Proliferating Cell Nuclear Antigen Recruits Cyclin-dependent Kinase Inhibitor Xic1 to DNA and Couples Its Proteolysis to DNA Polymerase Switching**

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The Xenopus cyclin-dependent kinase (CDK) inhibitor, p27Xic1 (Xic1), binds to CDK2-cyclins and proliferating cell nuclear antigen (PCNA), inhibits DNA synthesis in Xenopus extracts, and is targeted for ubiquitin-mediated proteolysis. Previous studies suggest that Xic1 ubiquitination and degradation are coupled to the initiation of DNA replication, but the precise timing and molecular mechanism of Xic1 proteolysis has not been determined. Here we demonstrate that Xic1 proteolysis is temporally restricted to late replication initiation following the requirements for DNA polymerase α-primase, replication factor C, and PCNA. Our studies also indicate that Xic1 degradation is absolutely dependent upon the binding of Xic1 to PCNA in both Xenopus egg and gastrulation stage extracts. Importantly, while the addition of recombinant wild-type PCNA alone restores Xic1 degradation, the addition of a PCNA mutant defective for trimer formation does not restore Xic1 proteolysis in PCNA-depleted extracts, suggesting Xic1 proteolysis requires both PCNA binding to Xic1 and the ability of PCNA to be loaded onto primed DNA by replication factor C. Taken together, our studies suggest that Xic1 is targeted for ubiquitination and degradation during DNA polymerase switching through its interaction with PCNA at a site of initiation.

During the eukaryotic cell cycle, the events of DNA replication are temporally coordinated to ensure the completion of DNA replication only once per cell cycle. The steps of DNA replication initiation are evolutionarily conserved from budding yeast to humans and require the assembly of the prereplication complex or pre-RC3 on DNA. The pre-RC is comprised of the origin recognition complex (ORC1–6), Cdc6, Cdt1, and the minichromosome maintenance (MCM2–7) proteins (1–15). The next step of replication initiation requires the activation of replication factor C, and PCNA. The progression of the vertebrate cell cycle is positively regulated by cyclin-dependent kinases (CDKs) associated with their cyclin partners and is negatively regulated by CDK inhibitors (reviewed in Refs. 33–36). Cyclin E in association with CDK2 is required for the G1 to S phase transition in mammals and for the initiation of DNA replication in Xenopus laevis, although its critical targets have yet to be identified (16, 37, 38). Cyclin A in mammals associates with CDK2 during G1 and S phases, localizes to sites of DNA replication, and is required for the onset of S phase, although its specific role in S phase has not been well defined, and its critical substrates are not clear (24, 25, 39, 40). Recent studies suggest that cyclin A activation must follow cyclin E activation and that cyclin A performs two roles during G1/S, the activation of DNA replication and the prevention of replication complex re-initiation (41–45).

The Cip/Kip family of mammalian CDK inhibitors is comprised of p21Cip1, p27Kip1, and p57Kip2 and exhibits a broad range of inhibitory activity toward many CDK-cyclin complexes (46–51). All Cip/Kip-type CDK inhibitors contain CDK-binding domains within their amino termini, while p21Cip1 and p57Kip2 also contain PCNA-binding domains within their carboxyl-terminal residues (52–56). In normal diploid cells, PCNA is found in multiple independent quaternary complexes with p21Cip1, CDKs, and cyclins A, B, and D (57, 58). Before the onset of S phase, both p27Kip1 and p21Cip1 are targeted for ubiquitin-dependent proteolysis, while p21Cip1 is also directly targeted for proteolysis by the proteasome (59–68). Expression of a non-degradable p27Kip1 mutant causes mammalian cells to arrest in G1, indicating that the proteolysis of p27Kip1 is a prerequisite for the entry into S phase (62). In mammals, CDK inhibitors are targeted for proteolysis at a point before the onset of DNA replication, but it is unclear when this turnover occurs in relation to the specific molecular events of DNA replication initiation. Studying the precise timing and mechanism of CDK inhibitor proteolysis can aid in understanding the role of CDKs and CDK inhibitors in regulating the start of processive DNA replication.

