroviral vector. The oligonucleotide sequences employed to direct the expression of shRNA from the H1 promoter to suppress parcs expression were as follows (5′ to 3′): g193, GAGGATTGATTCTCTGGCAGAT; and g325, GGTACAGATTGTTGTACATACA; and control g239, GCATGGAGTACTTTGCCAAG. Retroviruses were produced in 293T cells by transfecting the corresponding pSRP or pSRP-Parcs plasmid with gag-pol- and vsv-g-expressing plasmids. Cells were transfected by the calcium phosphate method. Supernatants from transfected 293 cells containing the retroviral particles were collected 48 h after transfection and diluted 1:3 with fresh culture medium to infect HeLa or MCF-10A cells in the presence of either 1.5 or 2 μg/ml (HeLa) or 2 μg/ml (MCF-10A) puromycin to select a resistant population of cells.

Analysis of Cell Cycle Distribution—MCF-10A cells were infected with the indicated retroviruses and selected by addition of 2 μg/ml puromycin. To evaluate cell cycle distribution, cells were detached from the tissue culture plate with trypsin, washed twice with phosphate-buffered saline, and fixed overnight with 80% ethanol. After cells were treated with RNAse and propidium iodide, cell cycle distribution was evaluated in 10,000 cells by fluorescence-activated cell sorting. Results are expressed as the percentage of cells in specific phases of the cell cycle taking 100% as the total number of cells examined.

Evaluation of Ki67- and Ki-Mcm6-positive Cells—MCF-10A cells were infected with the indicated retroviruses and selected with 2 μg/ml puromycin. Cells grown on glass coverslips were washed with phosphate-buffered saline and fixed with 4% formaldehyde. After cells were permeabilized with 0.1% Triton X-100 and blocked with fetal bovine serum, cells were incubated overnight with the primary antibodies for Ki67 or Ki-Mcm6. After incubation with fluorescently labeled secondary antibodies, Ki67- and Ki-Mcm6-positive cells were quantified under a regular fluorescence microscope.

RESULTS

Isolation of Parcs as an Apaf-1-interacting Protein—A small pool expression library made from mouse spleen (15) was screened using an in vitro binding assay for proteins with the ability to interact with the GST–Apaf-1(1–412) fusion protein including two functionally important domains of Apaf-1, the caspase recruitment domain and the nucleotide-binding and oligomerization domain, which are responsible for binding of caspase-9 and oligomerization of Apaf-1, respectively. We reasoned that in this fusion protein these domains would be readily available to bind putative regulators, in contrast to the inactive full-length protein, in which they are masked by the WD-40 repeats at the C-terminal part of Apaf-1. Individual cDNA pools of the mouse spleen expression library were used to direct the synthesis of proteins in vitro in rabbit reticulocyte in the presence of [35S]methionine/cysteine. From the screening of >1600 pools, we identified a pool containing an ~33-kDa protein that selectively bound to GST–Apaf-1 but not to GST alone. The bacterially expressed and purified GST fusion of the nucleotide-binding and oligomerization domain of Apaf-1 (amino acids 98–412) was sufficient to bind [35S]methionine/cysteine-labeled Parcs. Full-length Apaf-1 co-immunoprecipitated with Parcs in mammalian cells. Expression vectors of Parcs-FLAG and Apaf-1-HA were cotransfected into 293 cells. Apaf-1-HA was specifically detected in Parcs–FLAG immunoprecipitates obtained 24 h after transfection. Expression vectors of full-length or truncated (amino acids 1–530) FLAG-Apaf-1 were cotransfected with the expression vector of HA-Parcs into 293 cells. HA-Parcs was present in the immunoprecipitate of both forms of Apaf-1 but not in the control immunoprecipitate. Endogenous Apaf-1 was detected in the immunocomplex of endogenous Parcs from HeLa cells isolated with a rabbit anti-Parcs polyclonal antibody (Ab) but not with preimmune serum (Pre) as a control. The specificity of this antibody is demonstrated by the results shown in Fig. 2, where parcs expression was suppressed by shRNA. aa, amino acids; CMV, cytomegalovirus; IP, immunoprecipitation.
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A

B

UV Dose (J/m²):

20

50

75

FIGURE 2. Apaf-1-mediated caspase activation is defective in the cytosolic extract isolated from HeLa cells deficient in parcs expression. A, cytochrome c (Cyto c)- and cytochrome c/dATP-mediated caspase-3 (Casp-3) activation was assessed in cell extracts generated from HeLa cells expressing the empty vector or Parcs shRNA. The upper panel was probed with both anti-Parcs (the same rabbit antibody used in Fig. 1) and anti-full-length caspase-3 antibodies. The middle panel was probed with anti-active caspase-3, and the anti-tubulin antibody (lower panel) was used as a control. The S-100 cytosolic fraction was incubated for 1 h at 30 °C in the presence of the indicated additions, followed by Western blot analysis with the indicated antibodies. B, cytochrome c-induced activation of caspase-9 was reduced in a cytosolic extract prepared from HeLa cells deficient in parcs expression. Conditions were as in A, but cell lysates were probed with the indicated antibodies.

