FFA-1, a Protein That Promotes the Formation of Replication Centers Within Nuclei

Hong Yan and John Newport*

In the nuclei of eukaryotic cells, initiation of DNA replication occurs at a discrete number of foci. One component of these foci is the DNA replication factor RP-A. Here, the process leading to the association of RP-A with foci was reconstituted with cytosolic fractions derived from *Xenopus* eggs. With the use of this fractionated system, a 170-kilodalton protein required for the assembly of RP-A into foci was identified and purified. The protein appears to be an integral component of the foci at which replication of DNA is initiated in eukaryotic nuclei.

DNA replication is an essential and highly conserved process. Many proteins indispensable for DNA replication in eukaryotic cells have been identified with the use of a cell-free model system composed of an SV40 DNA template, viral T antigen protein, and fractionated cell extracts (*1*, *2*). Although this system has been valuable in the development of a model of replication, it does not recreate the steps that lead to the initiation of synthesis at a cellular origin of replication in the context of the native nuclear environment. This limitation is important because cell-free systems with eukaryotic chromatin encapsulated within a native nuclear structure show that the nucleus has an indispensable function in the replication process (*3*, *4*).

DNA replication within eukaryotic cells begins at a large number of discrete, subnuclear locations (*5*–*8*). It has been proposed that these replication centers are composed of 300 to 1000 DNA loops that are aggregated together around a single focal hub (*8*). Indirect immunofluorescent staining of nuclei indicates that a number of proteins involved in DNA replication, including the single-stranded DNA binding protein RP-A, aggregate at these foci (*9*–*11*). RP-A associates with foci before initiation (*9*–*11*), distributes over DNA surrounding foci during replication (*11*), and then localizes to foci again once replication is completed (*11*) (Fig. 1A). The persistent localization of RP-A at foci after DNA replication suggests that these centers may be a stable structural complex important for both replication and other DNA functions. In spite of the potential importance of these focal structures in the regulation of the initiation of DNA replication, little is known about either their structural organization or their protein composition.

Sperm chromatin with the membrane removed (*9*–*11*) or purified lambda DNA (*11*) incubated in cytosol made from *Xenopus* eggs binds RP-A in a focal distribution. Thus, focus formation is independent of nuclear formation, and all of the proteins required to form foci are present in the egg cytosol. Here, we used this cell-free system to initiate a search for proteins that are involved in the formation of these foci.

To identify proteins that are essential for either the formation or function of foci, high-speed cytosolic extracts derived from unfertilized eggs were prepared (*12*) and fractionated by ammonium sulfate (AS) precipitation (*13*). These fractions were then analyzed individually and in combination to determine which fractions were essential for the reconstitution of RP-A foci-forming activity on purified sperm chromatin template. AS fraction 1, which contained RP-A, could not form foci. However, when AS fractions 1 and 3 were combined, RP-A bound to sperm in a focal pattern identical to that of crude cytosol (Fig. 1B). Further characterization showed that this reaction, as in unfractionated cytosol, was dependent on adenosine triphosphate (ATP) but not on topoisomerase II (*9*, *10*, *14*). To determine whether AS fraction 3 contained all of the components needed for foci formation except RP-A, we combined purified RP-A with AS fraction 3 alone. This combination failed to form foci on sperm chromatin (Fig. 1C). This observation indicated that AS fraction 1 contained both RP-A and at least one other protein required for foci formation. We separated this new protein from RP-A by fractionating AS fraction 1 on a single-stranded DNA cellulose column. When a low salt fraction from this column, which lacked RP-A, was combined with purified RP-A and AS fraction 3, RP-A foci formed efficiently on sperm chromatin (Fig. 1D).

