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Identification and Characterization of a *Xenopus* Homolog of Dbf4, a Regulatory Subunit of the Cdc7 Protein Kinase Required for the Initiation of DNA Replication

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Dbf4 is a regulatory subunit for the Cdc7 protein kinase that is required for the initiation of eukaryotic DNA replication, but the precise roles of Dbf4-Cdc7 remain to be determined. Here we identified a Xenopus homolog of Dbf4 (XDbf4) and characterized XDbf4 and *Xenopus* Cdc7 (XCdc7) in *Xenopus* egg extracts. XDbf4 formed a complex with XCdc7 in egg extracts and activated XCdc7 kinase activity in vitro. In contrast with Dbf4 in yeast and mammalian cultured cells, the XDbf4 levels in egg extracts did not change during the cell cycle progression. XDbf4 was a phosphoprotein in interphase extracts, and was apparently hyperphosphorylated in cytostatic factor (CSF)-mediated, metaphase-arrested extracts and in mitotic extracts. However, the hyperphosphorylation of XDbf4 did not seem to affect the level of kinase activation, or chromatin binding of the XDbf4-XCdc7 complex. Upon release from CSF-arrest, XDbf4 was partially dephosphorylated and bound to chromatin. Interestingly, XDbf4 was loaded onto chromatin before XCdc7 during DNA replication in egg extracts. These results suggest that the function of XDbf4-XCdc7 during the early embryonic cell cycle is regulated in a manner distinct from that during the somatic cell cycle.

Key words: cell cycle, Dbf4-Cdc7 kinase, DNA replication, protein phosphorylation, *Xenopus* egg extract.

Abbreviations: ARS, autonomously replicating sequence; APC, anaphase-promoting complex; CDK, cyclin-dependent kinase; CSF-extracts, cytostatic factor-mediated, metaphase-arrested extracts; GST, glutathione S-transferase; MCM, minichromosome maintenance protein; ORC, origin recognition complex; PMSF, phenylmethanesulfonyl fluoride; PPase, protein phosphatase; pre-RC, pre-replicative complex.

The initiation of DNA replication in eukaryotic cells is a highly regulated process involving many replication factors (reviewed in Ref. *1*). Among them, the binding of origin recognition complex (ORC) to DNA is thought to be crucial for the selection of the origins of DNA replication. After ORC binding, a minichromosome maintenance 2–7 (MCM2-7) complex is loaded onto chromatin in a Cdc6- and Cdt1-dependent manner to form the pre-replicative complex (pre-RC) at the origins. The pre-RC then needs to be activated for DNA synthesis to start. The precise mechanisms of pre-RC activation remain to be determined, but several proteins that are involved in this step have been identified, including two distinct protein kinases, cyclin-dependent kinase (CDK) and Dbf4-Cdc7. However, the mechanism by which these kinases function in pre-RC activation is largely unknown.

Saccharomyces cerevisiae CDC7 encodes a catalytic subunit of this protein kinase complex (reviewed in Ref. *2*). Cdc7-related kinases are found in other species including human, mouse, frog, and fission yeast (*3–7*). Previous genetics and biochemical studies indicate that *S. cerevisiae* Cdc7 is required for entry into S phase of the cell cycle and for the activation of individual origins throughout the S phase (*8*, *9*, reviewed in Refs. *1* and *2*). The *in vitro* substrates for the Cdc7 kinases have been reported, including MCM2-4, MCM6, Cdc45, the p180 subunit of DNA polymerase α-primase complex, Dbf4 and Cdc7 itself (*3*, *10–14*). Among them, MCM2 is the most probable candidate as a physiological substrate, and its phosphorylation has been extensively characterized for human Cdc7 as well as for *S. cerevisiae* Cdc7 (*12*, *15–17*). However, the precise consequence of MCM2 phosphorylation is still unknown. The link between Dbf4-Cdc7 and MCM2-7 *in vivo* is also suggested by the observation that one allele of mcm5 in budding yeast can rescue the viability of a cdc7 (or dbf4) null mutant (*18*, *19*). Consistent with this, the loading of *Xenopus* Cdc7 on chromatin is dependent on the pre-RC formation (*13*, *20*). Biochemical studies in *Xenopus* and *S. cerevisiae* suggest that Cdc7 is required for the loading of Cdc45 onto chromatin (*13*, *21*).

The Dbf4 protein is a regulatory subunit required for the activation of Cdc7 kinase (reviewed in Refs. *1* and *2*).

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The protein levels of *S. cerevisiae* Dbf4 are cell cycle-regulated with a peak attained during S phase so that kinase activity also fluctuates during the cell cycle (*18*, *22–24*). The abundance of *S. cerevisiae* Dbf4 is regulated not only at the level of mRNA, but also by protein degradation directed by the anaphase-promoting complex (APC) (*11*, *23–25*). Human Dbf4 (ASK) is also most abundant during S phase of the cell cycle, and Cdc7 kinase activity is significantly increased in the S phase (*26*). By one-hybrid assay, *S. cerevisiae* Dbf4 was shown to interact with the autonomously replicating sequences (ARS), chromosomal origins of DNA replication (*27*). The analyses of Dbf4-ARS interaction and the association of Dbf4 with chromatin in budding yeast suggest that one of the functions of Dbf4 is to recruit Cdc7 kinase to the target (*27*, *28*)

Dbf4-Cdc7 is also a target for the control of cell cycle progression in response to S-phase perturbations such as nucleotide deprivation; *S. cerevisiae* Dbf4 is hyperphosphorylated by Rad53 kinase in response to early S phase arrest with hydroxyurea (HU), and Rad53-directed hyperphosphorylation of Dbf4 results in the attenuation of Cdc7 kinase activity (*11*, *16*, *29*).

Recently, another Dbf4-related protein, Drf1/ASKL1, was identified in human cultured cells (*30*; Masai, H. *et al*., submitted). Drf1/ASKL1 also binds to Cdc7 and activates its kinase activity. This identification implies complexity in the regulation of Cdc7 kinase activity in mammalian cells.

Toward the goal of understanding the mechanism by which the phosphorylation by Dbf4-Cdc7 kinase leads to activation of the pre-RC, here we have isolated and characterized a *Xenopus* homolog of Dbf4 (XDbf4). The biochemical characterization of XDbf4 and *Xenopus* Cdc7 (XCdc7) revealed that XDbf4 is a stable phosphoprotein in *Xenopus* egg extracts and its phosphorylation is regulated in a cell-cycle-dependent manner. Our results suggest that the function of Dbf4-Cdc7 during DNA replication in *Xenopus* egg extracts may be regulated in a manner different from that in somatic cells.

MATERIALS AND METHODS

Materials—Cycloheximide and lambda protein phosphatase were obtained from Sigma and NEB, respectively. Glutathione S-transferase (GST)-fused p21 was bacterially expressed and purified through glutathione-Sepharose 4B and SP Sepharose columns (Amersham Biosciences). The nondegradable mutant of *Xenopus* cyclin B (GST-fused cyclin B2 Δ85) was bacterially expressed and purified.

cDNA Cloning—The sequence in a *Xenopus* EST clone (AW635780) that showed similarity to the C-terminal region of human ASK was extended by nested PCR using a *Xenopus* ovary cDNA library (Stratagene) with vector primers and primers corresponding to the 5′-terminal region of the registered EST sequence. By this PCR, a 570-bp fragment containing the 5′-region of the EST sequence was obtained. A nested PCR was again carried out using the same library with vector primers and primers corresponding to the 5′-end of the extended sequence. By this PCR, a further extended DNA fragment (510 bp) was obtained that encoded a sequence similar to the Dbf4 motif-M and -C. At this point, a *Xenopus* ovary cDNA library was screened using the insert (650 bp) of the EST clone above as a probe, and one positive clone containing a cDNA insert of about 2.3 kb was obtained. The sequencing of this insert revealed that the clone encoded the full-length coding region of *Xenopus* Dbf4 (accession number AB095983).