In Xenopus, two CDK inhibitors have been identified that share sequence and functional similarity with both p27Kip1 and p21Cip1. Xenopus inhibitor of CDK (p27Xic1 or Xic1) and kinase inhibitor from Xenopus (p28Kix1 or Kix1) share ~90% amino acid sequence identity with...
Each other, preferentially inhibit the activity of CDK2-cyclins E and/or A, and bind all CDK-cyclins and PCNA (69, 70). Xic1 and Kix1 have been shown to inhibit nuclear DNA synthesis in egg extracts through their binding to CDK2-cyclins (69, 70), while Xic1 has also been shown to inhibit the replication of single-stranded DNA through its binding to PCNA (69). Studies have demonstrated that in Xenopus interphase egg extracts, Xic1 is targeted for nuclei-dependent ubiquitination and degradation in a manner that is independent of CDK2-cyclin binding and phosphorylation at CDK consensus sites (71–74). Recently, two additional CDK inhibitors have been identified in Xenopus, p16Xic2 and p17Xic, and these CDK inhibitors may be orthologs of the mammalian inhibitors, p21cip1 and p27kip1, respectively (75).

The cell-free interphase egg extract from Xenopus can recapitulate the synchronous temporal events of DNA replication initiation and elongation and can support the proteolysis of Xenopus CDK inhibitors (72, 76). Using this model extract system, past studies suggest that Xic1 ubiquitination and degradation are coupled to the initiation of DNA replication, but the molecular mechanism of Xic1 proteolysis has not been determined. Our studies indicate that Xic1 is degraded during late replication initiation following DNA unwinding, RFC, and PCNA function. Importantly, we demonstrate that the ubiquitination and degradation of Xic1 is critically dependent upon the binding of Xic1 to PCNA and the ability of PCNA to be loaded onto primed DNA by RFC. Taken together, our findings suggest that Xic1 is targeted for ubiquitination and degradation when recruited to a site of initiation by PCNA, thereby coupling the proteolysis of Xic1 to DNA polymerase switching.

**EXPERIMENTAL PROCEDURES**

**Preparation of Xenopus Interphase Egg Extracts, Embryonic Extracts, Xenopus Demembranated Sperm Chromatin, M13 Single-stranded DNA, and Methyl Ubiquitin—Xenopus egg interphase extract (low speed supernatant or LSS) and demembranated sperm chromatin were prepared as described previously (72, 74, 77, 78). The membrane-free high speed supernatant (HSS) was prepared from fresh LSS by centrifugation at 55,000 rpm for 1 h at 4 °C using a RPS55 rotor and a Sorvall RC M120 centrifuge. The clear yellowish supernatant was collected and then re-centrifuged for 25 min as above. The cleared HSS was supplemented with RPE and 4% and 4% of DNA and PCNA function. Importantly, we demonstrate that the ubiquitination and degradation of Xic1 is critically dependent upon the binding of Xic1 to PCNA and the ability of PCNA to be loaded onto primed DNA by RFC. Taken together, our findings suggest that Xic1 is targeted for ubiquitination and degradation when recruited to a site of initiation by PCNA, thereby coupling the proteolysis of Xic1 to DNA polymerase switching.

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**Recombinant Protein Expression and Purification—GST-Xic1 fusion proteins were expressed in HB101 (for GST pull-down assays) or in BL21(pLysS)DE3 for (for depletion studies). XPCNA cloned into pQE30 was expressed in M15 for antibody production, while XPCNA cloned into pET28a was expressed in BL21(pLysS)DE3 for rescue assays. GST- and His6-tagged proteins were purified using glutathione (Amersham Biosciences) or nickel-nitriilotriacetic acid (Qiagen)-Sepharose as described by the manufactures. Recombinant proteins were dialyzed into XB and concentrated before use.

**Inhibitors and Antibodies—Roscovitine (A. G. Scientific Inc.) and actinomycin D (Sigma) were dissolved in Me2SO at 50 mM and 10 mg/ml, respectively, and stored at −80 °C. Aphidicolin (Sigma) was dissolved in ethanol at 5 mg/ml and stored at −20 °C.