The proteins eluting from single-stranded DNA (ssDNA) cellulose with this new fraction were further fractionated on heparin sulfate and Mono Q columns and assayed for foci-forming activity. When proteins that eluted from the final Mono Q column were assayed for foci-forming activity in combination with purified RP-A and AS fraction 3, we found that activity was quantitatively associated with the amount of a single purified protein of 170 kD (Fig. 2). The specific activity of the foci-forming fraction increased 40,000-fold during purification, and 10 μg of p170 was purified from 50 ml of pure egg cytosol (yield = 20%). On the basis of these numbers, we

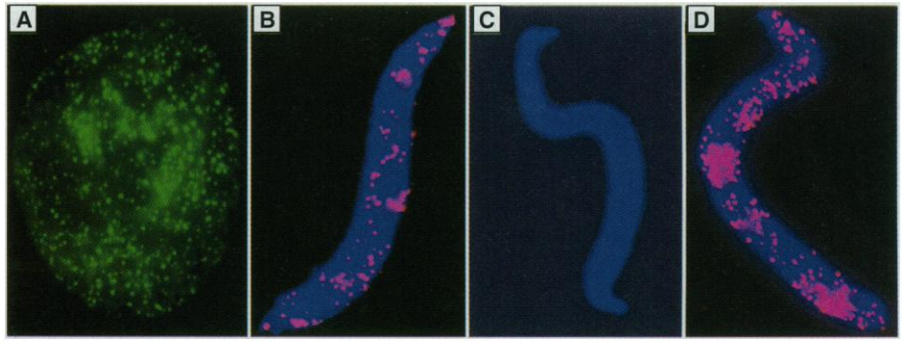

Fig. 1. Identification of RP-A foci-forming activity 1 (FFA-1). (**A**) RP-A foci in a nucleus that has completed DNA replication after a 150-min incubation in an interphase *Xenopus* egg extract (*11*). Biotin–deoxyuridine triphosphate (incorporated into DNA during replication) and total DNA distributed fairly uniformly throughout the nucleus (not shown in figure) (*21*), whereas RP-A was localized to about 300 discrete foci (green). (**B**) RP-A foci formation with AS fractions 1 and 3 (*13*). RP-A foci were formed with 2.5 μl of AS fraction 1, 2.5 μl of AS fraction 3, 1 μl of 10× ATP regeneration system, 5000 sperm, and 4 μl of ELB (*13*). (**C**) Absence of RP-A foci formation with RP-A and AS fraction 3. Sperm were incubated in the standard RP-A foci cocktail (*13*). (**D**) RP-A foci formation with an ssDNA column fraction containing the FFA-1 activity (*22*) in the standard RP-A foci cocktail (*13*). Sperm in (B) through (D) were incubated at room temperature for 60 min, fixed, and stained for RP-A with an RP-A antibody (*20*) followed by a rhodamine-labeled secondary antibody (red) and for DNA with bisbenzimide (blue) as described (*11*). The DNA and RP-A staining images were collected separately with different excitation emission filter combinations and then superimposed on each other to generate the multicolor composite. Because of its large size, the nucleus in (A) flattens onto the cover slip and most of the foci are observable in a single focal plane. In contrast, the sperm in (B) through (D) are fairly rigid, and at least three focal planes (not shown in figure) are required to observe and count all foci on a single sperm.

University of California, San Diego, Department of Biology, La Jolla, CA 92093, USA.

*To whom correspondence should be addressed.

estimate that the endogenous concentration of p170 in the initial cytosol (and egg) is 6 nM. Because the binding of RP-A to foci is absolutely dependent on the addition of this protein, we have named it *Xenopus* foci-forming activity 1 (FFA-1).