Expression of Recombinant Proteins—The full-length, coding regions of XCdc7 and XDbf4 were cloned into pFastBac vector (Invitrogen) to obtain recombinant baculoviruses for the expression of untagged or N-terminally, six histidine-tagged XCdc7 and XDbf4. To express both XCdc7 and XDbf4 simultaneously, the recombinant baculovirus was also made by cloning these cDNAs into a single plasmid pFastBacDual (Invitrogen). To express the proteins, infection with recombinant baculoviruses was performed at MOI 10 using High Five cells, and the cells were harvested 64 h after infection. To make the insect cell lysate for immunoblotting and IP-kinase assay, harvested cells were washed with ice-cold 10 mM HEPES-KOH, pH 7.7, 150 mM NaCl, and lysed in 10 mM HEPES-KOH, pH 7.7, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 μg/ml each of aprotinin, leupeptin and chymostatin. The lysate was cleared by centrifugation at 11,000 ×*g* for 5 min at 4°C and used for analyses.

GST-fused or untagged XDbf4 and XCdc7 were expressed in *E. coli* by transformation with plasmids constructed based on pGEX vectors (Amersham Biosciences) and pET vectors (Novagen). GST-fused p21 was expressed and purified using glutathione-Sepharose 4B and SP Sepharose columns (Amersham Biosciences).

Production of Antibodies—To prepare the antigen for the production of anti-XCdc7 antibodies, an N-terminally, ten histidine-tagged polypeptide corresponding to the C-terminal region (amino acid 377–483) of XCdc7 was expressed in *E. coli*. This histidine-tagged polypeptide was solubilized in buffer A (300 mM NaCl, 50 mM sodium phosphate, pH 7.0) containing 6 M guanidine-HCl and applied to a chelating TALON column (Clontech). After the column was washed with buffer A containing 8 M urea, the bound polypeptides were eluted with buffer A containing 8 M urea and 100 mM EDTA, dialyzed against phosphate-buffered saline (PBS), and used to immunize a rabbit. To raise anti-XDbf4 antibodies, the N-terminal (amino acid 1–120) and C-terminal (amino acid 524–661) polypeptides of XDbf4 were used separately as antigens. The N-terminal polypeptide was expressed as a histidine-tagged protein and purified under denaturing conditions as above, and the C-terminal polypeptide was expressed as a GST-fused protein and purified using glutathione-Sepharose 4B and HiTrapQ columns (Amersham Biosciences). The purified proteins were also dialyzed against PBS and used to immunize rabbits. The antibodies are referred to as anti-Dbf4-N and anti-Dbf4-C antibodies, respectively. Both the anti-Dbf4-N and anti Dbf4-C antibodies could be used to immunoprecipitate XDbf4, but only the anti-Dbf4-N antibodies worked well for immunoblotting.

The antibodies were affinity purified by immobilizing the C-terminal polypeptide of XCdc7 (purified under non-denaturing conditions) and GST-fused C-terminal polypeptide of XDbf4 (purified as above) to Affi-Gel 15

resin (Bio-Rad), and the bound antibodies were eluted with 100 mM glycine-HCl, pH 2.5. The purified antibodies were neutralized and concentrated in PBS.

Immunoprecipitation and Kinase Assay—For immunoprecipitation of XCdc7 and XDbf4 in egg extracts, the respective affinity-purified antibodies were crosslinked to Affi-Prep Protein A beads (Bio-Rad) with dimethylpymerimidate, and 10 μl (10 μg of immunoglobulin crosslinked) of the beads was mixed with each of the egg extracts for 2 h at 4°C. The beads were then washed twice with 10 mM HEPES-KOH, pH 7.7, 150 mM NaCl and 0.5% Nonidet P-40, once with PBS, and once with the lambda phosphatase-reaction buffer as recommended by the manufacture (NEB). The beads were treated with 150 units of lambda phosphatase for 30 min at 30°C, and then mixed with SDS sample buffer and subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Detection was carried out using an ECL detection kit (Amersham Biosciences).

For kinase assay, 10 μl of the beads crosslinked with affinity-purified anti-XCdc7 antibodies as above were incubated with the protein fractions or extracts for 2 h at 4°C, and washed twice with 10 mM HEPES-KOH, pH 7.7, 150 mM NaCl and 0.5% Nonidet P-40 and twice with kinase buffer (50 mM HEPES-KOH, pH 7.7, 10 mM $MgCl_2$, 1 mM dithiothreitol and 5 μM ATP). The beads were then incubated with 5 μCi of [γ-^{32}P]ATP and 0.6 μg of purified XMCM2 in the kinase buffer for 30 min at 25°C, unless specified otherwise, and subjected to SDS-PAGE and autoradiography. Phosphorylated XMCM2 was quantified using an image analyser FLA3000 with software Image Gauge Version 3.4 (Fuji Film). Histidine-tagged XMCM2 was purified from High Five cells infected with the recombinant baculovirus (Masuda, T. and Takisawa, H., unpublished) using a HiTrap chelating HP resin (Amersham Biosciences) essentially as described previously (*31*). The assay for the phosphorylation of histone H1 was performed as described elsewhere (*32*).

Egg Extracts and Sperm Nuclei—Crude interphase egg extracts were prepared as described previously (*33*) and supplemented with 0.25 mg/ml cycloheximide. Cytostatic factor (CSF)-mediated metaphase-arrested egg extracts were prepared essentially as described previously (*34*). Demembranated sperm nuclei were prepared as described elsewhere (*35*). For ORC2 depletion, interphase egg extracts were treated twice with the beads to which anti-ORC2 antibodies were bound.

Analysis of Proteins in Extracts and Bound to Chromatin—To analyze the proteins bound to chromatin during DNA replication, demembranated *Xenopus* sperm nuclei were mixed with egg extract (4,000 sperm nuclei/μl extract) and an ATP-regenerating system, and incubated at 23°C. At the indicated times, an aliquot (30 μl) of the mixture was withdrawn and diluted 10-fold with EB buffer (100 mM KCl, 2.5 mM $MgCl_2$ and 50 mM HEPES-KOH, pH 7.7) containing 0.25% Triton X-100. The mixture was overlaid on 100 μl of EB buffer containing 10% sucrose and centrifuged at 6,000 $\times g$ for 5 min at 4°C to collect chromatin. The chromatin pellet was washed with EB buffer, resuspended in EB buffer containing 2 mM $CaCl_2$ and 4 units/μl micrococcal nuclease (TAKARA), and incubated for 15 min at 30°C. The samples were then mixed with SDS sample buffer, boiled for 3 min and subjected to SDS-PAGE followed by immunoblotting.

For treatment with lambda protein phosphatase, chromatin samples (without micrococcal nuclease digestion) or extracts were supplemented with 100–200 units phosphatase and incubated for 30 min at 30°C prior to mixing with SDS loading buffer.