**DNA Constructs, Site-directed Mutagenesis, and DNA Sequencing—Xic1 deletion mutants were generated by PCR mutagenesis using pCS2+/Xic1-WT (72) as the template plasmid followed by subcloning into pCS2+/GST (74). For the generation of GST-Xic11–161–190, PCPR primers 5′-TCCGCCGAGACATCTACACGACGCCAGAAGAG and 5′-CGGATTCTCTATATACGCTATAGATC were used, and the PCR product was cloned into the SmaI and EcoRI sites of pCS2+/GST and pGEX2T (Amersham Biosciences). Xic1-WT was subcloned from the BamHI and EcoRI sites of pCS2+/ into pGEX2T. Xic11–161–210 was subcloned from pCS2+/GST-Xic11–161–210 as a blunt (filled-in XbaI)-BamHI fragment into the BamHI and SmaI sites of pGEX2T. Xenopus PCNA (XPCNA) was PCR-cloned from a stage 12 Xenopus embryonic cDNA library (Clontech) into the BamHI and Stul sites of pCS2+ using the following primers: 5′-CGCCGGATCCATGTGTTGAGGGTCGCTTGTTGCGAC and 5′-GAAGGCCTTAGAGCCTCTCTCTCAATCG. Point mutations in the Xic1 and XPCNA genes were generated using pCS2+/Xic1 or pCS2+/XPCNA plasmids, the QuikChange site-directed mutagenesis kit (Stratagene), and the following primers: Xic1:1-174A, 5′-GAGATCCACACTCCCCGACCGATTATTTCCCT and 5′-AGGGAAATACTGTCGGGGAGTTGGTATC; XPCNA:Y114A, 5′-CAAGAGAAGTTTTCTACGCGTGAAAATGACTGAT and 5′-CCATCGCTTCTTGTGCATGC. All mutations were confirmed by DNA sequencing using the Amplified sequencing kit (PerkinElmer Life Sciences). For bacterial expression, XPCNA was subcloned as a BamHI and Stul fragment from pCS2+/XPCNA into the BamHI and SmaI sites of pQEs30 (Qiagen). XPCNA was also subcloned from pCS2+/XPCNA as a BamHI and XhoI fragment into pET28a (Novogen). The construct, pCS2+/Xic1–16A, was kindly provided by P. F. Jackson (73).

__In Vitro Transcription and Translation—Wild-type and mutant Xic1 were in vitro transcribed and translated from the SB6 promoter in pCS2+ using [35S]methionine (PerkinElmer Life Sciences) and the TNT-coupled reticulocyte lysate system (Promega).

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SIIK132 hybridoma cells obtained from the ATCC were cultured, and hybridoma supernatant containing monoclonal antibody SIIK132 against DNA polymerase α was purified using protein G-Sepharose (Amersham Biosciences). Ascites against human RFC-c140 (mouse #6 and #11) were generously provided by B. Stillman (82). Purified rabbit IgG against human RFC-c140 (Chemicon) was precipitated in 50% ammonium sulfate and resuspended in 1× phosphate-buffered saline. Monoclonal antibody against human PCNA was purchased (Santa Cruz Biotechnology). Rabbit antibody generated against Xenopus PCNA (Covance) was affinity-purified using recombinant His6-XPCNA coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). Antibodies to hRPA-p70 (NeoMarkers), control mouse IgG, and rabbit IgG (Sigma) were purchased. Xic1-specific rabbit antibody was generated against Xic1 fused to maltose-binding protein (Covance) and affinity-purified against GST-Xic1.

__Degradation, DNA Replication, and Nuclei or Chromatin Spin Down Assays—Degradation, DNA replication, and nuclei or chromatin spin down assays were conducted as described previously with the following noted exceptions (74). [35S]Methionine-labeled Xic1 was added to a final concentration of ∼15 mM in degradation assays. For nuclei or chromatin spin down assays, the timing of Xic1 ubiquitination and PCNA chromatin association were performed in the absence of methyl ubiq-
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**FIGURE 1.** Xic1 is degraded during the late initiation events supported by the HSS supplemented with single-stranded DNA. A, Xic1 degradation assay in the LSS. [35S]Methionine-labeled Xic1 was incubated in the LSS with (+) or without (−) demembranated sperm chromatin (XSC) or M13 single-stranded DNA (ssDNA; 10 ng/μl), and double-stranded plasmid DNA (dsDNA; 10 ng/μl) for 0, 1, and 3 h followed by SDS-PAGE. B, Xic1 degradation assay in the HSS. [35S]Methionine-labeled Xic1 was incubated in the HSS in the absence of DNA (NO DNA) or in the presence of demembranated sperm chromatin (XSC; 10 ng/μl), M13 single-stranded DNA (ssDNA; 10 ng/μl), and double-stranded plasmid DNA (dsDNA; 10 ng/μl) for 0, 1, and 3 h followed by SDS-PAGE. C, Xic1 degradation assay in the HSS. Wild-type Xic1 (Xic1-WT), Xic1 bearing point mutations within the CDK-binding domain (Xic1-ck; R33A, L35A, F65A, F67A), and Xic1 bearing mutations at all six consensus CDK phosphorylation sites (Xic1-6A) were [35S]methionine-labeled and incubated in the HSS with (+) or without (−) M13 single-stranded DNA (ssDNA) for 0, 1, and 3 h followed by SDS-PAGE. D, Xic1 degradation assay in the HSS. GST, GST fused to wild-type Xic1 (GST-Xic1 WT), GST-Xic1 bearing point mutations within the CDK-binding domain (GST-Xic1-ck; R33A, L35A, F65A, F67A), and GST-Xic1 bearing mutations at all six consensus CDK phosphorylation sites (GST-Xic1-6A) were [35S]methionine-labeled and incubated in the HSS with (+) or without (−) M13 single-stranded DNA (ssDNA) for 0, 1, and 3 h followed by SDS-PAGE. The asterisks indicate mono-, di-, and triubiquitinated intermediaries of GST-Xic1 (161–190). The percentage of Xic1 remaining is shown where the 0-h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample.

