Geminin Cleavage during Apoptosis by Caspase-3 Alters Its Binding Ability to the SWI/SNF Subunit Brahma*

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Geminin has been proposed to coordinate cell cycle and differentiation events through balanced interactions with the cell cycle regulator Cdt1 and with homeobox transcription factors and chromatin remodeling activities implicated in cell fate decisions. Here we show that Geminin is cleaved in primary cells and cancer cell lines induced to undergo apoptosis by a variety of stimuli. Geminin targeting is mediated by caspase-3 both in vivo and in vitro. Two sites at the carboxyl terminus of Geminin (named C1 and C2) are cleaved by the caspase, producing truncated forms of Geminin. We provide evidence that Geminin cleavage is regulated by phosphorylation. Casein kinase II alters Geminin cleavage at site C1 in vitro, whereas mutating phosphorylation competent Ser/Thr residues proximal to site C1 affects Geminin cleavage in vivo. We show that truncated Geminin produced by cleavage at C1 can promote apoptosis. In contrast, Geminin cleaved at site C2 has lost the ability to interact with Brahma (Brm), a catalytic subunit of the SWI/SNF chromatin remodeling complex, while binding efficiently to Cdt1, indicating that targeting of Geminin during apoptosis differentially affects interactions with its binding partners.

Organogenesis and tissue homeostasis relies on an intricate balance between cell proliferation, differentiation, and apoptosis. Regulatory mechanisms must ensure coordination between these alternative cell fates depending on cell history and extracellular stimuli. Identifying factors linking these processes is therefore pivotal for an understanding of the control mechanisms acting over cell fate decisions.

Geminin has recently attracted attention as a possible link between the cell cycle and cell differentiation programs: through direct interactions with an increasing number of binding partners, Geminin has been proposed to affect the balance between proliferation and differentiation, thereby coordinating cell fate decisions in different developmental contexts (1, 2). Geminin is a small nuclear protein (25 kDa), which was initially identified as a substrate for proteolysis at the metaphase to anaphase transition by the anaphase promoting complex (3). Geminin was shown to negatively regulate DNA replication licensing by preventing the formation of the pre-replicative complex on origins of replication through physical association with the licensing factor Cdt1 (4, 5). Geminin is absent (6) or inactive (7) during the G1 phase, allowing licensing to take place, whereas its accumulation during the S, G2, and M phases is essential for preventing ectopic licensing from taking place until mitosis has been completed. Gain- and loss-of-function experiments revealed the essential role of Geminin in maintaining genome stability. High level expression of Geminin in mammalian cells arrests cells in G1 phase or pushes them into apoptosis (4, 8, 9), whereas the depletion of Geminin in Drosophila causes partial over-replication of the genome and giant nuclei formation (10–12). Consistently, inactivation of Geminin in several human normal and tumor cell lines leads to re-replication within the same cycle and activation of Chk1 and Chk2 protein kinases and checkpoint arrest, preventing cells with re-replicated DNA to enter into mitosis (12, 13). These results suggest that Geminin plays an important role in maintaining genomic integrity by preventing re-replication.

In addition to its cell cycle function, Geminin is implicated in cell fate decisions during development. Kroll et al. (14) identified Geminin as a molecule that promotes expansion of the neural plate of Xenopus embryos at the expense of adjacent non-neural ectoderm, suggesting a possible role in specifying neural territory during early embryogenesis. More recently, Geminin has been shown to interact with transcription factors involved in differentiation. Geminin impairs the transcriptional activity of the Hox homeobox proteins by directly interacting and displacing Hox proteins from target genes and also by a polycomb-like inhibition of Hox gene expression (15). In addition, during eye development in medaka fish, Geminin interacts with and acts antagonistically to the homeobox transcription factor Six3 (16). Competitive interactions of Geminin with Cdt1 and with homeobox transcription factors have been proposed to control the balance between proliferation and differentiation during vertebrate development. Geminin gain- and loss-of-function experiments in the medaka fish have demonstrated an anti-proliferation and pro-differentiation activity for Geminin (16). Surprisingly, gain- and loss-of-function experiments in Xenopus embryos and mouse P19 cells suggested an additional anti-differentiation activity for Gemi-
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Geminin (17). Geminin interacts with the two alternative catalytic subunits of the mammalian SWI/SNF chromatin remodeling complex, Brahma (Brm) 2 and Brahma related-gene 1 (Brg1), through its carboxyl-terminal region. The carboxyl-terminal interaction of Geminin with Brg1 has been suggested to block the association of Brg1 with proneural basic helix loop helix proteins, such as Neurogenin and NeuroD, preventing proneural gene target activation and subsequent neurogenesis (17). This study proposed that whereas Geminin promotes neural fate acquisition, it can block premature neuronal differentiation dependent on its interactions with Brg1/Brm, thereby regulating the transition from proliferating neural progenitors to differentiating neurons. Temporal and spatial regulation of Geminin ability to interact with several different partners appears therefore crucial for cell fate decisions.