RESULTS

A Xenopus Homolog of Dbf4—Our initial attempt to isolate the cDNA of a *Xenopus* homolog of Dbf4 by screening a *Xenopus* cDNA library using the human ASK sequence as a probe was not successful. We then searched the EST database for ASK-related sequences and found that one *Xenopus* EST clone (accession number AW635780) exhibited limited but significant similarity to the carboxyl terminal region of human ASK. Starting from this EST sequence, sequential nested PCRs and screening of a *Xenopus* cDNA library were carried out to obtain a full-length cDNA clone containing this Dbf4-like sequence (see "MATERIALS AND METHODS" for details). The initial methionine was assigned by comparison of the sequences with human ASK. The full-length cDNA encoded a 74K polypeptide comprising 661 amino acids.

The similarity of amino acid sequences between a *Xenopus* Dbf4-like polypeptide (referred to as XDbf4 in Fig. 1) and human Dbf4 (ASK) was not so high with overall identity of only 29%. However, the sequences of the Dbf4 motif-N, -M, and -C, which are important for Dbf4 functions (*36*), were well conserved in this *Xenopus* polypeptide (Fig. 1); especially, the motif-N and -C were highly conserved (72% and 68% identity, respectively). Recently, another Dbf4-related human protein, Drf1, has been reported (*30*). The polypeptide identified in this study was more closely related to ASK than to Drf1 in terms of sequence similarity (29% identity between the Xenopus polypeptide and human ASK, compared to 17% identity between the Xenopus polypeptide and human Drf1) (Fig. 1). Especially, the carboxyl terminal half of the *Xenopus* polypeptide was more similar to ASK than to Drf1 (Fig. 1). Furthermore, we have found that *Xenopus* EST clones (BG408573, BM191058) encode a Drf1-related sequence, although in the limited region (data not shown). Therefore, we conclude that the clone obtained in this study encodes a *Xenopus* homolog of ASK rather than Drf1, and thus we refer to this *Xenopus* polypeptide as XDbf4.

XDbf4 Is a Phosphoprotein and Is Not Abundant in Egg Extracts—Specific antibodies that recognize XDbf4 were prepared and used for immunoblot analysis of egg extracts. It was found that XDbf4 in interphase egg extracts migrated more slowly in the gel as smeared bands (migrated as 100–110 kDa) than bacterially expressed XDbf4 (migrated as 85 kD) (compare lanes 2 and 4, Fig. 2A). After phosphatase treatment, these bands were converted to faster migrating bands (lane 3, Fig. 2A), suggesting that XDbf4 is phosphorylated in interphase extracts. Two discrete bands of XDbf4 were detectable even after phosphatase treatment (lanes 3, Fig. 2A), but the reason for this is not clear at this time. Similar but less extensive phosphorylation of XDbf4 was also detected when XDbf4 and XCdc7 were co-expressed

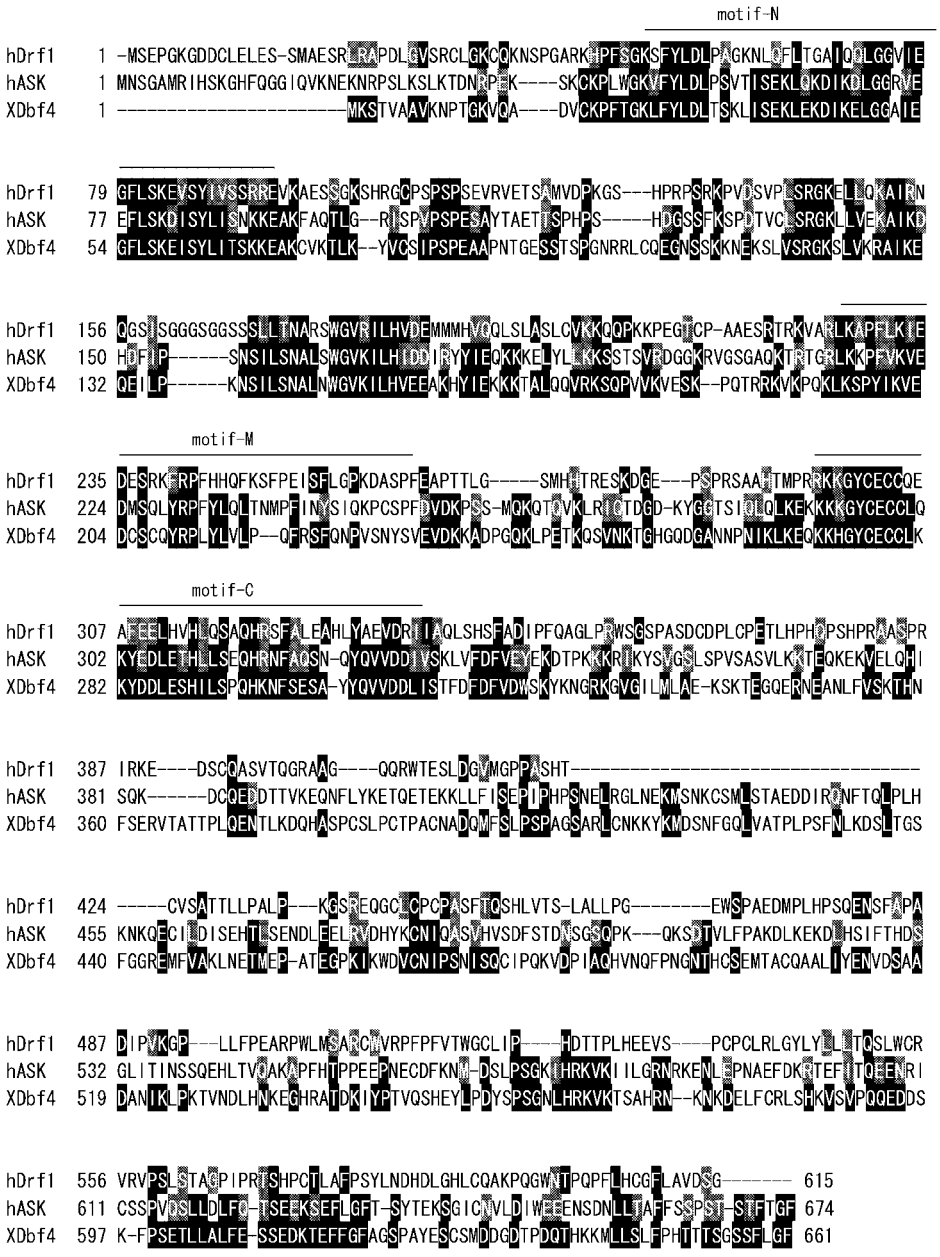

Fig. 1. **Amino acid sequence alignment of *Xenopus* Dbf4 (XDbf4), human ASK (hASK) and human Drf1 (hDrf1).** The amino acid sequences are compared between XDbf4 and human ASK and between XDbf4 and human Drf1. Identical and similar amino acids in these two comparisons are highlighted in black and gray, respectively. The regions corresponding to the conserved Dbf4 motif-N, -M, and -C are indicated above the alignment.

in baculovirus-infected insect cells (lanes 5 and 6, Fig. 2A). However, the slowly migrating bands of XDbf4 were not seen when XDbf4 alone was expressed (lanes 7 and 8, Fig. 2A), indicating that the phosphorylation of XDbf4 is due at least in part to XCdc7.

We quantified the XCdc7 and XDbf4 proteins in interphase extracts by comparative immunoblotting using recombinant proteins as standards. These data indicate that XDbf4 is much less abundant in crude interphase extracts (10 nM), compared to XCdc7 (60 nM) (data not shown).

