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Geminin is cleaved by caspase-3 during apoptosis in *Xenopus* egg extracts

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Abstract

Geminin is an important cell cycle regulator having a dual role in cell proliferation and differentiation. During proliferation, Geminin controls DNA synthesis by interacting with the licensing factor Cdt1 and interferes with the onset of differentiation by inhibiting the activity of transcription factors such as Hox and Six3. During early development Geminin also functions as neural inducer. Thus differential interaction of Geminin with Cdt1 or development-specific transcription factors influence the balance between proliferation and differentiation. Here, we report an additional feature of Geminin showing that it is a novel substrate of caspase-3 during apoptosis in *in vitro Xenopus* egg extracts. We also show that cleavage of Geminin occurs both in solution and on chromatin with distinct kinetics. In addition we show that cleavage of Geminin by caspase-3 is not relevant to its function as regulator of DNA synthesis, suggesting that its cleavage may be relevant to its role in differentiation.

Keywords: Cell cycle; Licensing; DNA replication; Chromatin; 6-DMAP

The development of an organism relies on a fine cross talk between cell proliferation and differentiation to ensure that a well-defined number of cells are generated that will be later remodelled into tissues and organs by the differentiation programmes. During early stages of development cell proliferation is dominant, while during differentiation proliferation is down regulated to prevent abnormal growth and unbalanced cellular homeostasis. In addition to this regulation, cellular homeostasis is also controlled by a highly regulated process of programmed cell death or apoptosis that ensures the elimination of unwanted or highly damaged cells. This process is first initiated by the activation of a family of aspartic-specific cysteine proteases, the caspases, leading to fragmentation of the DNA and subsequent cell death.

The Geminin protein was first identified in the frog *Xenopus laevis* as both an inhibitor of DNA synthesis and inducer of differentiation of neural tissues [1–3]. These two properties have made of Geminin an interesting factor that may have an important role in regulating the balance between cell proliferation and differentiation during development. At the molecular level, these properties lie into two distinct and functionally separable domains of the Geminin protein that have been characterized in several model organisms including mammalian cells. The DNA synthesis inhibition domain (coiled-coil) lies in the central part of Geminin and interacts with the essential DNA replication factor Cdt1 [4–6]. This interaction is essential but not sufficient for Cdt1 inhibition [5–7] as an additional step seems to be required that involves the oligomerization of Geminin on chromatin, at DNA replication origins [8]. The neuralization domain lies in the N-terminal part of Geminin [2], but the molecular mechanisms underlying this activity are still unknown. Geminin can also interact with a number of transcription factors, such as Six-6, Hox genes, and Polycomb proteins, as well as with the chromatin remodelling factor Brg1, a member of the SWI/SNF family of chromatin remodelling factors [9–11]. It has been suggested that Geminin, by interacting with different partners at a given time, may be a regulator of cell proliferation and

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differentiation. For instance, during differentiation, down regulation of Cdt1 may give the opportunity to Geminin to interact with other partners and therefore drive the cell towards a differentiation program [11].

Here, we report that Geminin is cleaved by caspase-3 during apoptosis stimulated by an inhibitor of serine–threonine protein kinases in *Xenopus* egg extracts. Cleavage of Geminin occurs both in the cytoplasm and on chromatin, although with different kinetics. Importantly, we show that the cleavage of Geminin by caspase-3 has no role in regulating the function of Geminin as inhibitor of DNA synthesis suggesting that its cleavage may be related to the function of Geminin in differentiation.

Materials and methods

Preparation of Xenopus egg extracts. Mitotic egg extracts were prepared as previously described [12,13] and frozen at –80 °C as 50 µl aliquots. For the Geminin cleavage assay, upon thawing extracts were supplemented with an energy regeneration system (10 µg/ml creatine kinase; 10 mM creatine phosphate, 1 mM ATP, and 1 mM $MgCl_2$) and released in interphase with 0.5 mM $CaCl_2$ in the presence or absence of the indicated concentrations of 6-DMAP (Sigma). Where specified 1 nM of the caspase-3 inhibitor *N*-acétyl-D-E-V-D-ala (Sigma) was used.

Replication reactions. DNA replication was monitored by incorporation of α-[^{32}P]dCTP (20 µCi/ml) into trichloric acetic acid (TCA) insoluble material precipitated onto GF/C filters (Whatmann) on ice. Reactions were set up at room temperature by addition of demembranated sperm nuclei (1000 nuclei/µl of egg extract) to mitotic egg extracts activated with 0.5 mM $CaCl_2$ and supplemented with 250 µg/ml of cycloheximide and an energy regeneration system (see above). Where stated, recombinant proteins were added to replication reactions at a concentration of 40 nM.

Antibodies and recombinant proteins. Antibodies against Geminin were described in [7]. MCM3 antibody was as previously described [14]. Recombinant Geminin and Cdt1 were prepared as previously described [15].