Geminin’s molecular structure offers clues to its multiple functions (18–20). Geminin contains a central coiled-coil domain, flanked by regions predicted to be more unstructured, where several regulatory functions have been mapped. Close to the NH2 terminus lies a destruction box sequence essential for anaphase promoting complex-mediated degradation during the cell cycle, whereas several phosphorylation sites have been mapped close to both the NH2 and COOH termini of the molecule. The central coiled-coil domain is essential for the dimerization of Geminin, Cdt1 binding, and replication inhibition (18). The Hox binding domain partly overlaps with the coiled-coil region, antagonizing Cdt1 for binding (15). In contrast, the observed in early embryogenesis “neuralizing” activity of Geminin was mapped to its amino-terminal domain (14), whereas the Brg1 binding site lies close to the carboxyl terminus of Geminin (17). Balanced interactions of Geminin with its multiple binding partners appear to form the basis for its ability to coordinate the cell cycle and cell differentiation.

We show here that this balance is affected when cells progress into apoptosis. During apoptosis, Geminin is cleaved by caspase-3 at two sites close to its COOH terminus. Geminin cleavage at one of these sites leads to loss of its ability to interact with the chromatin remodeling factor Brm, whereas interactions with the cell cycle regulator Cdt1 are maintained. Interestingly, cleavage at the other site promotes apoptosis and is regulated by phosphorylation, suggesting that overlapping pathways govern Geminin’s function to coordinate cell fate decisions.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Induction of Apoptosis—HeLa and MCF7 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% (v/v) fetal bovine serum (Invitrogen). Human umbilical vein endothelial cells (HUVEC) were cultured in M199 Earle’s medium (Biochrome) supplemented with 10% fetal calf serum, 0.01 M Hepes, 500 units of heparin (Sigma), and 0.03 mg/ml endothelial cell growth supplement (Sigma). MCF7 cells were transfected with a total of 1 μg of plasmid DNA for 22 h using FuGENE 6 (Roche), and HeLa cells were transfected with a total of 1 μg of plasmid DNA for 22–36 h using Lipofectamine 2000 (Invitrogen). Apoptosis was induced by treatment with staurosporine (5 μM; Cayman Chemicals), etoposide (85 μM; Sigma), or tumor necrosis factor-α (40 ng/ml; Peprotech) combined with cycloheximide (40 μg/ml; Sigma).

Western Blotting—Floating and attached cells were harvested separately as indicated and cell lysates were prepared lysing cell pellets directly in SDS-PAGE loading buffer and boiling. Western blot analysis was performed using specific polyclonal rabbit antibodies against Cdt1 (6) and Geminin (21) and a mouse monoclonal antibody raised against the middle region of Geminin (amino acids 76 to 160).3 Antibodies were used at the following dilutions for Western blotting: anti-hCdt1 1:500, polyclonal anti-hGeminin 1:3000, monoclonal anti-hGeminin 1:30, anti-hCdc6 1:1000 (Upstate), anti-cyclin A 1:500 (Neomarkers), anti-PARP 1:3000 (BD Pharmingen), anti-tubulin 1:10000 (Sigma), anti-MCM7 1:1000 (Santa Cruz), anti-γH2A.X 1:1000 (Upstate), and anti-HA (clone 12CA5) 1:500.

Plasmids—Pro-caspase-3 cDNA (a gift from Dr. B. Fang) was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen) at the EcoRI-XbaI site. The cDNA encoding human full-length Geminin was fused to the carboxyl terminus of green fluorescent protein (GFP) (vector EGFP-pcDNA3.1, Invitrogen) producing wild type GFP-Geminin. Based on this construct, a polymerase chain reaction-based strategy was used to construct mutant forms of Geminin. The nucleotide sequence of each PCR-generated construct was determined to verify the mutations. Geminin bearing an alanine instead of aspartic acid at position 170 in pcDNA3.1-EGFP was constructed by site-directed mutagenesis (Stratagene) and verified by sequencing. HA-tagged hBrm expression vector was kindly provided by Drs. H. Katoh and Shinya Tanaka.

Protein Expression, in Vitro Assays, and Mass Spectrometry—Full-length Geminin cloned in pET28 (Invitrogen) was kindly provided by Drs. V. De Marco and A. Perrakis. Geminin D170A was constructed by site-directed mutagenesis (Stratagene). Both mutant and wild type Geminin were expressed as amino-terminal-tagged His6 fusions in Escherichia coli strain Rosetta (DE3) pLysS (Novagen) and purified by Ni2+-NTA-agarose affinity chromatography (Qiagen).

For in vitro cleavage assays, 5 μg of full-length recombinant Geminin was incubated with 0.1 or 0.5 μg of recombinant caspase-3 (Upstate) in 50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM 1,4-dithiothreitol (caspase buffer), at 37 °C for the times indicated. The incubation products were analyzed by SDS-PAGE electrophoresis followed by staining with Coomassie Brilliant Blue R-250.

For the in vitro binding assay, 10 μg of His-tagged recombinant Geminin were incubated with 0.8 μg of recombinant caspase-3 for 6 h at 37 °C in assay buffer and allowed to interact.

2 The abbreviations used are: Brm, Brahma; STS, staurosporine; PARP, poly(ADP-ribose) polymerase; MCMs, minichromosome maintenance proteins; EGFP, enhanced green fluorescent protein; CKI, casein kinase I; CKII, casein kinase II; HUVEC, human umbilical vein endothelial cells; HA, hemagglutinin; Ni-NTA, nickel-nitrilotriacetic acid; CHAPS, 3-(3-Cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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with Ni²⁺-NTA-agarose beads (Qiagen) for 1 h at 4 °C. Bound fraction and flow-through were analyzed by SDS-PAGE electrophoresis followed by Coomassie Brilliant Blue staining.