XDbf4 Binds to XCdc7 and Activates Its Kinase Activity—XDbf4 co-precipitated with XCdc7 when immunoprecipitation with anti-XCdc7 antibodies was carried out against an insect cell lysate containing XCdc7 and XDbf4, indicating that XDbf4 indeed forms a complex with XCdc7 (Fig. 2B). The co-immunoprecipitation of XCdc7 with XDbf4 was also observed when the egg extracts were immunoprecipitated with anti-XCdc7 or anti-Dbf4-C antibodies, indicating that the complex containing XDbf4 and XCdc7 exists in egg extracts (Fig. 2C). More XCdc7 was precipitated with anti-XCdc7 antibodies from interphase egg extracts than with anti-Dbf4-C antibodies, whereas almost the same amounts of XDbf4 were precipitated (Fig. 2C), suggesting that the free form of XCdc7 may be also present in interphase egg extracts. Similarly, a fraction of XDbf4 may not associate with XCdc7 in interphase egg extracts, since part of the XDbf4 remained in XCdc7-immunodepleted extracts (data not shown).

To determine if XDbf4 is required for the kinase activity of XCdc7, as shown in other species, an immunoprecipitation-kinase assay (IP-kinase assay) with insect cell lysates was performed using *Xenopus* MCM2 (XMCM2) as an *in vitro* substrate. When the lysate containing XCdc7 alone was subjected to the IP-kinase assay, only

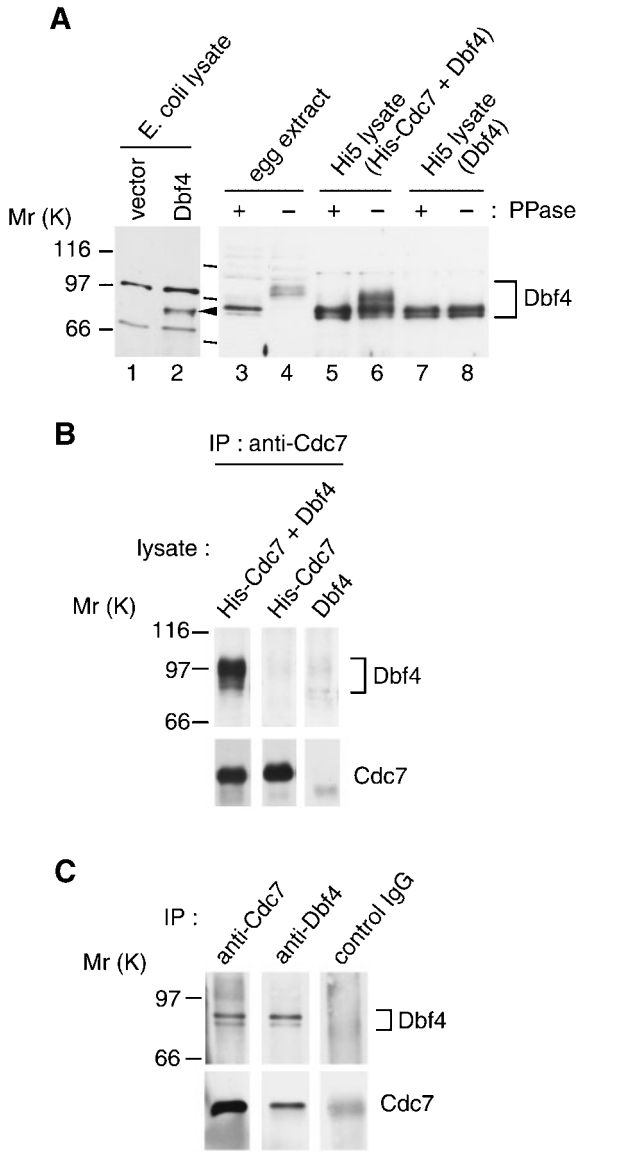

Fig. 2. **XDbf4 is a phosphoprotein in egg extracts and forms a complex with XCdc7.** A: The lysate prepared from *E. coli* cells transformed with empty vector (lane 1) or an XDbf4-expressing plasmid (lane 2), *Xenopus* egg extracts (lanes 3 and 4), and the insect cell lysate prepared from cells expressing both XDbf4 and histidine-tagged XCdc7 (lanes 5 and 6) or XDbf4 alone (lanes 7 and 8) were analyzed by immunoblotting with anti-Dbf4-N antibodies. The cell lysates were also pre-treated with lambda protein phosphatase (PPase) prior to mixing with SDS loading buffer (lanes 3, 5, and 7). The arrowhead and bracket indicate the bands corresponding to XDbf4. Note that two bands commonly seen in lanes 1 and 2 are non-specific. B: Insect cell lysates containing both XDbf4 and histidine-tagged XCdc7, histidine-tagged XCdc7 alone or XDbf4 alone were subjected to immunoprecipitation with anti-XCdc7 antibodies. The immunoprecipitates were then analyzed by immunoblotting with anti-Dbf4-N antibodies (top) and anti-XCdc7 antibodies (bottom). C: Egg extracts immunoprecipitated with anti-XCdc7 and anti-Dbf4-C antibodies or whole rabbit IgG (control IgG) were analyzed by immunoblotting with anti-Dbf4-N antibodies (top) or anti-XCdc7 antibodies (bottom). The immunoprecipitates in C were treated with lambda phosphatase before mixing with SDS loading buffer. A faint band seen in the "control IgG" lane with anti-XCdc7 antibodies is due to the heavy chains of IgG.

the background level of XMCM2 phosphorylation was seen (lane 2, Fig. 3A). On the other hand, a significant increase in the level of phosphorylation of XMCM2 was observed when the lysate contained both XCdc7 and XDbf4 (lane 1, Fig. 3A). The phosphorylations of XDbf4 and XCdc7 were also seen, likely representing autophosphorylation (lane 1, Fig. 3A).

The immunoprecipitates obtained with anti-XCdc7 antibodies from interphase egg extracts also possessed XMCM2 phosphorylation activity (lanes 3 and 4, Fig. 3B). Since this phosphorylation was seen even in the reaction including the cyclin-dependent kinase inhibitor p21 (lane 4, Fig. 3B), the phosphorylation of XMCM2 likely performed by XCdc7. Consistent with this, the immunoprecipitates obtained with anti-XDbf4 antibodies also exhibited XMCM2 phosphorylation kinase activity (lanes 5–8, Fig. 3B). Furthermore, when a mouse MCM2-4-6-7 complex containing a mutant MCM2, in which amino acid residues at the Cdc7 phosphorylation sites were converted to alanine (Masai, H. *et al*., in preparation), was used as a substrate for this IP-kinase assay, the mutant MCM2 protein was not phosphorylated, whereas the wild-type MCM2 in the MCM2-4-6-7 complex was phosphorylated as was seen in the assay with mammalian Cdc7 kinases (*12*; data not shown), indicating that the phosphorylation seen in the IP-kinase assay is Cdc7-specific. The results thus confirm that the cDNA cloned in this study encodes a *Xenopus* homolog of Dbf4 and also establish that XDbf4 is indeed an activator of XCdc7 kinase.

Interestingly, another phosphorylated band of about 160 kDa (p160) was also detected in the immunoprecipitates obtained with anti-XCdc7 antibodies from interphase extracts but not in the immunoprecipitates obtained with anti-Dbf4-C antibodies (see the bands indicated by the asterisk in lanes 3 and 4, Fig. 3B). The reason that phosphorylated p160 was not detected when anti-XDbf4 antibodies were used is not clear at this time. Nevertheless, since the phosphorylation of p160 was augmented when the kinase reaction was carried out in the absence of XMCM2 (Fig. 3C), MCM2 may compete with p160 in the XCdc7 kinase reaction. Thus, the p160 protein may be another substrate for XCdc7 kinase. The identification of p160 is currently under way.