To characterize the role of FFA-1 in foci formation, we added increasing amounts of FFA-1 to an assay mix containing AS fraction 3, sperm chromatin, ATP, and excess RP-A (Fig. 3). At low concentrations of FFA-1, a small number of brightly staining RP-A foci (Fig. 3B) and a large number of weakly staining foci (*15*) (not visible in Fig. 3B) were observed. At progressively higher concentrations of FFA-1, the number of bright foci increased linearly with FFA-1 concentration, eventually reaching a plateau at concentrations above 24 nM (Fig. 3, A and C). At the same time, the number of weakly staining foci decreased. At saturating amounts of FFA-1, we observed 270 ± 25 bright RP-A foci sites per sperm. These saturated sites were uniformly distributed over the surface area of the sperm and were uniform in size and fluorescent intensity. Each sperm genome was able to form a finite and conserved number of foci sites. The uniform size and intensity of the saturated foci suggest that the foci have a unique structural composition containing multiple RP-A binding sites. The numerical correlation between the maximum number of RP-A foci sites on sperm and the number of RP-A sites observed in intact nuclei (300 sites per nucleus) both before and after DNA replication (Fig. 1A) suggests that FFA-1 is essential for the formation of the focal centers associated with DNA replication in eukaryotic nuclei.

We investigated how FFA-1 is involved in the binding of RP-A to foci. Topoisomerase II (*16*) and the large subunit of DNA polymerase α (*17*), two proteins with similar molecular weights, were excluded as candidates for FFA-1 by protein immunoblot analysis with antibodies to these two proteins (*18*). A replication origin recognition complex (ORC) containing at least five subunits has been purified from the yeast *Saccharomyces cerevisiae* (*19*). Both the molecular weight of FFA-1 and its isolation as a single-subunit protein demonstrate that it is not a component of the core ORC complex. Thus, FFA-1 may not directly contact the replication origin sequences on sperm chromatin.

Therefore, we considered three alternative functions for FFA-1 in foci formation. (i) FFA-1 could be a chaperone protein that ferries and assembles RP-A into preexisting foci binding sites. (ii) FFA-1 could bind to preexisting foci sites and could act as an RP-A receptor. (iii) FFA-1 could be a structural component necessary for the assembly of foci.

To determine whether FFA-1 is a chaperone that ferries RP-A to foci sites, we examined whether sperm when first exposed to FFA-1 and then washed could subsequently bind RP-A or whether RP-A binding occurs only in the presence of FFA-1. Sperm chromatin was first treated with AS fraction 3 and then washed and pelleted onto glass cover slips. The bound sperm were then sequentially treated with either FFA-1 and then RP-A, or RP-A and then FFA-1. Sperm that were first treated with FFA-1 and then washed formed a large number of discrete foci after addition of RP-A (Fig. 4A). In contrast, when RP-A was added before FFA-1, foci were not observed (Fig. 4B). These results suggest that FFA-1 is not a chaperone protein. Rather, our results indicate that FFA-1 stably associates with foci elements and in so doing generates binding sites for RP-A at these foci.

Because RP-A plays an essential role in

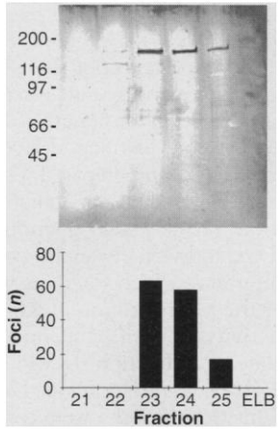

Fig. 2. Purification of the FFA-1 protein. About 10 μg of FFA-1 was purified from 50 ml of *Xenopus* egg cytosol (*22*). (**Top**) The final Mono Q fractions around the FFA-1 activity peak (15 μl each) were analyzed on a SDS-polyacrylamide gel (4 to 15% gradient). The proteins were stained by the silver procedure (*23*). The molecular markers are indicated on the left in kilodaltons. (**Bottom**) RP-A foci-forming activity of the corresponding fractions. Each fraction (0.5 μl) was assayed in the standard RP-A foci cocktail (*13*). The total number of brightly staining RP-A foci formed on sperm chromatin in each fraction were counted.