For *in vitro* phosphorylation of Geminin by casein kinase II described in Fig. 4D, 2.5 µg of recombinant wild type Geminin was incubated with recombinant casein kinase II (New England Biolabs) for 5 h at 30 °C, in 20 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, in the presence of 200 µM ATP. Recombinant Geminin was subsequently incubated with 0.5 µg of recombinant caspase-3 overnight at 37 °C and the products were analyzed by SDS-PAGE electrophoresis followed by Coomassie Brilliant Blue staining.

Casein kinase II (CKII) reactions described in Fig. 4A, were carried out in a final volume of 120 µl containing 200 µM ATP, 40 µCi/ml [γ⁻³²P]ATP, in 20 mM Tris–HCl, 50 mM KCl, and 10 mM MgCl₂, pH 7.5, at 30 °C. Full-length hGeminin, the middle part of hGeminin (76–160 amino acids) and the carboxy-terminal part of Geminin (115–209 amino acids) all expressed and purified from *E. coli* as His-tagged fusions, were used as substrates at respective final concentrations of 5.47, 16.45, and 14.45 µM. Synthetic peptides (WT, RRRGTVSSSTDAKPCI; S201A, RRRGTVSSSTDAKPCI; and SSST/A, RRRGTVAA-AADAKPCI) were synthesized by Peptide Specialty Laboratories GmbH (Heidelberg, Germany) and used at a final concentration of 400 µM. At the indicated time points, samples were stopped by addition of SDS sample buffer, boiled, and analyzed by SDS-PAGE electrophoresis followed by autoradiography or phosphorimager analysis (FLA3000, Fuji). Alternatively, the caspase buffer, incubated with 0.5 µg of recombinant caspase-3 in caspase buffer for 6 h at 37 °C, and analyzed by Western blotting using anti-Geminin monoclonal antibodies.

For immunoprecipitation experiments defining protein-protein interactions, HeLa cells were transfected with the constructs indicated for 22–36 h and total cell extracts were prepared after lysing cells in 20 mM Hepes, 150 mM NaCl, 0.2% Triton X-100, 5 mM MgCl₂, supplemented with protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1 µg/ml leupeptin, 1.4 µg/ml pepstatin, 1 µg/ml aprotinin, 1 µg/ml chymostatin, 0.1 mM sodium vanadate). Affinity purified polyclonal antibody against Geminin was conjugated to Protein A-agarose beads (GE Healthcare) and incubated with cell extracts for 4 h at 4 °C. The immunoprecipitates were washed with lysis buffer and caspase buffer, incubated with 0.5 µg of recombinant caspase-3 in caspase buffer for 6 h at 37 °C, and analyzed by Western blotting using anti-Geminin monoclonal antibodies.

**RESULTS**

**Human Geminin Is Targeted during Apoptosis—Cell proliferation, differentiation, and apoptosis are intricately linked during development. Given the involvement of Geminin in coordinating proliferation and differentiation, we wished to study its behavior when cells undergo apoptosis.**

To address whether hGeminin is targeted during apoptosis, HeLa cells were induced to undergo apoptosis with the microbial alkaloid staurosporine (STS) (24, 25) and Geminin was followed by Western blotting. As shown in Fig. 1A, after STS treatment for 6 h, a faster migrating form of Geminin appears, consistent with cleavage of Geminin producing a lower molecular weight product (Fig. 1A, upper panel, filled arrow). In addition, a longer film exposure reveals a second cleavage product (Fig. 1A, lower panel, open arrow). The cleavage of PARP protein is a distinct feature of cells undergoing apoptosis and it was observed after 3 h of STS treatment. In addition, as previously shown, human replication protein Cdc6 is also cleaved during apoptosis (26), whereas we observed no significant changes in the cell cycle regulatory protein cyclin A. Thus, in apoptotic HeLa cells treated with staurosporine, the electrophoretic mobility properties of Geminin were altered, indicative of a cleavage event.

176 amino acids of Geminin, in full agreement with the proposed sequences.

**Immunofluorescence and Immunoprecipitation—Immunofluorescence of HeLa cells was carried out as previously described (6), using an antibody against the phosphorylated form of the histone H2AX at serine 139 (γH2AX) (dilution 1:1000, Upstate).**

For the immunoprecipitation described in Fig. 3C, GFP-Geminin and GFP-GemD204A constructs were transfected in HeLa cells and total cell extracts were prepared by lysing cells in buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 5 mM MgCl₂, supplemented with protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1 µg/ml leupeptin, 1.4 µg/ml pepstatin, 1 µg/ml aprotinin, 1 µg/ml chymostatin, 0.1 mM sodium vanadate). Affinity purified polyclonal antibody against Geminin was conjugated to Protein A-agarose beads (GE Healthcare) and incubated with cell extracts for 4 h at 4 °C. The immunoprecipitates were washed with lysis buffer and caspase buffer, incubated with 0.5 µg of recombinant caspase-3 in caspase buffer for 6 h at 37 °C, and analyzed by Western blotting using anti-Geminin monoclonal antibodies.

