The Xenopus Xmus101 protein is required for the recruitment of Cdc45 to origins of DNA replication

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The initiation of eukaryotic DNA replication involves origin recruitment and activation of the MCM2-7 complex, the putative replicative helicase. Mini-chromosome maintenance (MCM2-7) recruitment to origins in G1 requires origin recognition complex (ORC), Cdt1, and Cdc6, and activation at G1/S requires MCM10 and the protein kinases Cdc7 and S-Cdk, which together recruit Cdc45, a putative MCM2-7 cofactor required for origin unwinding. Here, we show that the Xenopus BRCA1 COOH terminus repeat–containing Xmus101 protein is required for loading of Cdc45 onto the origin. Xmus101 chromatin association is dependent on ORC, and independent of S-Cdk and MCM2-7. These results define a new factor that is required for Cdc45 loading. Additionally, these findings indicate that the initiation complex assembly pathway bifurcates early, after ORC association with the origin, and that two parallel pathways, one controlled by MCM2-7, and the other by Xmus101, cooperate to load Cdc45 onto the origin.

Introduction

The initiation of DNA replication is tightly regulated so that origins of replication are normally utilized only once per cell cycle. Many of the factors and regulators that participate in the initiation phase of DNA replication are known, and the order in which these factors interact with one another and load onto origins is emerging. The process begins in late mitosis or G1, when origin recognition complex (ORC)* binds directly to chromosomal origins of replication (for review see Kelly and Brown, 2000; Bell and Dutta, 2002). Once bound, ORC recruits Cdc6 and Cdt1, which in turn allow assembly of the mini-chromosome maintenance (MCM2-7) complex onto the DNA. Recruitment of MCM2-7, the presumptive replicative helicase (Labib and Diffley, 2001), completes assembly of the prereplication complex (pre-RC). At the G1/S transition, the pre-RC is converted to a preinitiation complex (pre-IC) through the actions of two protein kinases, S phase–specific cyclin dependent kinase (S-Cdk) and the Cdc7-Dbf4 protein kinase, which cooperate to recruit the initiation factor Cdc45 (Mimura and Takisawa, 1998; Zou and Stillman, 1998, 2000; Jares and Blow, 2000; Walter, 2000). In addition to S-Cdk and Cdc7, another factor, MCM10, has been shown in Xenopus extracts to act after MCM2-7 to recruit Cdc45 (Wohlschlegel et al., 2002). After Cdc45 binding, the next discernible step in the initiation process is origin unwinding and recruitment of the single-stranded DNA binding protein RPA, followed by DNA polymerase α (pol α; Mimura and Takisawa, 1998; Tanaka and Nasmyth, 1998; Zou and Stillman, 1998, 2000; Mimura et al., 2000; Walter and Newport, 2000). Because Cdc45 loading is the last known step before origin unwinding and the commencement of DNA synthesis, and because it has been shown to be rate limiting for DNA replication in Xenopus egg extracts (Edwards et al., 2002), this event is considered to be critical for the regulation of initiation.

The Drosophila mus101 gene was isolated 25 years ago in a screen for mutants showing hypersensitivity to DNA damaging agents (Boyd et al., 1976). Further genetic analysis revealed that mus101 is an essential gene. Hypomorphic alleles have been isolated that exhibit defects in the kinetics of progression through S phase, and in chorion gene amplification (Orr et al., 1984). Therefore, these two phenotypes are consistent with a role for mus101 in DNA replication. Molecular cloning of mus101 (Yamamoto et al., 2000)
Figure 1. Xmus101 is required for DNA replication in Xenopus. (A) The Mus101 protein family. Shown are schematic depictions of Mus101-related proteins from divergent organisms. The shaded gray boxes indicate the position of the BRCT domains, and the numbers to the right indicate the size of the protein, in kD, of molecular mass markers. (B) Immunoblot analysis of Xenopus egg extract (XEE), or egg extract that had been immunodepleted of Xmus101 (α-Xmus101), probed with affinity-purified anti-Xmus101 antibodies. The asterisk denotes a background band recognized by the antibody that is not diminished in the depleted extract. The numbers to the left of the gel denote the migration position and molecular mass, in kD, of molecular mass markers. (C) Egg extracts were prepared, and then depleted of Xmus101 protein (α-Xmus101) or mock-depleted (mock). Additionally, Xmus101-depleted extracts were supplemented with in vitro transcription and translation (IVT) reactions programmed by the Xmus101 cDNA. In vitro transcription was performed in either the sense, or anti-sense orientation. Sperm chromatin was then added, along with 32P-dATP, and DNA replication in the given extract was measured at the indicated times. The graph depicts the amount of DNA replication observed after quantification of the dried gels by PhosphoImager analysis. The data shown are from a single experiment, and are representative of four independent trials. The amount of replication observed in the mock-depleted sample at the 90-min time point was set to 100, and all other values adjusted accordingly.