XDbf4 Levels in Egg Extracts Do Not Change during Cell-Cycle Progression, and XDbf4 Is Further Phosphorylated Mitotically—It has been reported that the protein levels of Dbf4 in yeast and mammalian cultured cells are regulated in a cell cycle-dependent manner with a peak in S phase of the cell cycle (*18*, *22–24*, *26*). Thus, we wanted to know if the XDbf4 level in egg extracts would fluctuate during the cell cycle. To this end, a nondegradable form of cyclin B (cyclin B2 Δ85) (*37*, *38*) was added to interphase extracts to convert them to mitotic extracts, and the Dbf4 levels in these extracts were compared by immunoblotting. The histone H1 kinase activity, mainly reflecting cyclin B-Cdc2 kinase activity, started to rise beginning 20 min after the addition of cyclin B Δ85 under these conditions. In parallel with an increase in H1 kinase activity, the bands of XDbf4 disappeared (lanes 3–7, Fig. 4A), while the band intensity did not change throughout the incubation when control buffer was added (lanes 8–14, Fig. 4A). Furthermore, XCdc7 was also con-

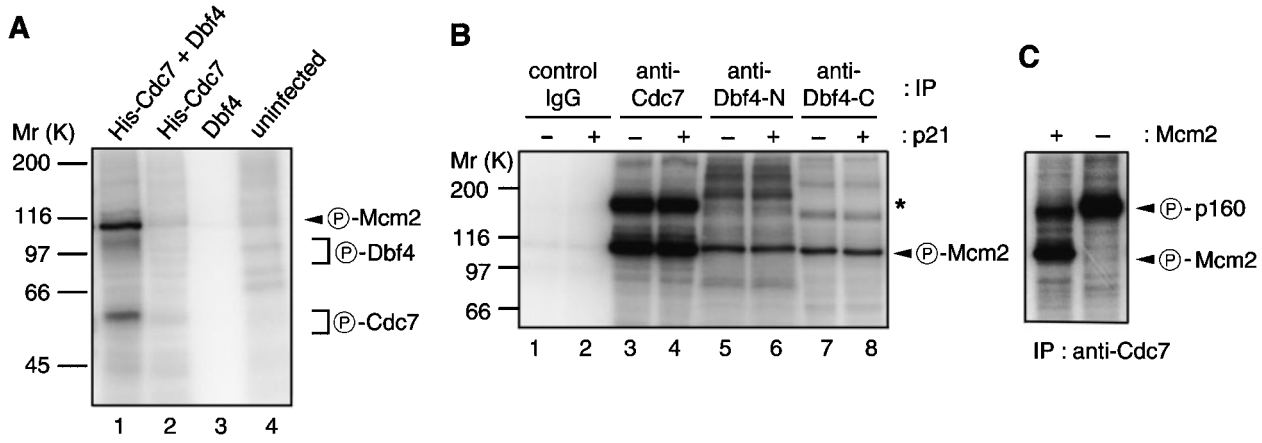

Fig. 3. **Characterization of the kinase activity of XDbf4-XCdc7.** A: An IP-kinase assay with anti-XCdc7 antibodies was carried out using insect cell lysates prepared from uninfected cells (lane 4) or cells infected with recombinant baculoviruses expressing both XDbf4 and histidine-tagged XCdc7 (lane 1), histidine-tagged XCdc7 alone (lane 2) or XDbf4 alone (lane 3). XMCM2 was added as a substrate to the kinase reaction. The positions of phosphorylated XMCM2, XDbf4 and XCdc7 are indicated by the arrowhead and brackets. B: An IP-kinase assay using interphase extracts was performed with anti-XCdc7 (lanes 3 and 4), anti-Dbf4-N (lanes 5 and 6), anti-Dbf4-C antibodies (lanes 7 and 8) or whole rabbit IgG (lanes 1 and 2). The assay was also performed using extracts supplemented with 500 nM of the CDK inhibitor p21 (lanes 2, 4, 6 and 8). The kinase reaction was carried out for 10 min. Note that the amount of p21 used severely inhibited DNA replication (data not shown). Phosphorylated XMCM2 and p160 protein (lanes 3 and 4) are indicated by the arrowhead and the asterisk, respectively. C: An IP-kinase assay using interphase extracts and anti-XCdc7 antibodies was carried out with or without exogenously added XMCM2. Phosphorylated XMCM2 and the p160 protein are indicated.

verted to a slowly migrating form in parallel with the disappearance of the XDbf4 bands, while no shifted band was seen when control buffer was added (middle panel, Fig. 4A).

We found that treating the extracts with phosphatase prior to SDS gel analysis led to the appearance of XDbf4 bands with similar intensity to those in a control (lane 3, Fig. 4B). This indicates that XDbf4 is actually present in mitotic extracts and highly likely to be hyperphosphorylated upon activation of cyclin B-Cdc2 kinase. Since the extracts used in this experiment were supplemented with cycloheximide, an inhibitor of protein synthesis, little or no *de novo* protein synthesis occurred. Thus, the results shown in Fig. 4A indicate that XDbf4 in the extracts is likely to remain stable during the transition from interphase to mitosis. More importantly, the XDbf4 level did not change during release from metaphase arrest (see below) when the anaphase-promoting complex (APC) is known to be active. Therefore, it is most likely that the XDbf4 level is not significantly altered at least during the first cycle of the early embryonic cell cycle.

Our data strongly suggest the existence of mitotic phase-specific phosphorylation of XDbf4. The failure to detect XDbf4 bands without phosphatase treatment implies that phosphorylation in mitotic extracts may occur in the region that overlaps with the epitopes for the XDbf4 antibodies (against the N-terminal region of XDbf4), so that the antibodies do not recognize the mitotically phosphorylated form of XDbf4 during immunoblotting. The shift in the Cdc7 band is also most likely due to its phosphorylation in a mitotic phase-dependent manner, since phosphatase treatment resulted in the loss of this shifted band (lane 3, Fig. 4B). Based on the correlation between the phosphorylation of both XDbf4 and XCdc7 and an increase in H1 kinase activity, the phosphorylation of XDbf4-XCdc7 is likely to depend on the activation of Cdc2 kinase.

Mitotically Phosphorylated XDbf4 Binds to XCdc7 and Activatse Its Kinase Activity—We next examined whether mitotically phosphorylated XDbf4 forms a complex with phosphorylated XCdc7 and activates its kinase activity in the mitotic phase. Immunoprecipitation experiments using anti-Dbf4-C antibodies and extracts supplemented with cyclin B Δ85 showed that phosphorylated XDbf4 binds to phosphorylated XCdc7; a slowly migrating band of XCdc7 was detected in the immunoprecipitates (lane 8, Fig. 4B), and XDbf4 bands appeared in the immunoprecipitates after phosphatase treatment (lane 7, Fig. 4B). In addition to complex formation, the XDbf4-XCdc7 complex in mitotic extracts phosphorylated exogenous XMCM2 at levels similar to those seen in interphase extracts (Fig. 4C).