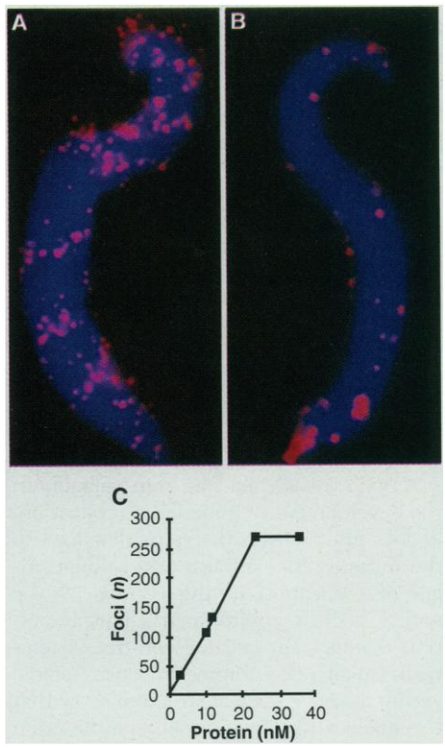

Fig. 3. Dependence of RP-A foci on the concentration of FFA-1. RP-A foci were formed with different amounts of purified FFA-1 protein in the standard RP-A foci cocktail (*13*). (**A**) RP-A foci formed on sperm chromatin with 24 nM FFA-1. Red, RP-A; blue, DNA. (**B**) RP-A foci formed on sperm chromatin with 3 nM FFA-1. Colors are as in (A). (**C**) Numbers of brightly staining RP-A foci formed on sperm chromatin plotted against the concentration of FFA-1 in the reaction. Total foci numbers were derived by counting the total number of foci in three separate focal planes. Weakly staining foci cannot be observed at the exposure presented, and apparent differences in the size of brightly staining foci are largely a result of the location of foci in slightly different focal planes.

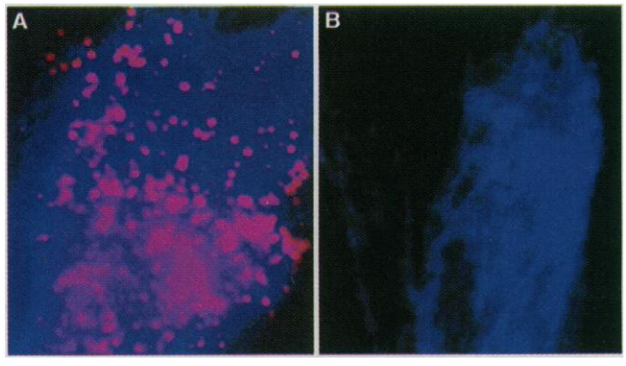

Fig. 4. Stepwise assembly of RP-A foci. Sperm (500 per microliter) were first incubated with AS fraction 3 at room temperature for 60 min and then spun onto glass cover slips. The cover slips were rinsed with ELB and inverted over a 40-μl drop of 24 nM FFA-1 (**A**) or 120 nM RP-A (**B**) on a sheet of parafilm in a humidified plastic box for 60 min. This step was then repeated with a 40-μl drop of 120 nM RP-A (A) or 24 nM FFA-1 (B). The ATP regeneration system was present in all three steps. Sperm, which became extensively decondensed, were finally fixed and stained for RP-A (red) and DNA (blue) as described (*11*).

the earliest step of replication initiation (*1*, *2*), FFA-1 is likely to be a factor involved in the establishment of functional origin complexes within nuclei. The purification of quantities of FFA-1 able to be sequenced should allow for a direct molecular dissection of its role in foci formation. Moreover, the in vitro system and the assay procedures used for purification of FFA-1 should facilitate the purification of the remaining factors required for the assembly of foci. Purification of these factors will provide a biochemically tractable system for understanding how the spatial aggregation of DNA into discrete, intranuclear domains participates in the initiation of DNA replication, in particular, and in DNA function, in general, in eukaryotic cells.