Results and discussion

The metazoan Mus101 protein family includes human TopBP1, Drosophila Mus101, and an uncharacterized locus in C. elegans, F37D6.1 (Fig. 1 A). To initiate an analysis of this protein family in DNA replication, we isolated a Xenopus homologue of Mus101, named Xmus101. The full-length Xmus101 cDNA encodes a 1,513 amino acid protein with significant similarity to TopBP1 (75% amino acid similarity), to Drosophila Mus101 (43% similarity), and to C. elegans F37D6.1 (39% similarity). The similarity between Xmus101 and Cut5 is restricted to the BRCT domains, whereas no significant sequence similarity between Xmus101 and Dpb11 was detected.

Rabbit polyclonal antibodies were raised against a recombinant protein containing the NH2-terminal 333 amino acids of Xmus101, and affinity purified. These antibodies recognize a 180-kD protein in Xenopus egg extract (Fig. 1 B), and can immunoprecipitate the 180 kD in vitro transcription and translation product of the cloned Xmus101 cDNA (unpublished data). To determine if Xmus101 functions in DNA replication in Xenopus, the protein was removed from egg extracts, by immunodepletion, and the ability of the depleted extract to replicate sperm chromatin was assessed. As shown in Fig. 1 B, the immunodepletion procedure removed all detectable Xmus101 from the extract. To control for nonspecific effects of immunodepletion, a mock-depleted extract was also prepared, and replication was measured in both the mock- and Xmus101-depleted extracts. DNA replication was severely compromised in the Xmus101-depleted extract, relative to the mock-depleted control extract (Fig. 1 C, compare “mock” sample to “α-Xmus101” sample). Importantly, addition of recombinant Xmus101 protein, produced by transcription and translation in vitro, fully rescued the replication defect in the Mus101-depleted extract (Fig. 1 C, “α-Xmus101+IVT sense” sample). Rescue was not observed when transcription was performed in...
the opposite orientation, to produce the anti-sense Xmus101 mRNA (Fig. 1 C, “α-Xmus101+IVT anti-sense” sample). This rescue by the sense, but not anti-sense Xmus101 mRNA, demonstrates that the Xmus101-depleted extracts failed to replicate their DNA because Xmus101 was removed, and not because of nonspecific inactivation of the extract. These data show that Xmus101 is an essential DNA replication protein in *Xenopus*.

Chromatin immuno precipitation-based methodologies in budding yeast indicate that Dpb11 acts after RPA has bound to the origin to recruit pol α and pol ε to the assembling replication complex (Masumoto et al., 2000). Because Xmus101 is distantly related to Dpb11, we asked if Xmus101 was required for replication complex assembly in egg extracts. For this, sperm chromatin was added to either mock- or Xmus101-depleted extract, and after 45 min the chromatin was isolated and probed, by immunoblotting, with antibodies against Cdc45 and Xmus101. Lane 1 shows a reaction lacking sperm chromatin.

The data in Fig. 2 (B and C) demonstrate that Xmus101 replication of DNA initiation.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Xmus101 is required for the loading of Cdc45 onto chromatin during the initiation of DNA replication. (A) Immunoblot of either total extract samples from mock-depleted (mock, lane 1), or Xmus101-depleted (α-Xmus101, lane 2) extract, or chromatin isolated after a 45-min incubation in either mock- (mock, lane 3), or Xmus101-depleted (α-Xmus101, lane 4) extract. The blots were probed with antibodies against Xenopus Xmus101 (panel I), pol α (pol α p60 subunit, panel II), RPA (the 34-kD subunit, panel III), pol ε (pol ε p70 subunit, panel IV), Cdc45 (panel V), MCM10 (panel VI), Cdc7 (panel VII), or the MCM7 component of the MCM2-7 complex (panel VIII). (B) Immunoblot of complete egg extract, both total extract (Tot.) and the isolated chromatin fraction (Chr.), and membrane-free egg extract, or egg cytosol, both total extract and the isolated chromatin fraction. (C) Egg extracts were prepared and supplemented with sperm chromatin (lane 2), or sperm chromatin plus recombinant geminin (500 nM, lane 3), or sperm chromatin plus recombinant p27Kip (500 nM, lane 4). After a 45-min incubation, the chromatin was isolated and probed, by immunoblotting, for the presence of Cdc45 and Xmus101. Lane 1 shows a reaction lacking sperm chromatin.