XDbf4 Is Partially Dephosphorylated Upon Release from CSF-Arrest—The results shown above indicate that XDbf4 in egg extracts is phosphorylated in a cell-cycle dependent manner. To investigate further the phosphorylation of XDbf4 and XCdc7 under more physiological conditions, we utilized CSF-mediated, metaphase-arrested extracts (CSF-extracts) (reviewed in *39*). Adding free calcium ions to CSF-extracts caused the release from metaphase arrest and entrance into interphase. As shown in Fig. 5A, XDbf4 was not detected in CSF extracts without phosphatase treatment (0 min, Fig. 5A), but phosphatase treatment resulted in the re-appearance of XDbf4 (see Fig. 6A), indicating that XDbf4 in CSF-extracts is phosphorylated in a way similar to that seen in extracts supplemented with cyclin B Δ85. Consistent with this, slow migrating forms of XCdc7 were detected in CSF-extracts. Importantly, these phosphorylated forms were converted to the forms seen in interphase extracts when the

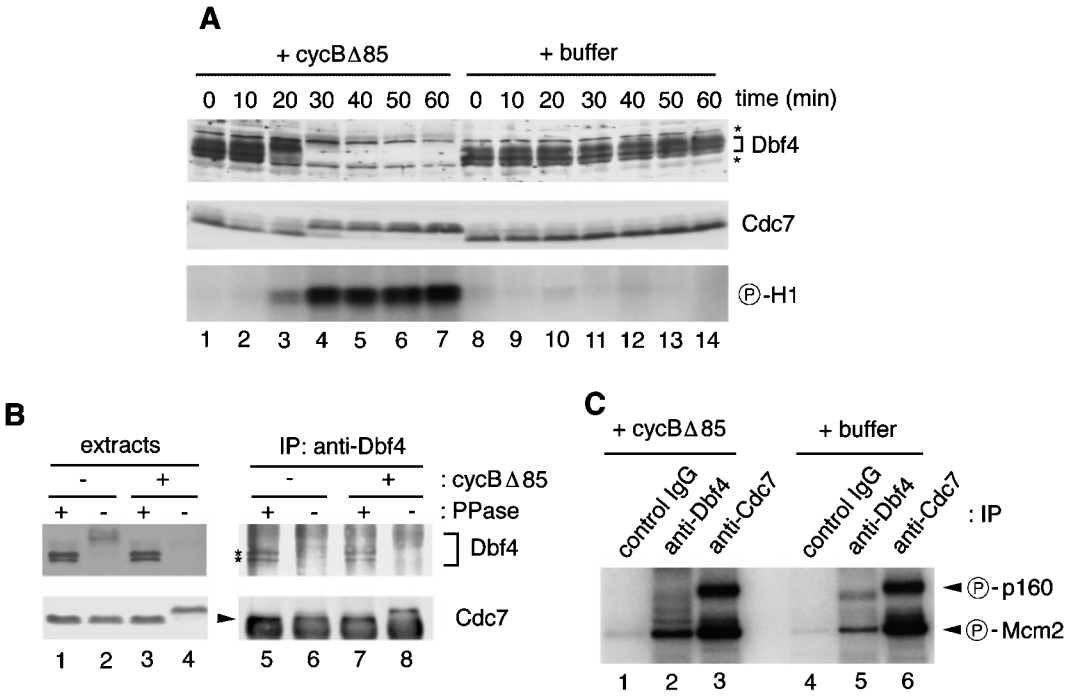

Fig. 4. **XDbf4 and XCdc7 are phosphorylated in a cyclin B-dependent manner.** A: Interphase extracts were supplemented with cyclin B Δ85 (lanes 1–7) or control buffer (lanes 8–14) and incubated for the indicated time. Aliquots of extracts were subjected to histone H1 kinase assay and immunoblotting with anti-Dbf4-N and anti-XCdc7 antibodies. The bands indicated by asterisks are non-specific. B: Interphase extracts were incubated in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of cyclin B Δ85 for 60 min. Aliquots of extracts treated with (lanes 1 and 3) or without (lanes 2 and 4) lambda protein phosphatase (PPase) were analyzed directly by SDS-PAGE/immunoblotting. The same extracts were also immunoprecipitated with anti-Dbf4-C antibodies and analyzed as above (lanes 5–8). Asterisks indicate the bands of dephosphorylated XDbf4 seen in lanes 5 and 7. Note that XDbf4 in the immunoprecipitates (lane 6) was not clear due to a high background. The arrowhead indicates the XCdc7 band just above the IgG background. C: An IP-kinase assay with anti-Dbf4-C antibodies, anti-XCdc7 antibodies or control IgG was done with the extracts prepared as in B. The assay was carried out as described for Fig. 3.

extracts entered interphase (30–50 min, Fig. 5A). These findings indicate that both XDbf4 and XCdc7 are dephosphorylated upon release from CSF-metaphase arrest.

After release from CSF-arrest, interphase extracts are known to enter the mitotic phase when newly synthesized cyclin B accumulates (*40*). As shown in Fig. 5B, the interphase forms of both XDbf4 and XCdc7 were converted again to the mitotic forms 90 min after the addition of calcium; that is, the XDbf4 bands disappeared with the appearance of a slowly migrating XCdc7 band (Fig. 5B). When the incubation was carried out in the presence of cycloheximide, under which the synthesis of cyclin B was suppressed, those changes in XDbf4/XCdc7 were not observed (Fig. 5C). These results further confirm that both XDbf4 and XCdc7 are phosphorylated in a cell-cycle dependent manner. In particular, XDbf4 is phosphorylated throughout the cell cycle, but its phosphorylation state in interphase is distinct from that in the mitotic phase and during CSF-metaphase arrest.

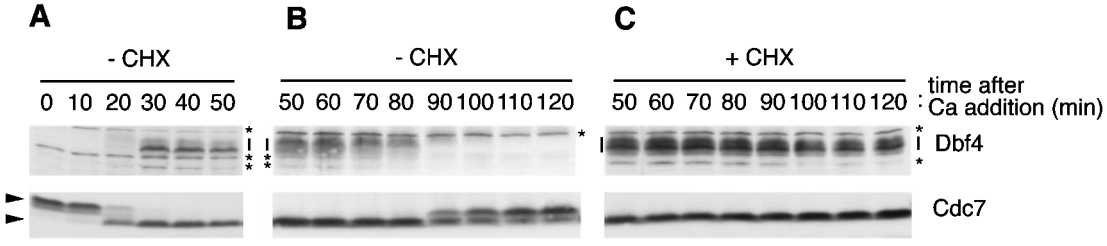

Fig. 5. **XDbf4 and XCdc7 are dephosphorylated upon release from CSF-metaphase arrest and re-phosphorylated upon entering mitosis.** A, B: CSF-extracts were supplemented with free calcium ions and incubated in the absence of cycloheximide (–CHX) for the indicated times. An aliquot of each extract was subjected to SDS-PAGE/immunoblotting with anti-Dbf4-N and XCdc7 antibodies. C: The experiment was carried out under the same conditions as in A and B, except that the incubation of CSF-extracts supplemented with free calcium ions was carried out in the presence of cycloheximide (+CHX). Bars and arrowheads indicate XDbf4 and XCdc7, respectively. Asterisks indicate non-specific bands. Note that the experiments shown in B and C were done using the same extracts, while the experiment in A was done with separate extracts.