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To investigate whether targeting of Geminin during apoptosis was also observed in primary cells, human endothelial cells isolated from umbilical vein (HUVEC cells), were treated with STS over a time course of 12 h. As shown in Fig. 1B, Geminin is cleaved after 9 h of STS treatment and this targeting occurs with a similar time course to the cleavage of PARP.

To investigate whether the cleavage of Geminin occurs in response to treatment by other apoptotic stimuli, we subjected HeLa cells to treatment with tumor necrosis factor-α (lanes 1–4), apoptosis is induced, evidenced by PARP cleavage, and Geminin is cleaved, producing the cleavage pattern observed during staurosporine-induced apoptosis.

Similarly, treatment of HeLa cells with the topoisomerase-II inhibitor etoposide (Fig. 1D), which induces DNA double strand breaks and leads to apoptosis (28) results in a similar cleavage pattern for Geminin (Fig. 1D), which is most obvious in cells that have detached from the culture dish (lanes 6, 8, and 10). Cdc6 and PARP are also cleaved as previously shown. In contrast, the member of the minichromosome maintenance complex (MCM), MCM7, remains intact. The phosphorylated form of the histone H2AX (γ-H2AX) accumulates following the treatment, serving as a control for the induction of DNA double strand breaks. Because Geminin is cleaved by a number of apoptotic stimuli in different cell lines, we conclude that Geminin is targeted for cleavage during apoptosis.

**Caspase-3 Mediates Cleavage of Geminin**—Caspases 3, 6, and 7 are the effector caspase executioners of apoptosis that are responsible for the sequence-specific proteolytic cleavage of a broad spectrum of cellular protein targets, leading to the apoptotic phenotype. Among them, caspase-3 catalyzes the specific cleavage of many key cellular proteins (29). To investigate a possible targeting of Geminin during apoptosis by caspase-3, we monitored the electrophoretic mobility of Geminin in apoptotic MCF7 cells. The MCF7 breast carcinoma cell line is deficient in caspase-3 because of a functional deletion of the CASP-3 gene (30). To this end, MCF7 cells were treated with staurosporine for 0, 3, 6, 9, and 12 h and a possible targeting of Geminin was followed by Western blotting. As shown in Fig. 2A, the characteristic pattern of cleaved Geminin is not present in staurosporine-treated MCF7 cells, whereas, as previously reported, PARP is cleaved (31). This result suggests that caspase-3 is required for Geminin cleavage during apoptosis.

To further examine this suggestion, we transiently transfected the caspase-3-deficient MCF7 cells with the pro-caspase-3 cDNA. After transfection and treatment with stauro-
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**A**

[Diagram of Geminin, PARP, and Tubulin bands on Western blots with arrows indicating cleavage products]

**B**

[Diagram showing transfection and cleavage products of Geminin and PARP]

**C**

[Diagram showing Geminin/caspase-3 cleavage products with molecular weight markers]

**D**

[Diagram illustrating in vitro and in vivo cleavage of Geminin]

**FIGURE 2. Caspase-3 mediates Geminin cleavage in vivo and in vitro.** (A) geminin is not cleaved in caspase-3–deficient apoptotic MCF7 cells. MCF7 cells were induced to undergo apoptosis by treatment with 5 μM STS for the time indicated. Total protein extracts were prepared and subjected to Western blot analysis, using antibodies against Geminin, PARP, and Tubulin. The band marked with an asterisk (*) in the PARP blot corresponds to a cross-reacting band, whereas the arrow marks the apoptosis-induced PARP cleavage product. The decrease in total Geminin levels observed following the treatment may be due to proteolytic degradation of Geminin in the absence of caspase cleavage, as previously reported for Cdc6 (42). (B) transfection of Caspase-3 into MCF7 cells restores the cleavage pattern of Geminin. MCF7 cells were transiently transfected with procaspase-3 (lanes 5–7) or the vector alone (lanes 3 and 4). 22 h following transfection, cells were treated with 5 μM STS for 6 h (lanes 4, 6, and 7). A floating population was present in procaspase-3-transfected staurosporine-treated cells, but not in vector-transfected cells, and was collected separately (lane 7, F). Protein extracts were subjected to Western blot analysis, using antibodies against Geminin and PARP. Extracts from untreated (lane 1) or STS-treated (lane 2) HeLa cells were used as controls. C, Geminin is cleaved by caspase-3 in vitro. Recombinant Geminin was incubated with 0.1 μg of recombinant caspase-3 for 30 min (lane 6) or 0.5 μg of recombinant caspase-3 for 30 min, 2.7, and 16 h (lanes 7–10). Geminin was incubated in parallel in the absence of caspase-3 (lanes 1–4). Lane 5 contains 0.5 μg of caspase-3 without recombinant Geminin. The incubation products were analyzed by SDS-PAGE electrophoresis in 10% (upper panel) and 18% (lower panel) polyacrylamide gels and proteins were visualized by staining with Coomassie Brilliant Blue. Filled and open arrowheads mark the major (C1) and the minor (C2) cleavage products, respectively. D, Geminin fragment C1 produced by caspase-3 cleavage in vitro corresponds to the cleavage product observed in vivo during apoptosis. Geminin present in untreated HeLa cell extracts (lane 2) was immunoprecipitated with anti-Geminin-specific rabbit antibody (immunoprecipitation supernatants are shown in lane 3 and anti-Geminin beads in lane 1) and incubated in the presence (lane 5) or absence (lane 4) of 0.5 μg of recombinant caspase-3 for 3 h (in vitro). In parallel, HeLa cells were incubated for 8 h in the presence (lane 7) or absence (lane 6) of 5 μM STS, to induce cleavage of Geminin in vivo. The electrophoretic mobility of the cleavage fragments was compared by Western blotting, using a specific anti-Geminin monoclonal antibody. A long SDS-PAGE gel was used to better resolve the cleavage products.

