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Inhibition of initiation of DNA replication in Xenopus egg extracts by a phosphatase inhibitor, calyculin A

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The protein phosphatase inhibitor calyculin A was found to strongly inhibit the DNA replication activity of Xenopus egg extracts in a dose-dependent manner. Calyculin A (0.8nM) completely prevented the oscillation in DNA replication activity of mitotic extracts released by the addition of $CaCl_2$. In contrast, in the case of extracts prepared from eggs activated by calcium ionophore A23187, calyculin A (0.8nM) had no effect on the first peak of the oscillation in activity but inhibited the second peak of the oscillation. These results strongly suggest that calyculin A inhibits a protein phosphatase, which is involved in the initiation of DNA replication in the Xenopus egg extracts.

Very little is known about how the initiation of eukaryotic DNA replication is coordinated so that the genome is replicated precisely once per cell cycle. The eggs of Xenopus laevis have proved to be an exceptionally good source of a cell-free system for studying the initiation of chromosomal DNA replication in vitro.

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Abbreviations: PP2A, protein phosphatase 2A; PP1, protein phosphatase 1.

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The initiation of DNA replication can only occur in interphase, and not in Xenopus egg extracts arrested in mitosis(1). If mitotic extracts are released into interphase by adding $CaCl_2$, DNA replication can occur. Unfertilized (M phase-arrested) eggs can also be induced into interphase (activated state) by treating them with the calcium ionophore A23187. When demembranated Xenopus sperm nuclei are added to such an extract, they begin semiconservative DNA replication, and ultimately undergo chromosomal condensation and nuclear envelope breakdown, thus demonstrating all the events of a single cell cycle in vitro. Moreover, Hutchison et al(2) and Murray and Kirschner(3) developed extracts that can undergo multiple cell cycles in vitro.

The simian virus 40 (SV40) large T antigen-dependent DNA replication system has been used to study the initiation of mechanisms of DNA replication in vitro(4). In this report, protein phosphatase 2A (PP2A) was suggested to be involved in DNA replication, and inhibition of phosphatase activity was assumed to cause the inhibition of SV40 DNA replication.

In the present communication, using the Xenopus cell-free system that undergoes multiple cell cycles in vitro, we investigated the effects of a phosphatase inhibitor, calyculin A, which was isolated from Discodermia calyx(5), on the initiation of DNA replication in demembranated sperm nuclei and the oscillation in activity in Xenopus cell-free extracts.

Materials and Methods

Preparation of egg extracts Mitotic extracts were prepared by a modified method as described by Blow and Nurse (6). Eggs were collected in high salt Barth solution (110mM NaCl, 15mM Tris-HCl [pH7.6], 2mM KCl, 2mM

$NaHCO_3$, 1mM $MgSO_4$, 0.5mM Na_2HPO_4) and were washed in high-salt Barth solution containing 2mM EGTA. Any degenerated or activated eggs were removed at this stage. The eggs were dejellied in 2% cysteine-HCl(pH7.8), 2mM EGTA. The dejellied eggs were washed first in high-salt Barth solution containing 2mM EGTA, and then in extraction buffer (250mM sucrose, 50mM HEPES-KOH [pH7.6] 5mM EGTA) containing protease inhibitors (leupeptin, chymostatin and pepstatin 20μg/ml) and cytochalasin B 10μg/ml. The eggs were then packed into 2 ml microtubes by centrifugation in an Eppendorf centrifuge at 1000rpm for 1min. All excess buffer was removed. The eggs were then spin-crushed in the centrifuge at 15000 rpm for 10 min at 4°C. The cytoplasmic layer was taken and centrifuged again. Activated extracts were prepared by the modified method of Smythe and Newport (7). The dejellied eggs were washed in 20% modified Barth solution and then activated by incubation in 0.5μg/ml calcium ionophore A23187 (Sigma) for about 5 min. Following activation, the eggs were extensively rinsed and spin-crushed as mentioned above in ice-cold extraction buffer (250mM sucrose, 2.5mM $MgCl_2$ 50mM KCl, 1mM dithiothreitol, 50mM HEPES-KOH, pH7.6) containing the protease inhibitors and cytochalasin B as previously mentioned.