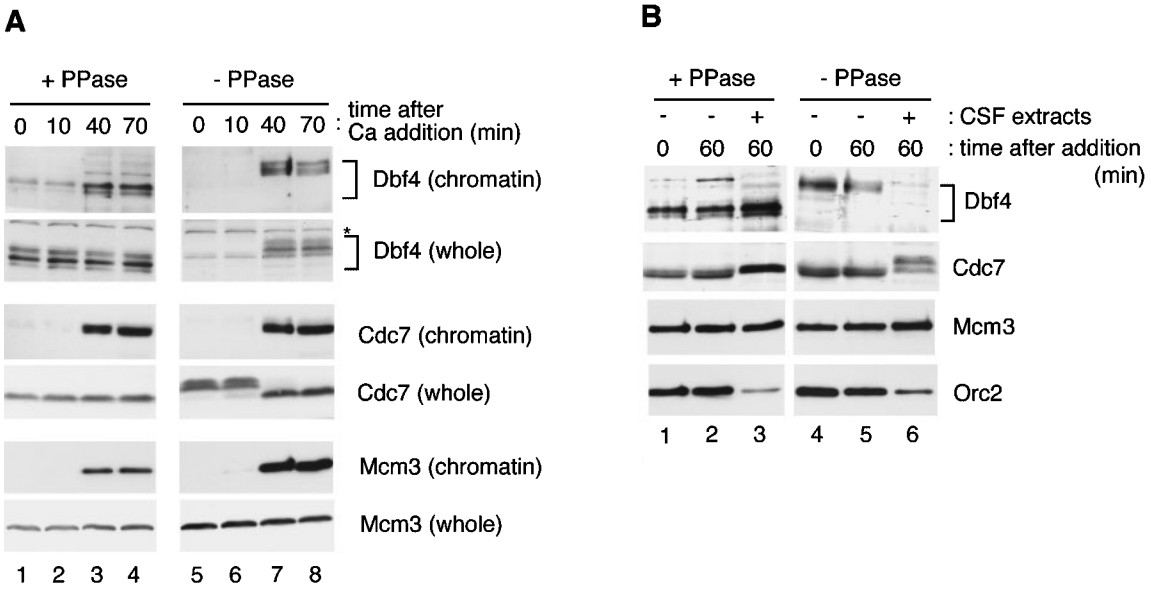

Fig. 6. **Chromatin binding of mitotically phosphorylated XDbf4 and XCdc7.** A: CSF-extracts were mixed with free calcium ions and demembranated sperm chromatin (4,000 nuclei/μl). After incubation for the indicated times, chromatin was isolated from the mixture. Aliquots of the whole mixture and chromatin fractions were treated with or without lambda phosphatase (+/–PPase) and then subjected to SDS-PAGE/immunoblotting. The asterisk indicates non-specific bands in the XDbf4 blots. B. CSF-extracts were mixed with free calcium ions and demembranated sperm chromatin (500 nuclei/μl). After a 170-min incubation, two volumes of CSF-extract without calcium were added and the samples were incubated for a further 60 min (lanes 3 and 6). After incubation, chromatin was isolated, treated with or without lambda phosphatase, and subjected to SDS-PAGE/immunoblotting with appropriate antibodies.

Chromatin Binding of Mitotically Phosphorylated XDbf4 and XCdc7—The data shown above indicate that XDbf4/XCdc7 in the mitotic phase has kinase activity. We next examined whether the mitotic phosphorylation of XDbf4/XCdc7 affects the binding of these proteins to chromatin. First, demembranated sperm chromatin was mixed with CSF-extracts and then free calcium ions were added to release the arrest. As shown in Fig. 6A, mitotically phosphorylated forms of XDbf4 and XCdc7 were present in CSF-extracts and in the extracts 10 min after the addition of calcium (prior to entering interphase), and these failed to bind to chromatin. XMCM3 was also not loaded onto chromatin at this time point. At 40-min, the XDbf4/XCdc7 in the extracts had been converted to the interphase forms, and XDbf4/XCdc7 as well as XMCM3 were loaded onto chromatin. Also, as mentioned above, the XDbf4 level in the extracts did not change during the transition from metaphase arrest to interphase.

Although these results show the correlation between chromatin binding and the dephosphorylation of XDbf4/XCdc7, these experiments do not prove that mitotic phosphorylation is a cause for the inability of XDbf4/XCdc7 to bind to chromatin. Thus, we examined whether XDbf4 and XCdc7 are dissociated from chromatin when XDbf4/XCdc7-bound chromatin is exposed to the mitotic state in which cyclin B-Cdc2 kinase is activated. To this end, demembranated sperm nuclei were first mixed with CSF-extracts and free calcium, and incubated for 170 min (at this time, replication was not yet completed), and then CSF-arrested extracts without calcium were added as a source of active cyclin B-Cdc2 and the mixture was further incubated for 60 min. As shown in Fig. 6B, just before the addition of the CSF-extracts, XDbf4, XCdc7, XMCM3 as well as XORC2 were bound to chromatin (lanes 1 and 4, Fig. 6B). After a 60-min incubation following the addition of the CSF extracts, both XDbf4 and XCdc7 remained bound to chromatin even though majorities of both proteins were converted to the mitotically phosphorylated forms, whereas part of XORC2 dissociated from chromatin as shown previously (lanes 3 and 6, Fig. 6B) (*41*). On the other hand, XMCM3 as well as XDbf4/XCdc7 remained on chromatin when the CSF extracts were added. These results suggest that XDbf4/XCdc7 bound to chromatin can be mitotically phosphorylated and that these phosphorylated forms can remain bound to chromatin.

XDbf4 Binds to Chromatin Earlier than XMCM2-7 and XCdc7 during DNA Replication—The above results show that XDbf4 binds to chromatin in interphase extracts. As to the binding of Dbf4/Cdc7 to chromatin, it was previously shown that XORC, XMCM2-7 and XCdc7 are sequentially loaded on sperm chromatin during incubation with egg extracts (*13*). On the other hand, it has also been shown that yeast Dbf4 may be loaded onto chromatin in an ORC- dependent but Cdc6-independent manner (*28*). Thus, we examined the timing of XDbf4 binding to chromatin during DNA replication in more details using interphase extracts. We found that XDbf4 loaded onto chromatin at almost the same time as XORC2, clearly before XMCM2-7 and XCdc7 (Fig. 7A). These results suggest that XDbf4 in interphase extracts binds to chromatin independent of pre-RC formation. Consistently, XDbf4 was loaded onto chromatin even in the presence of geminin, where the chromatin loading of MCM2-7 and XCdc7 is suppressed (data not shown). Moreover, the level of chromatin-bound XDbf4 was not altered signifi-

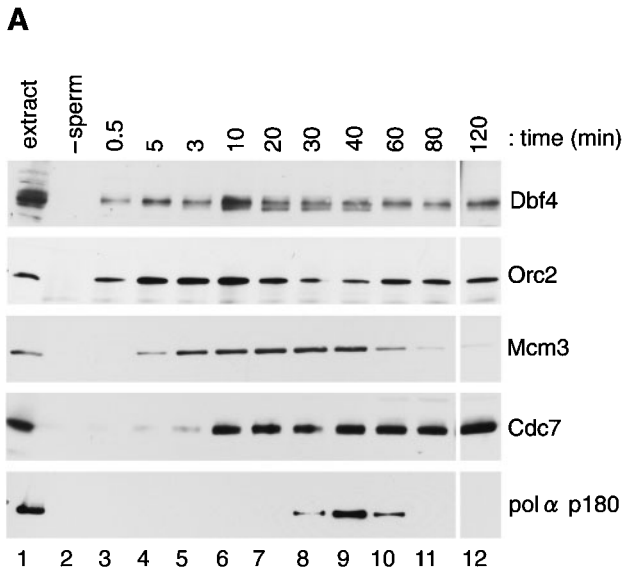

Fig. 7. **XDbf4 is loaded on chromatin before XMCM3 and XCdc7.** A: Demembranated sperm nuclei were incubated with interphase extracts (+ cycloheximide) for the indicated times, and the proteins bound to chromatin were analyzed by SDS-PAGE/immunoblotting with the indicated antibodies. A negative control without sperm nuclei (0.5-min incubation) is also shown (–sperm). An interphase egg extract (1 μl) was loaded in the left lane. B: Demembranated sperm nuclei were incubated in XORC2-depleted or mock-depleted interphase extracts for 5 min, and chromatin-bound XDbf4, XORC2, XORC1 and XMCM3 were then analyzed by SDS-PAGE/immunoblotting. The chromatin samples in B but not A were treated with lambsda protein phosphatase before mixing with SDS loading buffer.

cantly during DNA replication (Fig. 7A); in some cases, a slight increase in chromatin binding of XDbf4 was seen during the period of DNA synthesis (data not shown). Curiously, XCdc7 and XDbf4 remained bound to chromatin even after XMCM6 and DNA polymerase α dissociated, which is indicative of the completion of DNA replication (lanes 10–12, Fig. 7A).