To better understand how Xmus101 participates in Cdc45 recruitment, we characterized its chromatin-binding properties. We found that Xmus101 did not load onto chromatin in membrane-free egg extracts (egg cytosol) that are competent to form pre-RCs, but incompetent to form nuclei or execute subsequent initiation events (Fig. 2 B, lane 4). In contrast, in nuclear assembly extracts that undergo initiation of replication, Xmus101 bound to chromatin (Fig. 2 B, lane 2). The inability to bind to chromatin in egg cytosol indicates that Xmus101 is not a constituent of the pre-RC. To ask if Xmus101 chromatin binding is dependent upon an intact pre-RC once nuclear formation occurs, we assessed Xmus101 chromatin-association in extracts treated with geminin, an inhibitor of the Cdt1 protein (Wohlschlegel et al., 2000; Tada et al., 2001). Interestingly, we found that Xmus101 chromatin binding was insensitive to geminin (Fig. 2 C, lane 3). To ensure that the geminin was active in this experiment, we also probed the purified chromatin fraction for the presence of Cdc45, and found, as expected (Jares and Blow, 2000), that geminin blocked Cdc45 association with chromatin (Fig. 2 C, lane 3). Next, we determined if Xmus101 chromatin association required S-Cdk activity. For this we used p27Kip (Toyoshima and Hunter, 1994), an S-Cdk inhibitor that has previously been shown to block DNA replication in frog egg extracts (Michael et al., 2000). We found that p27Kip prevented Cdc45 from loading onto chromatin, as expected, but that it could not prevent loading of Xmus101 (Fig. 2 C, lane 4).

The data in Fig. 2 (B and C) demonstrate that Xmus101 requires nuclear formation to bind to chromatin, but that it binds in a S-Cdk– and pre-RC–independent manner. Therefore, there was a possibility that Xmus101 bound to chroma-
Figure 3. Xmus101 requires ORC for association with sperm chromatin. In lanes 1–4, egg cytosol (EC) that either contained or lacked ORC, as indicated, was mixed with sperm chromatin and incubated for 30 min. The chromatin was then isolated and probed by immunoblotting for the presence of Xmus101, MCM10, MCM7, or ORC2. In lanes 5–8, egg cytosol that either contained or lacked ORC, as indicated, was mixed with sperm chromatin and incubated for 30 min. After incubation, NPE that either contained or lacked ORC, as indicated, was then added and incubation continued for an additional 30 min before isolation of the chromatin and immunoblotting for the indicated factor. The NPE contained the replication inhibitor aphidicolin, to trap assembled replication complexes and prevent disassembly following the completion of DNA replication.

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port pre-RC formation, but only after chromatin has been exposed to an initiation-competent nuclear environment. These data imply that, besides ORC, Xmus101 chromatin association also requires an unknown condition that is only met inside the nucleus. This could result from loss of a cytosolic inhibitor of Xmus101 chromatin association, or be due to a positive influence of the nucleus, such as an unknown bridging factor, or a nucleus-specific alteration of Xmus101 activity. Alternatively, because the concentration of DNA replication factors is much higher within nuclei (or in NPE), relative to cytosol, the nuclear-dependence of Xmus101 chromatin association might simply result from a concentration effect. Consistent with this, we find that the concentration of Xmus101 in NPE is at least 20-fold higher than in egg cytosol (unpublished data).

Although origin binding of Xmus101 requires ORC, it is independent of MCM2-7 and S-Cdk. This MCM2-7 independence distinguishes Mus101 from the other known Cdc45 loading factors, Cdc7 and MCM10, both of which require a fully intact pre-RC for chromatin loading (Jares and Blow, 2000; Walter, 2000; Wohlschlegel et al., 2002). Furthermore, we have shown that MCM2-7, Cdc7, and MCM10 all load onto chromatin independent of Xmus101. Thus, the mutual independence of Xmus101 and MCM2-7/Cdc7/MCM10 origin association strongly suggests that the assembly pathway that ultimately culminates in origin loading of Cdc45 bifurcates at a step soon after chromatin-loading of ORC (Fig. 5). One pathway leads via MCM2-7 to the recruitment of Cdc7 and MCM10, and the other pathway works through Xmus101. Besides MCM2-7, MCM10, Cdc7, and Mus101, the other major requirement for Cdc45 loading is S-Cdk activity. The model in Fig. 5 depicts two pathways emanating from ORC, and it is not yet clear which pathway requires S-Cdk. We have found that Xmus101 loads onto chromatin in the absence of S-Cdk activity, suggesting that if S-Cdk acts in the Mus101 branch, then it would be downstream of Mus101. If S-Cdk does act in the Mus101 branch of the initiation pathway, then both branches would be under the control of protein kinases, as the Cdc7 kinase functions in the parallel, MCM2-7 dependent branch. Having each branch of the initiation pathway under the control of a protein kinase would thus afford tight regulation and coordination of the two branches, and perhaps explain why initiation requires two protein kinase activities.