REFERENCES AND NOTES

1. B. Stillman, *Annu. Rev. Cell Biol.* **5**, 197 (1989).
2. S. Waga and B. Stillman, *Nature* **369**, 207 (1994).
3. J. Newport, *Cell* **48**, 205 (1987).
4. M. A. Sheehan, A. D. Mills, A. M. Sleeman, R. A. Laskey, J. J. Blow, *J. Cell Biol.* **106**, 1 (1988).
5. H. Nakamura, T. Morita, C. Sato, *Exp. Cell Res.* **165**, 291 (1986).
6. H. Nakayasu and R. Berezney, *J. Cell Biol.* **108**, 1 (1989).
7. A. D. Mills *et al.*, *J. Cell Sci.* **94**, 471 (1989).
8. L. S. Cox and R. A. Laskey, *Cell* **66**, 271 (1991).
9. Y. Adachi and U. K. Laemmli, *J. Cell Biol.* **119**, 1 (1992).
10. ———, *EMBO J.* **13**, 4153 (1994).
11. H. Yan and J. Newport, *J. Cell Biol.* **129**, 1 (1995).
12. C. Smythe and J. W. Newport, *Methods Cell Biol.* **35**, 449 (1991).
13. Cytosol from unfertilized *Xenopus* eggs was diluted two times with ELB [250 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol (DTT), and 10 mM Hepes (pH 7.7)] and precipitated with AS at the following saturations: 37.5% (AS fraction 1), 37.5 to 52.5% (AS fraction 2), and 52.5 to 75% (AS fraction 3). These fractions were resuspended in ELB (1/4 of the starting cytosol volume) and dialyzed extensively against ELB. The standard RP-A foci formation cocktail contains 2 μl of AS fraction 3, 1 μl of 10× ATP regeneration system (20 mM ATP, 200 mM phosphocreatine, 50 μg/ml creatine phosphokinase, and 20 mM $MgCl_2$), 120 nM RP-A (*20*), and 500 μl of sperm with membranes removed. The fraction to be assayed and ELB were then added to the cocktail to make a final volume of 10 μl. After 60 min at room temperature, sperm were fixed and stained for RP-A with an RP-A antibody and for DNA with bisbenzimide as described (*11*).
14. H. Yan and J. Newport, unpublished data.
15. ———, unpublished data.
16. M. Benedetti, I. Baldi, E. Mottoccia, G. P. Tocchini-Valentini, *EMBO J.* **2**, 1303 (1983).
17. M. Shioda, E. M. Nelson, M. L. Bayne, R. M. Benbow, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7209 (1982).
18. H. Yan and J. Newport, unpublished data.
19. S. P. Bell and B. Stillman, *Nature* **357**, 128 (1992).
20. F. Fang and J. W. Newport, *J. Cell Sci.* **106**, 983 (1993).
21. H. Yan and J. Newport, unpublished data.
22. The FFA-1 protein was purified by the following procedure (performed at 4°C). AS fraction 1 from 50 ml of cytosol was resuspended in buffer A [25 mM tris·HCl (pH 7.5), 1 mM EDTA, and 1 mM DTT] supplemented with 1 μg of aprotinin per milliliter and leupeptin. After dialysis against the same buffer, the sample was adjusted to an NaCl concentration of 200 mM and then loaded onto a ssDNA cellulose column (1 cm by 8 cm; Amersham) equilibrated with buffer A200 (buffer A plus 200 mM NaCl). The column was washed with A250 (buffer A plus 250 mM NaCl) and eluted with A600 (buffer A plus 600 mM NaCl). The A600 fraction was adjusted to an NaCl concentration of 50 mM with A0 and applied to a HiTrap heparin column (2 ml; Pharmacia), which was then eluted with a 24-ml linear gradient of NaCl (50 to 650 mM) in buffer A. The active fractions (around 450 mM) were pooled, adjusted to an NaCl concentration of 100 mM with A0, and loaded onto a Mono Q column (1 ml; Pharmacia). Bound proteins were eluted with a 16-ml linear gradient of NaCl (100 to 500 mM) in buffer A; FFA-1 eluted around 300 mM. This procedure yielded about 10 μg of FFA-1, with a yield of about 20%.
23. B. R. Oakley, D. R. Kirsch, N. R. Morris, *Anal. Biochem.* **105**, 361 (1980).