Preparation of Xenopus sperm nuclei Demembranated sperm nuclei were prepared by the method described by Smythe and Newport (7). A freshly isolated testis was minced with forceps. The released sperm were suspended in Buffer T (15mM PIPES, 15mM NaCl, 5mM EDTA, 7mM $MgCl_2$, 80mM KCl, 0.2M sucrose, pH7.4) and were recovered by centrifugation at 1500g for 5 min. The suspended sperm were demembranated with Buffer S (Buffer T+20mM maltose+0.05% lysolecithin) and washed with Buffer R (Buffer T+3% bovine serum albumin). The demembranated sperm were stored at -70°C. The addition of an ATP regenerating system containing 0.5mM ATP, 10mM creatine phosphate and 10μg/ml creatine phosphokinase to cycling extracts is necessary to cause the oscillation of DNA replication activity.

Replication assays The Xenopus cell-free DNA replication assay was performed by the modified method of Hutchison et al (2). For the labeling of sperm nuclear DNA, the extracts were incubated at 23°C in the presence of 0.1μCi of [α-32P]dCTP (3000Ci/mmole) per/μl extract with demembranated sperm nuclei for 40 min. The reactions were stopped by adding stopping buffer containing 80mM Tris-HCl [pH8], 8mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% sodium dodecyl sulfate (SDS) and 0.2% bromophenol blue. For pulse labeling, aliquots(20 μl) of unlabeled extracts containing sperm nuclei were removed from the reaction mixture treated with or without calyculin A at 20-min intervals, and added to 2uCi of dCTP. The incorporation of labeled dCTP into nascent DNA was carried out by incubating the mixture for 20 min and then terminating the reaction by adding 10μl of stopping buffer. These samples were further treated with proteinase-K (0.5mg/ml) for 1 h before agarose gel electrophoresis on 0.6% agarose gels. The dried gels were exposed to X-ray films at -70°C

with intensifying screens for 2 or 3 days. The density of the bands corresponding to sperm chromosomal DNA in the developed X-ray films was quantitatively measured by a densitometer as the relative DNA replication activity.

Results

Inhibition of DNA replication of sperm nuclei by calyculin A We wanted to determine whether protein phosphatases play an essential role in the control of the initiation of DNA replication in the Xenopus cell-free system. To do this, mitotic extracts were treated first with calyculin A and then released into interphase by the addition of $CaCl_2$. The extracts were then assayed for their DNA replication activity as described in Materials and Methods. Treatment with calyculin A inhibited this activity in a dose-dependent manner as shown in Fig. 1. Almost complete inhibition was observed at a concentration of 1 nM.

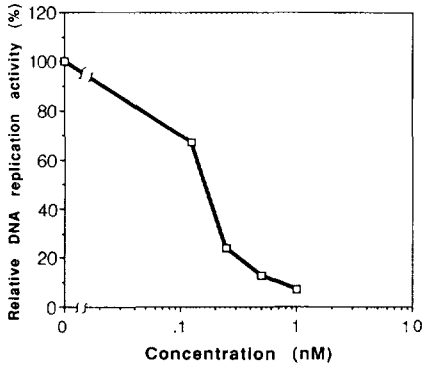

Fig. 1.

Inhibitory effect of calyculin A on DNA replication in Xenopus egg extracts. Mitotic extracts were treated with calyculin A as indicated and were then released into interphase by the addition of $CaCl_2$ (0.4mM). The extracts were assayed for their ability to replicate demembranated sperm nuclei (about 10/μl extract). DNA replication activity was expressed as a percentage of that obtained in the absence of calyculin A.