**RESULTS**

**The Temporal Requirements for Xic1 Proteolysis: Xic1 Is Degraded during Late Initiation**—Previous studies have characterized Xic1 proteolysis in membrane-containing cytosolic egg extracts often called the low speed supernatant, or LSS, supplemented with sperm chromatin or in membrane-free nucleoplasmic extracts called NPE (71–74, 83). The LSS supports nuclei formation and all the temporal events of DNA replication including pre-RC and pre-IC formation, DNA unwinding, DNA polymerase switching, and elongation (76, 77). The NPE supplemented with chromatin supports the events of DNA replication in the absence of nuclei formation (84). High speed centrifugation of the LSS results in a soluble, cytoplasmic, membrane-free extract called the high speed supernatant, or HSS (77, 85). Due to the absence of nuclear membrane precursors, the HSS will not support the formation of nuclei and thus will not support the events of pre-RC and pre-IC formation or origin unwinding (85). However, the HSS will support DNA replication processes that are not dependent upon origin unwinding, namely single-stranded DNA synthesis, which requires only the late initiation events of primer synthesis, DNA polymerase switching, and elongation (85). Thus, the HSS has been used as a model for the study of single-stranded DNA replication and the activities of DNA polymerase α, primase, RFC, PCNA, and DNA polymerase δ.

To determine whether Xic1 degradation was supported by the single-stranded DNA replication events supported in the HSS, we used the HSS supplemented with single-stranded DNA. We found that while Xic1 was efficiently degraded in the LSS in the presence of either single- or double-stranded DNA, Xic1 was only degraded in the HSS in the presence of single-stranded DNA (Fig. 1, A and B). This result suggests that Xic1 proteolysis is triggered by the presence of single-stranded DNA or unwound DNA and is triggered during DNA polymerase switching or elongation, replication events supported by the HSS.

We next addressed whether the HSS extract system recapitulates the same regulation of Xic1 proteolysis previously demonstrated in the LSS and the NPE. Because we use both the LSS and the HSS in the following studies to examine Xic1 proteolysis, we tested whether the mechanism of Xic1 degradation is the same in the LSS, the NPE, and the HSS. In the LSS containing nuclei and the NPE containing chromatin, Xic1 proteolysis is not dependent upon CDK2-cyclin binding (71, 74). Additionally, in the LSS, Xic1 proteolysis is not dependent upon phosphorylation at

### GST Pull-down Assay

GST fusion proteins (5 μg) were coupled to glutathione-Sepharose 4B (Amersham Biosciences) and then incubated in 5–10 μl of egg extract for 1 h at 23–24 °C. The beads were washed extensively with NETN buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM EDTA, and 0.5% Nonidet P-40) and then analyzed by SDS-PAGE and immunoblotting.