sporine, which acted as the stimulus for processing of transfected procaspase-3 to the active heterodimer of caspase-3, we monitored the mobility pattern of Geminin by Western blotting. In cells transfected with procaspase-3, but not with vector alone, a floating apoptotic population of cells was evident, and in these cells cleavage of Geminin was restored (Fig. 2B, lane 7). Thus, Geminin was targeted in MCF7 cells transfected with procaspase-3 cDNA, supporting the idea that Geminin cleavage during apoptosis is mediated by caspase-3.

To determine whether caspase-3 can cleave Geminin in vitro, we produced NH2-terminal His-tagged recombinant Geminin. Consistently, amino-terminal sequencing of the C1 product revealed that its NH2 terminus was intact (data not shown).

To identify the exact sites of Geminin cleavage by caspase-3, we used matrix-assisted laser desorption ionization-time of flight mass spectrometry to accurately determine the molecular weight of the major (C1) and minor (C2) cleavage products. Given these accurate molecular weight determinations, and considering that caspases require aspartic acid for cleavage, we were able to specify the major cleavage site at Asp-204 and the minor cleavage site at Asp-170.
To confirm that the SSTD204 is the site being recognized by caspase-3, we substituted the aspartic acid at position 204 for alanine using site-directed mutagenesis and cloned wild-type Geminin and mutant GemD204A, both EGFP tagged at the NH2 terminus, into the eukaryotic expression vector pCDNA3.1. HeLa cells were transfected with the empty pCDNA3.1-GFP vector, the GFP-Geminin, and GFP-GemD204A constructs and apoptosis was induced by treatment with staurosporine for 7 h. As shown in Fig. 3B, wild-type Geminin tagged to GFP is cleaved during apoptosis, similar to endogenous Geminin. The major cleavage product was not detected in apoptotic cells expressing the D204A mutant form of Geminin, suggesting that Asp-204 is the major site of the major caspase-3 cleavage product overlapping with the cleavage product, we therefore produced recombinant Geminin with Asp-170 changed to alanine and we incubated both wild type and the mutated forms of recombinant Geminin with recombinant caspase-3. As shown in Fig. 3D, wild type Geminin is cleaved after incubation with caspase-3 in vitro, producing both major and minor cleavage products (black and open arrows). In contrast, after the incubation of recombinant Gem-D170A with caspase-3, the minor cleavage product disappeared, suggesting that the site ESLD170 is the second site of Geminin recognized by caspase-3.

We therefore conclude that Geminin is cleaved by caspase-3 at two sites during apoptosis, both of which are located toward the COOH terminus of Geminin. Fig. 3E shows a multiple sequence alignment of Geminin from human, mouse, and Xenopus laevis (Geminin H and L) using Align X (Invitrogen). Caspase-3 cleavage sites are indicated with arrows.

**Cleavage of Geminin Is Regulated by Phosphorylation**—It was recently found that Geminin is a target for phosphorylation by CKII and this phosphorylation occurs at its carboxy-terminal region (32). Considering that phosphorylation by CKII has been shown to regulate caspase-mediated cleavage events in a number of proteins (33–36) and that close to the major cleavage site, a putative site of phosphorylation by CKII (S201) is located, we investigated a possible regulation of Geminin cleavage by CKII. The COOH terminus of Geminin. Fig. 3E shows a multiple sequence alignment of Geminin from human, mouse, and Xenopus, where the two aspartic acid residues targeted for cleavage have been marked. Despite the weak overall conservation of the carboxy-terminal region, the two aspartic acid residues that serve as sites for apoptosis-mediated cleavage in human cells are conserved at equivalent positions in mouse and Xenopus.