B

FIGURE 3. parcs deficiency prevents full activation of Apaf-1 by UV light in vivo. A, the same HeLa cells expressing the empty pSRP vector or Parcs shRNA described in the legend to Fig. 2 were exposed to the indicated doses of UV light, and caspase-9 (Casp-9) autoprocessing was assessed by Western blotting using a selective antibody for this product, which was completely absent in untreated cells. B, in parallel experiments, HeLa cell survival was evaluated at the indicated time points after exposure to UV light using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl-2H-tetrazolium (inner salt) method. The results expressed correspond to the signal of UV light-treated vector or Parcs-deficient cells compared with the corresponding untreated vector or Parcs-deficient cells, respectively.

This cell-free system was originally employed to isolate Apaf-1 (1) and constitutes the most direct approach to assess Apaf-1 function after the addition of its two activating factors, viz. dATP and cytochrome c. Parcs protein levels were successfully decreased by using a retroviral vector encoding a Parcs shRNA (Fig. 2A). The activation of Apaf-1 initiated by the addition of cytochrome c and dATP led to a marked reduction in the levels of full-length caspase-3 and a simultaneous increase in the levels of activated caspase-3 in the control cell extract (Fig. 2A). In contrast, in extracts from cells expressing Parcs shRNA, the activation of caspase-3 induced by the addition of cytochrome c and dATP was markedly inhibited (Fig. 2A). The Apaf-1-mediated cleavage of caspase-9 was also reduced in extracts from cells expressing Parcs shRNA (Fig. 2B). Notably, Apaf-1, caspase-9, and caspase-3 protein levels were not affected by suppressing parcs expression (Fig. 2, A and B). These results suggest that Parcs is required to maintain Apaf-1 in an optimal competent state for its activation by cytochrome c and dATP. To evaluate a role of Parcs in vivo, apoptosis was induced by exposure to UV light in HeLa cells with suppressed parcs expression. A small reduction in caspase-9 autoprocessing

Parcs Is Required to Maintain Apaf-1 in a Competent State—

Next, we tested for a possible functional regulation of Apaf-1 by Parcs. For this purpose, we directly examined Apaf-1-mediated caspase cleavage in a cytosolic extract isolated from HeLa cells inhibited for expression of parcs by shRNA.

This CDNA encodes a novel protein of 284 amino acids characterized by a hypothetical ATP-binding domain (amino acids 8–254; pfam03029). This protein is highly conserved from yeast to mammals, suggesting that it may serve a fundamental cellular function. Its mRNA and protein were ubiquitously expressed in mouse tissues, and the mRNA and protein expression patterns agreed mostly with each other (data not shown). Because the ortholog of this protein in yeast (YLR243W) is essential for cell viability (16), the inactivation of its ortholog in C. elegans (Y75B8A.14) by RNA interference causes embryonic lethality (17), and it is required for caspase activation (see below), we termed this protein Parcs for pro-apoptotic protein required for cell survival.

We first expressed tagged Parcs to study its interaction with Apaf-1. Hemagglutinin (HA)-tagged full-length Apaf-1 (Apaf-1-HA) co-immunoprecipitated with FLAG-tagged Parcs in human 293 cells (Fig. 1C). Conversely, HA-Parcs was specifically detected in immunoprecipitates of full-length or truncated (amino acids 1–530) FLAG-Apaf-1 (Fig. 1D). These results demonstrate that full-length Parcs and Apaf-1 interact with each other when overexpressed in mammalian cells. To determine whether Parcs and Apaf-1 associate at endogenous levels, we immunoprecipitated Parcs with a rabbit polyclonal antibody produced against a recombinant His-Parcs protein. Endogenous Apaf-1 was reproducibly detected in anti-Parcs immunoprecipitates, but not when preimmune rabbit serum was used (Fig. 1E), demonstrating that endogenous Parcs also interacts with Apaf-1.

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was observed at all UV doses, revealing a defect in Apaf-1 (Fig. 3A). However, the residual Apaf-1 activity seemed enough for apoptosis, as no difference in cell survival could be detected (Fig. 3B).