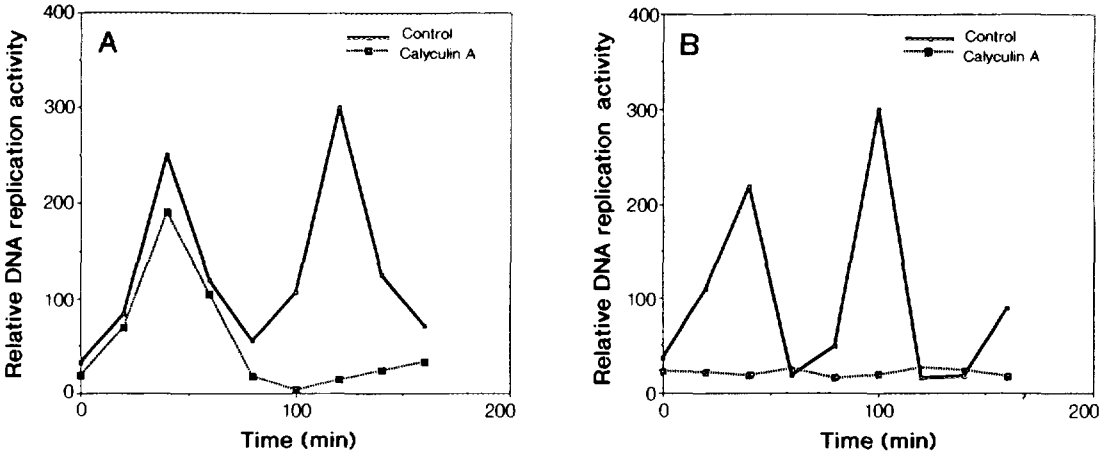

Fig. 2.

Effects of calyculin A on different cycling extracts in which oscillation in DNA replication activity occurs.

DNA replication activity was measured in the absence (□) or presence (■) of calyculin A (0.8nM) in the case of activated extract (Fig. 2A) and mitotic extract (Fig. 2B) as follows. Activated extract or mitotic extract was first treated with calyculin A. The mitotic extract was then released into interphase by the addition of $CaCl_2$. Aliquots (20μl) of the unlabeled extracts containing sperm nuclei were removed at 20-min intervals and added to 2μCi of [α-32P]dCTP. Labeling was started at the time indicated (shown in minutes) at 23°C. After 20 min, the reactions were stopped in individual tubes by adding stopping buffer (see Materials and Methods).

Effects of calyculin A on the cycling extracts derived from eggs in different states To confirm the involvement of protein phosphatase in the initiation of DNA replication, we used extracts of different types. Untreated control extracts showed periodic DNA replication, which had cycle lengths of 60 to 80 min, as shown in Fig. 2. In the case of activated extracts, which had already entered into interphase, calyculin A (0.8nM) had no effect on the first peak of oscillation in DNA replication activity but greatly reduced the second peak in comparison with the untreated control extracts (Fig. 2A). In contrast, the mitotic extracts prepared from eggs that were arrested by EGTA, calyculin A (0.8nM) completely inhibited the oscillation in activity from first to last as shown in Fig. 2B. These

results strongly suggest that calyculin A prevents the initiation of DNA replication in the transition from the M phase to the S phase.

Discussion

We clearly showed that calyculin A at a concentration of less than 1nM strongly inhibited DNA replication in the Xenopus cell-free system. Moreover, we demonstrated that calyculin A had no effect on the first peak of oscillation in DNA replication activity in cycling extracts activated by A23187. Therefore, we assumed that the inhibition of DNA replication by calyculin A is due to the inhibition of phosphatases involved in the initiation of DNA replication in the Xenopus cell-free system.

Calyculin A specifically inactivates PP2A and PP1 in vitro(8). The complete inhibition of their phosphatase activity by calyculin A was observed at a concentration of about 1 nM (8). The specificity of calyculin A allows us to predict that the protein phosphatase involved in the initiation of DNA replication in Xenopus extracts is PP1 and/or PP2A.

Thus, the dephosphorylation of certain proteins involved in the initiation of DNA replication seems to be essential to initiate DNA replication in the Xenopus cell-free system. Based on these results, calyculin A have the potent inhibitory effect in this system, we assume that dephosphorylation induced by PP1 and/or PP2A control(s) the activity of the key proteins that promote the completion of DNA replication in the system.

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