**FIGURE 3. Two sites at the carboxyl terminus of Geminin are targeted during apoptosis.** A, His-tagged recombinant Geminin was incubated with recombinant caspase-3 (lanes 2, 4, and 6) and cleavage products mixed with Ni2+-NTA-agarose beads. Bound and unbound fractions were analyzed by SDS-PAGE electrophoresis, followed by staining with Coomassie Brilliant Blue. B, HeLa cells were transfected with empty pCDNA3.1-GFP vector, the GFP-Geminin, and GFP-GemD204A constructs and apoptosis was induced by incubating with 5 μM STS for 7 h. Protein extracts were analyzed by Western blotting, using antibodies against Geminin and PARP. C, HeLa cells were transfected with GFP-Geminin and GFP-GemD204A constructs. 22 h following transfection, cell extracts were prepared, Geminin immunoprecipitated using a rabbit anti-Geminin antibody, and increased amounts of immunoprecipitate incubated with 0.5 μg of recombinant caspase-3 for 6 h, followed by Western blotting using a monoclonal anti-Geminin antibody. D, wild-type recombinant Geminin and Geminin with a substituted aspartic acid at position Asp-170 for an alanine (GemininD170A) were incubated with 0.5 μg of recombinant caspase-3 for 16 h. The incubation products were analyzed by SDS-PAGE and proteins were visualized by Coomassie Brilliant Blue staining. Filled and open arrowheads mark the C1 and the C2 cleavage products, respectively. E, multiple sequence alignment of Geminin from human, mouse, and Xenopus laevis (Geminin H and L) using Align X (Invitrogen). Caspase-3 cleavage sites are indicated with arrows.
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(A) Geminin is phosphorylated by casein kinase II at its carboxyl terminus. Recombinant His$_6$-tagged proteins corresponding to full-length (amino acids 1–209), the middle domain (amino acids 76–160), or the carboxyl-terminal domain (amino acids 115–209) of hGeminin, were incubated in the presence of [$\gamma$-32P]ATP with 30 units of CKII at 30 °C for the times indicated. Control phosphorylation reactions of full-length Geminin were carried out in parallel in the absence of CKII. The samples were subsequently assayed for SDS-PAGE electrophoresis and radioactivity quantified by phosphorimager analysis, whereas total protein were estimated by Coomassie Brilliant Blue staining and the Quant-iT™ (Molecular Probes) protein determination assay (data not shown). Band intensities normalized to protein amounts for each construct are plotted below their corresponding lanes. The middle part of Geminin showed no phosphate incorporation above background.

(B) Total incorporated radioactivity at the end point of the reactions shown in A was measured by spotting the reactions on phosphocellulose paper and measuring with a $\gamma$-counter (22). Counts/min obtained for each reaction normalized to the amount of substrate added is shown.

(C) Synthetic peptides corresponding to the wild type Geminin sequence surrounding site C1 (WT) or mutant forms substituting Ser-201 alone or Ser-200, Ser-201, Ser-202, and Thr-203 simultaneously to alanines (peptides S201A and SSST/A, respectively), were assayed for in vitro phosphorylation by casein kinase II. 400 µM of each peptide were incubated in the presence of 4 µCi of [$\gamma$-32P]ATP and 50 units of CKII for 1 h at 30 °C and radioactivity incorporated was measured with a $\gamma$-counter (22). Counts per minute of incorporated radioactivity obtained for each peptide are shown, whereas a reaction without peptide was carried out in parallel to estimate phosphate incorporation due to autophosphorylation of CKII. D, casein kinase II phosphorylation reduces cleavage of recombinant Geminin by caspase-3 in vitro. Recombinant Geminin (2.5 µg) was incubated with 500 units of recombinant casein kinase II for 5 h (lanes 5–8) in the presence (lanes 5–6) or absence (lanes 7–8) of ATP, and subjected to overnight incubation in the presence (lanes 6 and 7) or absence (lanes 8) of 0.5 µg of recombinant caspase-3. Parallel incubations in the absence of CKII are shown in lanes 1–4. Filled and open arrows indicate the major and minor cleavage products, respectively. The band marked with the asterisk (*) just below the minor cleavage product represents a nonspecific band of the casein kinase II preparation.

(E) HeLa cells were transfected with pcDNA3.1-GFP, GFP-Geminin, GFP-GemS201A, GFP-GemS201E, GFP-GemS200A,S201A,S202A,T203A, and GFP-GemS200E,S201E,S202E,T203E constructs. 22 h following transfection, cells were incubated in the presence (+) or absence (−) of 5 µM STS for 7 h. Protein extracts were analyzed by Western blotting, using antibodies against Geminin and PARP. F, schematic representation of Geminin mutants used in this study and their ability to be cleaved at site C1 during apoptosis in vivo.

(Gem-(115–209)) but not the middle part of Geminin (Gem-(76–160)). Full-length Geminin and Gem-(115–209) appear equally good substrates and the observed phosphorylation is very efficient with approximately 1 mol of phosphate transferred per mol of substrate on average (data not shown). In addition, a peptide corresponding to amino acids 198–209 of Geminin is phosphorylated by CKII in vitro (Fig. 4C). A mutant peptide where Ser-201 has been substituted by alanine shows markedly reduced, but still significant phosphorylation, which is abolished by concomitantly substituting the four competent for phosphorylation Ser-200, -201, -202, and Thr-203 residues by alanines. These data suggest that Geminin is indeed phos-
phorylated by CKII at sites adjacent to the major caspase-3 cleavage site.
To investigate the effects of CKII phosphorylation of Geminin on its ability to be cleaved by caspase-3 in vitro, recombinant Geminin was incubated with CKII prior to incubation with caspase-3 (Fig. 4D). As shown above, incubation of Geminin with caspase-3 results in cleavage of Geminin producing the major and minor cleavage products (lane 1). Interestingly, pre-incubation with recombinant CKII led to a pronounced decrease in the major cleavage product (lane 5), without affecting the minor cleavage production. In addition, the cleavage phenotype was restored in the absence of ATP, suggesting that regulation of cleavage depends on kinase activity (compare lanes 5 and 7).