Parcs Is Essential for the Proliferation of Normal but Not Tumorigenic Cells—Because the Parcs ortholog is essential for cell viability in yeast (16) and its inactivation by RNA interference causes embryonic lethality in C. elegans (17), we hypothesized that Parcs function may extend beyond the regulation of Apaf-1. To explore Parcs function in a more general manner, we evaluated the involvement of Parcs in cell proliferation in cell lines other than HeLa cells. The expression of parcs was successfully reduced in MCF-10A mammary epithelial cells after infection with retroviruses encoding two different Parcs shRNAs, g193 and g325, but not with a third shRNA, g239 (Fig. 4A). To assess the effect of Parcs RNA interference on cell proliferation, an equal number of cells infected with retroviruses expressing the Parcs shRNA indicated above were trypsinized and replated 3 days after retroviral infection and counted again 3 days later. In contrast to the proliferation of MCF-10A cells infected with the control Parcs shRNA, which proliferated normally, the proliferation of MCF-10A cells infected with the two effective Parcs shRNAs, g193 and g325, but not with a third shRNA, g239 (Fig. 4A). To assess the effect of Parcs RNA interference on cell proliferation, an equal number of cells infected with retroviruses expressing the Parcs shRNA indicated above were trypsinized and replated 3 days after retroviral infection and counted again 3 days later. In contrast to the proliferation of MCF-10A cells infected with the control Parcs shRNA, which proliferated normally, the proliferation of MCF-10A cells infected with the two effective Parcs shRNAs was completely blocked. This result demonstrates that Parcs is an essential protein for cell proliferation in MCF-10A cells, although HeLa cells proliferate normally in the absence of Parcs (Fig. 4B).

To determine whether Parcs plays a wider role in cell proliferation, we reduced the expression of parcs using the effective Parcs shRNA in other cell lines. Surprisingly, Parcs was also dispensable for cell proliferation in the MDA-MB-231 and H1229 cell lines (data not shown). These results suggest the intriguing possibility that although Parcs is an essential pro-

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**FIGURE 4.** parcs deficiency inhibits cell proliferation in MCF-10A cells but not in HeLa cells. A, inhibition of parcs expression with two different Parcs shRNAs (lower panel; g325 and g193) but not with the control shRNA (g239) inhibited proliferation in MCF-10A cells (upper panel). B, inhibition of parcs expression (lower panel; g325) did not affect cell proliferation in HeLa cells (upper panel). RNAi, RNA interference.

**FIGURE 5.** Parcs is required for cell proliferation in primary MEFs. A, Parcs protein levels were successfully decreased by Cre recombinase in Parcs loxP/loxP MEFs but not in Parcs loxP/+ MEFs. Both types of cells contain one Parcs allele flanked by LoxP sites and another allele that is either wild-type (+) or constitutively inactivated (−). Parcs protein levels were assessed by Western blotting in total cell lysates prepared 4 days after infection of MEFs with either an empty adenovirus or one encoding Cre recombinase (Adeno-cre). B, proliferation of Parcs loxP/loxP MEFs but not Parcs loxP/+ MEFs was decreased by Cre recombinase. Cell proliferation was evaluated at the indicated time points after infection of MEFs with either an empty adenovirus or one expressing Cre recombinase. C, shown are the results from microscopic assessment of Parcs loxP/+ or loxP/+ MEFs infected with either an empty adenovirus or one expressing Cre recombinase.
Parcs Is a Dual Regulator

![Image](http://www.jbc.org/content/jbc/283/36/24404/F6.large.jpg)

**FIGURE 6.** MCF-10A cells arrest in the early G₁ phase after suppression of parcs expression by shRNA. **A**, suppression of parcs expression led to an increase in the fraction of MCF-10A cells in the G₁ phase and a parallel reduction of cells in the S and G₂/M phases of the cell cycle. **B**, suppression of parcs expression significantly decreased the fraction of MCF-10A cells expressing Ki67 without affecting the expression of Ki-Mcm6. **C**, the protein levels of the proliferation markers cyclin A₁ and cyclin B₁ were markedly reduced after the suppression of parcs expression by two different Parcs shRNAs that efficiently decreased Parcs protein levels (g325 and g193) but not by one control shRNA that had no effect on Parcs protein levels (g239).

In an attempt to more precisely define the point in the cell cycle where Parcs knockdown MCF-10A cells arrest, we took advantage of the differences in the expression pattern between Ki67 and Ki-Mcm6 during the cell cycle. Ki67 and Ki-Mcm6 are both expressed in proliferating cells, but whereas Ki-Mcm6 is expressed in both the S and G₂/M phases of the cell cycle, which was not observed in cells expressing the control Parcs shRNA (Fig. 6A). Concomitant with the observed increase in the cells in the G₁ phase, the expression of the two effective Parcs shRNAs led to a strong reduction in the fraction of cells in both the S and G₂/M phases of the cell cycle, which was not observed in cells expressing the control Parcs shRNA (Fig. 6A).