Isolation of chromatin fractions. Detergent-extracted chromatin was obtained by five times dilution of replication reaction with ice-cold CPB buffer (50 mM KCl; 20 mM Hepes–KOH, pH 7.7; 5 mM $MgCl_2$; 2% sucrose; leupeptin, aprotinin, and pepstatin, 5 µg/ml each) supplemented with 0.1 % NP-40 followed incubation on ice for 1 min. Chromatin was then isolated by centrifugation at 6000*g* for 5 min at 4 °C through a 0.7 M sucrose cushion made in CPB. Chromatin-bound proteins were eluted in Laemmli buffer.

TUNEL assay. Nuclei assembled in egg extracts were recovered on coverslips by 10-fold dilution in CPB followed by centrifugation at 1500*g* for 10 min at 4 °C through a 0.35 M sucrose cushion made in CPB. Nuclei were permeabilized on cover slips and processed for TUNEL assay as recommended by the supplier using the Cell Death kit® (Roche). For visualization of DNA, nuclei were stained with Hoechst.

Detection of DNA fragmentation by agarose gel electrophoresis. Replication reactions assembled as described above were diluted 10-fold in STOP mix (0.5% SDS, 10 mM EDTA) and incubated for 1 h at 52 °C with proteinase K (500 µg/ml). Samples were phenol/chlorophorm-extracted and analyzed by 1.5% agarose gel electrophoresis in TAE buffer followed by SyberGold staining.

Results and discussion

Geminin is cleaved upon mitotic exit in the presence of protein kinases inhibitor 6-DMAP

6-DMAP is a purine analogue that inhibits serine/threonine protein kinases such as cyclin-dependent kinases (CDKs). When added to mitotic-arrested *Xenopus* egg extracts 6-DMAP strongly inhibits DNA synthesis [16] by blocking the activity of the essential DNA replication factor Cdt1 [17]. In these conditions a large amount of Geminin accumulates on chromatin suggesting that the inhibition of DNA synthesis (licensing) is very likely due to failure to degrade Geminin [17], which is normally targeted for proteolysis by the Anaphase Promoting Complex (APC) upon mitotic exit [1]. Accumulation of Geminin on chromatin would explain the observed block of DNA synthesis by inhibition of the Cdt1 protein. In the attempt to test this hypothesis, we sought to determine the stability of Geminin in egg cytoplasm in the presence of 6-DMAP. For this purpose mitotic-arrested egg extracts were synchronously released into interphase by addition of $CaCl_2$ in the absence or presence of 6-DMAP. Samples were taken at different intervals during a time course of the experiment and the stability of Geminin was assessed by Western blot with specific antibodies. As shown in Fig. 1A (−6DMAP) upon mitotic exit about 60% of Geminin is degraded, consistent with previous results showing that in *Xenopus* egg extracts Geminin is not completely degraded by the APC [7,18]. However, in the presence of 5 mM 6-DMAP (Fig. 1A, +6DMAP), degradation of Geminin was strongly inhibited for 10–20 min (Figs. 1B and 2B, +6DMAP) upon entry into interphase. Later on, Geminin degradation was followed by the appearance of two polypeptides of faster mobility (+6-DMAP, arrows). These faster migrating forms of Geminin remained fairly stable throughout the time course of the experiment. In the same time course no detectable faster migrating poly-

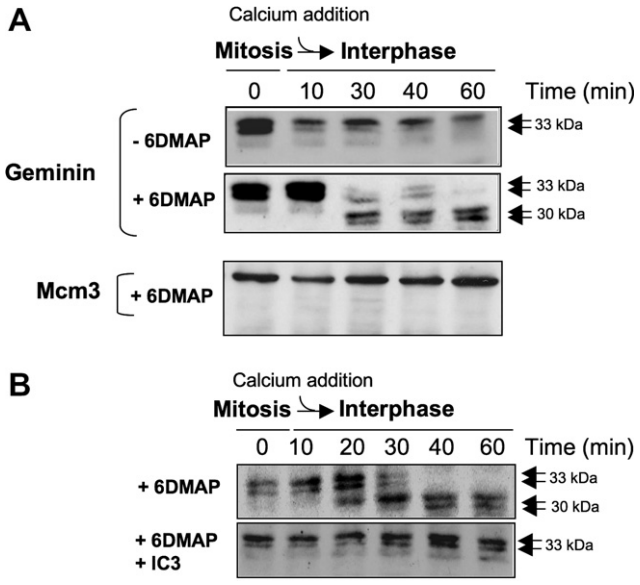

Fig. 1. Geminin is cleaved by Caspase-3 in *Xenopus* egg extracts. (A) Western blot of mitotic egg extracts released in interphase in the presence (+6-DMAP) or absence (−6-DMAP) of 5 mM 6-DMAP. Geminin, or Mcm3, were detected with specific antibodies. Arrows indicate the different mobility forms of Geminin. (B) Western blot of egg extracts processed as in (A) but in the presence of the Caspase-3 inhibitor (+IC3).