To further investigate the involvement of CKII phosphorylation in Geminin targeting during apoptosis in vivo, mutated forms of Geminin in putative phosphorylation sites close to the caspase-3 cleavage site were constructed. Serine 201 was substituted with the non-phosphorylatable amino acid alanine (GemS201A) or with glutamic acid (GemS201E), mimicking the phosphorylation. Additionally, the adjacent to the major cleavage site and competent for phosphorylation serines 200, 201, 202, and the threonine 203, were simultaneously substituted to alanines (GemS200A,S201A,S202A,T203A) or glutamic acids (GemS200E,S201E,S202E,T203E). HeLa cells were transfected with wild type Geminin and the above mutants tagged to GFP and apoptosis was induced by treatment with staurosporine. Fig. 4E clearly shows the cleavage of wild type Geminin in STS-treated HeLa cells (lane 4). In contrast, the mutated GemS201A form showed less efficient cleavage, whereas GemS201E produced an enhanced cleavage phenotype (lanes 6 and 8, respectively). These results indicate that a phosphorylation event in Ser-201 accelerates the caspase-3-mediated cleavage of Geminin at the Asp-204 site. In contrast, when, in addition to Ser-201, the remaining serine and threonine residues adjacent to Asp-204 were simultaneously mutated to glutamic acid, in vivo Geminin cleavage was abolished, suggesting that possible additional phosphorylation events regulate the cleavage in a negative way. As expected from the low efficiency of the S201A mutant, the GemS200A,S201A,S202A,T203A mutant also failed to be cleaved in vivo. Fig. 4F summarizes the constructed mutated forms at the carboxyl terminus of human Geminin and their effects on the cleavage of Geminin at site C1 during apoptosis.

We therefore conclude that Geminin cleavage at site C1 is regulated by phosphorylation, in vitro and in vivo. Both positive
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FIGURE 6. Cleavage of Geminin at Asp-170 alters its ability to interact with hBrm. A, Geminin deletion mutant Δ171–209 loses its ability to bind hBrm protein. HeLa cells were co-transfected with HA-hBrm and pcDNA3.1-GFP, GFP-Geminin, GFP-Geminin Δ171–209, or GFP-Geminin Δ205–209 constructs. 36 h following transfection, total cell extracts were subjected to immunoprecipitation with a specific anti-HA antibody and the presence of Geminin constructs in immunoprecipitates assessed by Western blotting using an antibody against Geminin. B, Geminin mutated constructs Δ171–209 and Δ205–209 bind to Cdt1. HeLa cells were transfected with pcDNA3.1-GFP, GFP-Geminin, GFP-Geminin Δ171–209, and GFP-Geminin Δ205–209 constructs for 22 h. Total cell extracts were immunoprecipitated (IP) with a specific anti-GFP antibody and the presence of endogenous Cdt1 in the immunoprecipitates assessed by Western blotting using a Cdt1-specific antibody. GFP-transfected cells and mouse IgG immunoprecipitates served as negative controls.

DISCUSSION

Several studies have demonstrated the essential role of Geminin for the maintenance of genome integrity, regulating once per cell cycle replication by binding to and inhibiting Cdt1 activity (38, 39). In addition, Geminin has been characterized as a regulator of cell fate and differentiation, and it has been suggested to coordinate cell cycle and developmental controls (1, 2).

Herein, we show that Geminin is regulated during apoptosis by site-specific cleavage, resulting in the generation of two lower molecular weight cleavage products. This targeting occurred in tumor cells treated with a variety of apoptotic triggers as well as in primary cells, suggesting that Geminin cleavage is a general feature of human cells undergoing apoptosis. Programmed cell death, a fundamental process for normal development and maintenance of homeostasis in multicellular organisms, is driven by the activation of specific cysteine proteases known as caspases, which cleave selected target proteins to execute cell death (40). Among these proteases, caspase-3 mediates the cleavage of many key cellular proteins, contributing to the apoptotic phenotype (29). We showed that Geminin is not cleaved in caspase-3-deficient MCF7 cells, whereas the
introduction of caspase-3 into MCF7 cells restored the cleavage event. Moreover, recombinant caspase-3 can cleave recombinant Geminin in vitro and this targeting resembles the one observed in vivo, suggesting that Geminin is targeted during apoptosis by caspase-3. Many caspase-3 targets have been identified including the structural protein fodrin, the p21-activated kinase 2, the cytoskeletal protein gelsolin, and the inhibitor of the CAD endonuclease ICAD/DFF-45. Interestingly, recent studies revealed that the member of the pre-replicative complex Cdc6 is also selectively cleaved by caspase-3 during apoptosis (26, 42). This cleavage event generates an NH2-terminal truncated form of Cdc6 that lacks the nuclear export sequence, resulting in an increased nuclear retention of the truncated fragment and has been suggested to promote apoptosis acting as dominant negative inhibitor of DNA replication (43).