To begin exploring the mechanism by which Parcs regulates cell proliferation, we investigated whether the absence of Parcs is associated with a specific defect in cell cycle progression. First, we infected MCF-10A cells with viruses expressing the control Parcs shRNA or either one of the two effective Parcs shRNAs for 3 days to suppress parcs expression, followed by trypsinization and reseeding of the cells and finally evaluation of cell cycle distribution by fluorescence-activated cell sorting after an additional 2 days in culture. MCF-10A cells infected with either one of the two effective Parcs shRNAs displayed a marked increase in the fraction of cells in the G₁ phase of the cell cycle, whereas cells infected with the control shRNA had a cell cycle distribution indistinguishable from that of cells infected with the empty vector (Fig. 6A). Concomitant with the observed increase in the cells in the G₁ phase, the expression of the two effective Parcs shRNAs led to a strong reduction in the fraction of cells in both the S and G₂/M phases of the cell cycle, which was not observed in cells expressing the control Parcs shRNA (Fig. 6A).

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**DISCUSSION**

In this work, we identified Parcs, a novel protein with a nucleotide-binding domain, by its ability to interact with the proapoptotic protein Apaf-1. Parcs is required to maintain Apaf-1 in an activable state, as the cell extract isolated from cells deficient in parcs expression was functionally defective in mediating caspase activation in response to the addition of cytochrome...
c and dATP. In addition, we have shown that Parcs is essential for cell proliferation in normal cells, including MCF-10A breast epithelial cells and primary MEFs, but dispensable for the proliferation of tumorigenic cells such as HeLa, MDA-MB-231, and H1229. We have further demonstrated that MCF-10A cells deficient in parcs expression have an increased fraction of cells in the G1 phase and a simultaneous reduction in the S and G2/M phases of the cell cycle. These G1-arrested cells are positive for Ki-Mcm6 but negative for Ki67, indicating that in the absence of Parcs, MCF-10A cells undergo cell cycle arrest in the early G1 phase.

It is interesting to note that whereas cytochrome c-induced, Apaf-1-mediated processing of caspase-9 and caspase-3 was defective in cell extracts isolated from cells with suppressed parcs expression by shRNA (Fig. 2), the acute removal of Parcs from a cell lysate isolated from control cells by antibody-mediated depletion did not have a negative impact on any of these parameters (data not shown). This implies that Parcs is not directly involved in apoptosome assembly. Accordingly, Parcs was not detected as a component of the apoptosome (data not shown). We believe that this requirement of Parcs for Apaf-1 function may reflect the involvement of Parcs in a step leading to a particular protein conformation or post-translational modification in Apaf-1, which is subsequently critical for apoptosome formation. Alternatively, Parcs could be needed to maintain Apaf-1 in a specific conformational state that is important for its optimal activation during apoptotic cell death. Further studies are needed to differentiate these possibilities. Interestingly, a regulatory action of Parcs on Apaf-1 function was also observed in vivo (Fig. 3).

Our results demonstrate that Parcs is essential for cell proliferation in certain mammalian cells. A role in a fundamental process such as cell proliferation may explain the conservation of this gene during evolution and the reported results from its inactivation in genome-wide studies as an essential gene in yeast (16) and for embryonic development in C. elegans (17, 19, 20). Unexpectedly, the tumorigenic cell lines examined so far (HeLa, MDA-MB-231, and H1229) proliferated normally in the absence of Parcs. These results suggest that Parcs may define a molecular checkpoint in the control of cell proliferation in normal cells that is lost in tumor cells.

Blockage of parcs-deficient MCF-10A cells in the early G1 phase suggests that Parcs may be involved in the competency for preparing entry into the S phase when DNA is synthesized. Interestingly, Kroemer and co-workers (13, 14) demonstrated that apaf-1 deficiency and loss-of-function mutations in ced-4 compromise the arrest of DNA synthesis and sensitize cells to chromosomal instability in response to DNA damage. It will be interesting to examine in future experiments if the interaction of Parcs with Apaf-1 is relevant for the role of Apaf-1 in mediating the arrest of DNA synthesis upon DNA damage.

Taken together, we have demonstrated that Parcs is a molecular checkpoint protein in normal but not tumorigenic mammalian cells in the early G1 phase of the cell cycle. Parcs is also important for maintaining Apaf-1 in an optimal activatable state. Because Parcs is involved in both cell cycle arrest and apoptosis, the two most basic cellular responses to DNA damage, we propose that Parcs is part of an evolutionarily conserved molecular checkpoint apparatus that is inactivated in cancer cells.

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