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Inhibition of ICE Family Proteases by Baculovirus Antiapoptotic Protein p35

Nancy J. Bump, Maria Hackett, Margaret Hugunin, Somasekar Seshagiri, Kenneth Brady, Patrick Chen, Catherine Ferenz, Simon Franklin, Tariq Ghayur, Ping Li, Peter Licari, John Mankovich, Lianfa Shi, Arnold H. Greenberg, Lois K. Miller, Winnie W. Wong*

The baculovirus antiapoptotic protein p35 inhibited the proteolytic activity of human interleukin-1β converting enzyme (ICE) and three of its homologs in enzymatic assays. Coexpression of p35 prevented the autoproteolytic activation of ICE from its precursor form and blocked ICE-induced apoptosis. Inhibition of enzymatic activity correlated with the cleavage of p35 and the formation of a stable ICE-p35 complex. The ability of p35 to block apoptosis in different pathways and in distantly related organisms suggests a central and conserved role for ICE-like proteases in the induction of apoptosis.

Apoptosis (programmed cell death) is essential for normal development and homeostasis in multicellular organisms and provides a defense against viral invasion and oncogenesis (*1*). The *p35* gene from *Autographa californica* nuclear polyhedrosis virus (AcMNPV) encodes an inhibitor that blocks apoptosis in mammalian and insect cell lines (*2*–*3*) and in developing embryos and eyes of transgenic *Drosophila melanogaster* (*4*) and partially substitutes for *ced*-9 function in *Caenorhabditis elegans* (*5*). The ability of p35 to block apoptosis induced by different signals in diverse organisms suggests that it acts at a central and phylogenetically conserved point in the apoptotic pathway. The mechanism by which p35 blocks apoptosis is not known, but it may be an inhibitor of the proteases of the ICE family. The involvement of these proteases in apoptosis was suggested when ICE or the related proteins ICE–CED-3 homolog-1 (ICH-1), ICH-2, or CPP32 induced cell death when each was overexpressed in mammalian or insect cell lines (*6*–*9*). However, because ICE-deficient mice do not exhibit the hallmarks of defective apoptosis, the apoptotic process may require the involvement of more than one member of the *ICE* gene family (*10*).

We tested the ability of p35 to inhibit ICE activity in a COS-1–derived cell line that constitutively expressed human prointerleukin-1β (proIL-1β) (*11*). Cotransfection of these cells with increasing amounts of plasmids encoding p35 and encoding either the p45 form (residues 1 to 404) or the p32 form (residues 120 to 404) of ICE resulted in decreases of as much as 90% in the release of mature IL-1β (Fig. 1A). To control for the possibility that p35 interfered with the expression of ICE or proIL-1β, we immunoprecipitated these proteins and showed that the amounts of p32 ICE precursor and proIL-1β were similar in the presence or absence of p35 (*12*). These findings suggest that p35 inhibits the enzymatic activity of ICE.

To confirm more directly that p35 is an ICE inhibitor, we purified recombinant p35 and tested it in a colorimetric assay that measured the activity of purified recombinant human ICE on a tetrapeptide substrate. In addition, we tested p35 with three other purified recombinant human ICE homologs, ICH-1, ICH-2, and CPP32 (*13*, *14*). Cleavage of the respective tetrapeptide substrates by ICE, ICH-1, ICH-2, and CPP32 was inhibited in a concentration-dependent manner when the enzymes were

N. J. Bump, M. Hackett, M. Hugunin, K. Brady, P. Chen, C. Ferenz, S. Franklin, T. Ghayur, P. Li, P. Licari, J. Mankovich, W. W. Wong, BASF Bioresearch Corporation, 100 Research Drive, Worcester, MA 01605, USA.
S. Seshagiri and L. K. Miller, Departments of Entomology and Genetics, University of Georgia, Athens, GA 30602, USA.
L. Shi and A. H. Greenberg, Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba R3E 0V9, Canada.

*To whom correspondence should be addressed.