Based on data obtained from mass spectrometry analysis we identified the exact sites of Geminin cleavage by caspase-3 in the carboxyl-terminal portion of Geminin at sites ESLD170 and SSTD204. Consistently, GFP-GemD204A mutant was not cleaved in apoptotic extracts in vitro and recombinant protein Geminin D170A was not cleaved by caspase-3 in vitro. In addition, similar caspase-3 cleavage site motifs have been identified previously. The sex determination protein FEM-1 is cleaved by the caspase-3 homologue in Caenorhabditis elegans at an ELLD motif (44), whereas the terminal region of pro-interleukin-16 is cleaved by caspase-3 recognizing the SSTD motif, thereby releasing bioactive interleukin-16 (45). Whereas the COOH-terminal region of Geminin is not highly conserved during evolution, aspartic acid residues have been conserved at the corresponding sites in mouse and Xenopus, suggesting that similar cleavage events may take place in other vertebrates.

A previous study suggested that Geminin is phosphorylated by CKII at its carboxyl terminus: Geminin is a CKII substrate in vitro, Geminin phosphorylation is abrogated in cells treated with the specific CKII inhibitor 4,5,6,7-tetabromobenzotriazole (TBB) in vivo, whereas a great reduction of phosphorylation was observed in a Geminin mutant lacking the last 34 amino acids, where CKII consensus sites are located (32). However, the function of this phosphorylation remained unknown. We show here that Geminin is efficiently phosphorylated in vitro by CKII and that this phosphorylation prevents its cleavage by caspase-3. In addition, phosphorylation mutants of Geminin showed altered susceptibility to cleavage by caspase-3 in vivo. We suggest that the caspase-3-mediated cleavage of Geminin during apoptosis is regulated by phosphorylation, and that CKII is a likely mediator of this effect. The enhanced cleavage of the GemS201E mutant, which mimics phosphorylation at a CKII consensus site, suggests that phosphorylation of Geminin by CKII in this site would regulate cleavage of Geminin in a positive way. In contrast, the abolished cleavage of Geminin observed in the GemS200E,S201E,S202E,T203E mutant, which is consistent with the noticed decrease of Geminin cleavage after the phosphorylation of Geminin in the presence of excess recombinant CKII in vitro, suggests that additional phosphorylation events negatively regulate cleavage by caspase-3. This idea is supported by the presence of additional phosphorylation competent serine and threonine residues adjacent to the caspase-3 cleavage site. We show using synthetic peptides that, indeed, Ser-201, as well as additional Ser/Thr residues proximal to the caspase-3 C1 cleavage site can be phosphorylated by CKII in vitro.

In accordance with our observations, similar enhancement or resistance to caspase-mediated proteolysis conferred by phosphorylation has been previously reported for proteins cleaved during apoptosis and notably many of these protein substrates were also found to be phosphorylated by CKII. The phosphorylation of protein kinase PITSRE p110 has been shown to enhance its cleavage by caspases (46), whereas CKI- and CKII-mediated phosphorylation of the pro-apoptotic protein Bid prevents its cleavage by caspase-8 (33). Caspase-3-mediated cleavage of phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome Ten) is negatively regulated by CKII-mediated phosphorylation (35), whereas the phosphorylation of the nucleolar factor acinus by Akt kinase
results in its resistance to caspase-mediated cleavage (47). Interestingly, although many of the caspase substrates identified to date (41) contain within the sequence recognized by caspses competent for phosphorylation serine or threonine residues, only a few studies have revealed regulation of caspase-mediated cleavage events by phosphorylation. The exact mechanism whereby phosphorylation confers this regulation is unclear; nevertheless, one likely possibility is that phosphorylation events alter the accessibility of the protease to the recognition site. It would be interesting to investigate which conditions lead to phosphorylation-mediated enhancement and which to inhibition of Geminin cleavage and how these two opposing possibilities are coordinated.

Cleavage of Geminin by caspase-3 during apoptosis results in generation of two truncated forms. A major cleavage product is produced by cleavage at site 204, whereas a minor cleavage product is produced by cleavage at site 170. Fig. 7A presents the known interactions of Geminin with Cdt1, Siz3, and Brg1 or Brm and the sites of its cleavage by caspase-3 during apoptosis. We show here a pro-apoptotic effect of the major cleavage product: an apoptotic phenotype is induced when this form is ectopically expressed in cultured cells. The minor cleavage product in contrast has no such activity, suggesting a gain-of-function role for the major product which is lost upon further cleavage. Interestingly, whereas both cleaved products bind strongly to Cdt1, the deletion mutant of Geminin (GemΔ171–209), which mimics the cleavage event at site C2, loses its interaction with the catalytic site C2 results in reduced interactions with the chromatin remodeling factors Brm or Brg1. Previous findings have highlighted the importance of an intricate balance of Geminin interactions with its multiple partners for accurate coordination of cell cycle and cell differentiation pathways, whereas affecting this balance has pronounced consequences for cell fate decisions. Our demonstration of Geminin cleavage during apoptosis suggests that this balance may be interrupted when certain cells initiate an apoptotic pathway (Fig. 7B). Whereas in cultured cells, cleavage at site C2 is a minor cleavage event, we would like to speculate that it may constitute a prominent event in certain cell types or developmental stages. Apoptosis, proliferation, and differentiation are closely linked and must be precisely coordinated during development and in the adult. Further analysis will be required to determine how Geminin cleavage during apoptosis affects cell fate decisions in specific tissues and physiological contexts.

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