Budding yeast Dpb11 and fission yeast Cut5 are considered homologues of one another because they are both required for DNA replication, they are both required for replication checkpoint control, and they are both composed of reiterated BRCT domains. These arguments have also been...
extended to TopBP1/Mus101, as it has been suggested that Mus101 represents the metazoan homolog of Dpb11/Cut5 (Yamamoto et al., 2000; Makiniemi et al., 2001; Wang and Elledge, 2002). The data reported here suggest a reconsideration of this designation, as we have shown that Mus101 acts in a fundamentally different capacity than does Dpb11 during the initiation of DNA replication. Mus101 is required for Cdc45 loading, whereas Dpb11 has been reported to act much later, after Cdc45 has been loaded and, indeed, after the helicase has been activated and RPA has been recruited to the origin (Masumoto et al., 2000). Another difference is that Dpb11 function during initiation is tightly connected to pol ε, as Dpb11 was initially isolated as in a synthetic lethal screen for genes interacting with pol ε, and Dpb11 has since been shown to physically interact with the pol ε complex. Such interaction with pol epsilon is not likely to be the case for Xmus101, as it has been shown that Cdc45 is required for pol ε chromatin association in frog egg extracts (Mimura et al., 2000); therefore, our data place Xmus101 minimally two steps upstream of pol ε in the initiation pathway. Thus, it is likely that Mus101 is not the metazoan orthologue of Dpb11/Cut5, and that the Dpb11/Cut5 orthologue still awaits identification.

Materials and methods

Cloning of the Xenopus Xmus101 cDNA

BLAST searching of NCBI databases using Drosophila mus101 sequences revealed several overlapping Xenopus ESTs containing significant sequence homology to the fly mus101 gene. Based on the sequence of the frog ESTs, primers were designed to amplify a 1,000 nucleotide fragment corresponding to nucleotides 3,566–4,566 of the Xmus101 open reading frame. The PCR fragment was then used as a probe to screen, by hybridization, a Xenopus cDNA CDNA library. Library screening resulted in the isolation of a full-length Xmus101 cDNA, which was then sequenced on both strands according to standard methods.

Antibody production

PCR primers were designed to amplify fragments corresponding to the COOH-terminal 333 amino acids of Xmus101. The fragment was cloned into the pET24a bacterial expression vector (Novagen) and used to clone into the pET24a bacterial expression vector (Novagen) and used to produce recombinant protein. Six histidine-tagged recombinant protein was purified on a nickel agarose column, under native conditions. Purified protein was then used as antigen to immunize rabbits. Polyclonal antibodies were obtained, and affinity purified according to standard procedures (Harlow and Lane, 1988). Antibodies against Xenopus Cdc45, RPA, MCM10, and Cdc7 have been described (Walter and Newport, 2000; Walter, 2000; Wohlschlegel et al., 2002). Additionally, antibodies against Xenopus MCM7 were obtained from John Newport (University of California San Diego, San Diego, CA), and against Xenopus pol α and pol ε from Shou Waga (Osaka University, Osaka, Japan).

Xenopus egg extract methodologies

Preparation of frog egg extracts, preparation of sperm chromatin, and DNA replication analysis was all performed according to Walter and Newport (1999). NPE was prepared as described (Walter et al., 1998). For immunodepletion, anti-Xmus101 antibodies were coupled to 50 µL of protein A Sepharose beads, and then incubated with 200 µL of frog egg extract on ice for 1 h. After incubation, the beads were pelleted, and the extract collected for an additional round of depletion. After the second round of depletion, the extract was used for the given experiment. For mock depletion, the procedure was identical except that anti-Xmus101 antibodies were replaced by phosphate-buffered saline. ORC depletion of NPE was performed as described (Walter and Newport, 2000).

Chromatin isolations

For the experiments shown in Fig. 2, chromatin was isolated as described (Michael et al., 2000). For the experiments shown in Fig. 3, chromatin isolation from NPE was performed as described (Wohlschlegel et al., 2002).

For the experiment shown in Fig. 4, the chromatin was isolated according to Jares and Blow (2000), using Nuclear Isolation Buffer containing 100 mM KCl.

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