Next, to examine whether the binding of XDbf4 to chromatin requires XORC, XORC2 was first depleted from interphase extracts and then demembranated sperm nuclei were added to the depleted extracts to follow the chromatin binding of XDbf4. At least 94% of XORC1 was also removed by XORC2-depletion, whereas XORC2 was hardly detected by immunoblotting. Consistently, XMCM3 was hardly loaded on chromatin in the XORC2-depleted extracts (Fig. 7B). However, the depletion of XORC2 did not cause any significant change in the level of chromatin-bound XDbf4, suggesting that the binding of XDbf4 to chromatin is independent of a functional XORC complex.

DISCUSSION

In this study we identified a *Xenopus* homolog of Dbf4 (XDbf4), and characterized XDbf4 and XCdc7 in egg extracts. The overall similarity of the amino acid sequences of human Dbf4 (ASK) and *Xenopus* Dbf4 is relatively low, but all the Dbf4-motifs are conserved and the protein encoded by this *Xenopus* cDNA exhibited essentially the characteristics as Dbf4; that is, it binds to Cdc7 and activates Cdc7 kinase activity. Therefore, we conclude that the *Xenopus* cDNA clone obtained in this study indeed encodes a *Xenopus* ortholog of Dbf4.

The data in this study indicate that the XDbf4 level remains constant in the different stages of the cell cycle in egg extracts. This feature of XDbf4 is in striking contrast to the stability of Dbf4 proteins in yeast and mammalian cultured cells. *S. cerevisiae* Dbf4 is degraded approximately at the metaphase-anaphase transition in an APC-dependent manner, and *S. cerevisiae* Dbf4 is known to have destruction box-like sequences that are necessary for its degradation in G1 phase (*11*, *23–25*). The protein levels of mammalian Dbf4 (ASK) also oscillate through the cell cycle (*26*). On the other hand, we did not detect a decline in the XDbf4 level during release from CSF-mediated metaphase arrest. In addition, the levels of both the chromatin-bound and unbound forms of XDbf4 did not decline from the start of DNA replication to the mitotic phase, even when protein synthesis was suppressed. Consistent with these results, we failed to find any amino acid sequence involved in protein degradation, such as a destruction box (*38*), a PEST sequence (*42*), or a KEN box (*43*). Thus, it seems likely that the function of XDbf4 may be regulated in the *Xenopus* early embryonic cell cycle, if any, by a mechanism different from that in yeast cells or mammalian cultured cells.

Instead of proteolysis, our study revealed that XDbf4 is phosphorylated in a cell cycle-dependent manner; in mitotic phase, both XDbf4 and XCdc7 are phosphorylated by a mechanism in which the Cdc2 kinase may be involved directly or indirectly. Although we did not see any effect of the mitotic phosphorylation of XDbf4 on XCdc7 kinase activity under our conditions, it is not clear whether mitotically phosphorylated forms of XDbf4-XCdc7 can also phosphorylate physiological substrates, one of which is presumably an MCM2-7 complex bound to chromatin. Furthermore, it is still possible that the mitotic phosphorylation of XDbf4 and/or XCdc7 may play additional, unknown roles in the regulation of DNA replication during the mitotic phase. Thus, it will be necessary to pursue the functional consequences of the mitotic phosphorylation of XDbf4/XCdc7.

With respect to the regulation of XDbf4-XCdc7, Costanzo *et al.* have recently shown that the activation of an ATR-dependent, DNA damage checkpoint pathway may lead to the inhibition of XDbf4-XCdc7 and, consequently, inhibit the initiation of DNA replication (*44*). Although it is not known exactly what causes such downregulation of XDbf4-XCdc7, the phosphorylation of XDbf4 might be involved in the checkpoint pathway, as in the case of the Rad53-directed hyperphosphorylation of *S. cerevisiae* Dbf4 (*11*, *16*).

Our study revealed also that XDbf4 is loaded on chromatin before MCM2-7. On this point, the analysis of *in vitro* replication using isolated nuclei from *S. cerevisiae* suggests that Dbf4 targets Cdc7 to pre-RC independent of MCM2-7 loading on chromatin, and that the tight association of *S. cerevisiae* Dbf4 with chromatin is dependent on an ORC but not Cdc6 (*28*). A recent study of mammalian Cdc7 and ASK also suggested that the nuclear localization and chromatin binding of Cdc7 and ASK may be regulated independently (*45*). In *Xenopus* egg extracts, the binding of XDbf4 to chromatin seems to be independent of functional XORC as shown in this study, but it cannot be excluded that residual level of XORC1 (6% of the original amount in egg extracts) in XORC2-depleted extracts might be sufficient for XDbf4 loading onto chromatin (Fig. 7B). Moreover, although the binding of XCdc7 onto chromatin requires pre-RC formation (*13*), it is still possible that the loading of XDbf4 onto chromatin prior to pre-RC formation requires XCdc7. Conversely, it also remains to be determined whether the loading of XCdc7 onto chromatin requires XDbf4 and whether each protein is loaded independently. Thus, in order to gain insight into the interdependence of XDbf4 and XCdc7 in terms of their binding to chromatin, it will be necessary to analyze their chromatin binding in XDbf4-depleted and XCdc7-depleted extracts.

Another interesting feature of the binding of XDbf4-XCdc7 to chromatin is that these proteins remain bound to chromatin even after the dissociation of XMCM2-7. This implies that they may have functions in processes other than the initiation of DNA replication, as suggested previously (*25*, *46*, *47*). In addition, it is also possible that the sustained binding of XDbf4-XCdc7 onto chromatin even in mitotic phase may be required for the subsequent S phase in order to allow rapid progression of the early embryonic cell cycle. Similarly, the feature of XDbf4 in the egg extracts, *i.e.*, it is stable and is not regulated by proteolysis, may also be involved in the early embryo-specific mechanism, if any, which would allow the rapid progression of the cell cycle without the G1 and G2 phases.

Our data indicate that XDbf4 can form a complex with XCdc7 and activate its kinase activity. However, it has not been demonstrated formally that XDbf4 is required for DNA replication in egg extracts. Although we could immunodeplete at least 94% of XDbf4 from egg extracts and the depletion caused a significant inhibition of DNA replication, it was not possible to restore DNA replication by adding back recombinant proteins to the depleted extracts (Furukohri, A., Sugino, A., and Waga, S., unpublished results).

In conclusion, the XDbf4 identified in this study exhibits some features that are not seen in Dbf4 from yeast and mammalian cultured cells; namely, XDbf4 is stable in egg extracts, binds to chromatin before MCM2-7 and XCdc7, and is phosphorylated differentially in a cell cycle-dependent manner. It seems possible that these features of XDbf4 in egg extracts are required for the rapid progression of the early embryonic cell cycle. Since it is likely that a homolog of Drf1 is also present in egg extracts, it will be necessary in the future to investigate the existence of a functional interrelationship between XDbf4 and *Xenopus* Drf1 in order to determine the precise role of XDbf4-XCdc7 in DNA replication and cell cycle progression in early embryos.

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