**Depletion and Rescuing**—GST-Xic1-(161–190) was coupled to glutathione-Sepharose 4B to a final concentration of 80 μM and then incubated in the egg extract for 1 h at 4 °C. The depleted extracts were then separated from the beads by centrifugation at 4 °C. Control depletions were performed using GST coupled to glutathione-Sepharose beads. The depleted extracts were rescued with XB buffer or bacterially expressed and purified wild-type or Y114A mutant His6-T7-XPCNA.
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FIGURE 2. Xic1 degradation requires RNA and DNA priming. A, Xic1 degradation assay. LSS (left panel) and HSS (right panel) were treated with buffer (dimethyl sulfoxide [DMSO]), 500 μM roscovitine (ROS), or actinomycin D (AMD) at the indicated concentrations followed by the addition of [35S]methionine-labeled Xic1. Extracts were incubated in the presence (+) or absence (−) of demembranated sperm chromatin (XSC, LSS) or M13 single-stranded DNA (ssDNA, HSS) for 0, 1, and 3 h followed by SDS-PAGE. B, Xic1 degradation assay. LSS (left panel) and HSS (right panel) were treated with ethanol (ETOH) or aphidicolin (APHID) resuspended in ethanol at the indicated concentrations, supplemented with [35S]methionine-labeled Xic1, and incubated with (+) or without (−) demembranated sperm chromatin (XSC, LSS) or M13 single-stranded DNA (ssDNA, HSS) for 0, 1, and 3 h followed by SDS-PAGE. C, immunoblot analysis of LSS probed with SJK132 antibody. Molecular mass markers (M) in kilodaltons are indicated. The arrow designates the protein band for DNA polymerase α. D, Xic1 degradation assay. LSS (left panel) and HSS (right panel) were treated with control mouse IgG (mIgG) or anti-DNA polymerase α IgG (SJK132) at the indicated concentrations. [35S]Methionine-labeled Xic1 was incubated in treated extracts with (+) or without (−) demembranated sperm chromatin (XSC, LSS) or M13 single-stranded DNA (ssDNA, HSS) for 0, 1, and 3 h followed by SDS-PAGE. The percentage of Xic1 remaining is shown where the 0-h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample (% Xic1 Remaining). DNA replication in the treated extracts at the 3-h time point was normalized to 100% of the replication observed with the appropriate control buffer (% DNA Replication).

six consensus CDK2 sites but is critically dependent upon basic residues within amino acids 180–183 (73, 74). Using the HSS supplemented with single-stranded DNA, we examined the degradation of three key Xic1 mutants: ck (unable to efficiently bind CDK2-cyclin) (74), 6A (substituted at all six consensus CDK2 phosphorylation sites) (73), and NLS2 (mutated at basic amino acids 180–183) (74). Identical to published results obtained in the LSS and the NPE (71, 73, 74), our results demonstrate that Xic1 proteolysis in the HSS with single-stranded DNA is not dependent upon CDK2-cyclin binding or phosphorylation by CDK2-cyclin but is dependent upon the small domain within amino acids 180–183 (Fig. 1C and Fig. 4C, right panel). To further study whether Xic1 proteolysis in the LSS and the HSS is mechanistically the same, we identified the minimal domain that was necessary and sufficient for Xic1 proteolysis in both extracts. Our studies demonstrate that in both the LSS and the HSS, the carboxyl-terminal 50 amino acids of Xic1 are necessary and sufficient for efficient Xic1 proteolysis (Fig. 1D; Ref. 86). Moreover, we find that 30 amino acids (161–190) of Xic1 are sufficient for Xic1 ubiquitination in the LSS and the HSS (Fig. 1D; Ref. 86), similar to a finding by You et al. (71) using the NPE. These results suggest that Xic1 degradation occurs during the late initiation events of primer synthesis, DNA polymerase switching, or elongation and that the mechanism of Xic1 proteolysis in the LSS, the HSS, and the NPE is the same.

Xic1 Degradation Requires the Synthesis of RNA and DNA Primers—The HSS supports Xic1 proteolysis in the presence of single-stranded DNA, but not double-stranded DNA, implying that beyond the requirement for DNA, Xic1 degradation may require DNA unwinding, which results in the generation of single-stranded DNA. This result also implies that Xic1 proteolysis occurs during either late initiation or elongation, when origin unwinding would be completed. To determine the timing of Xic1 proteolysis more precisely, we added inhibitors of different steps of late replication initiation and then tested whether Xic1 proteolysis was supported in the treated extracts. CDK2-cyclin E activity is required prior to origin unwinding during late replication initiation (16, 17, 87). Our studies show that the addition of the CDK inhibitor, roscovitine, blocks Xic1 degradation in the LSS, a result previously reported by Furstenthal et al. (73), but does not inhibit Xic1 degradation in the HSS (Fig. 2A). This result suggests that Xic1 degradation is dependent upon CDK2-cyclin-mediated DNA unwinding but does not require CDK2-cyclin kinase activity once the DNA has been un wound. This indicates that Xic1 degradation occurs after the CDK2-cyclin E requirement and DNA unwinding.

Following origin unwinding, a RNA primer is synthesized by the primase subunit of the DNA polymerase α-primase complex (23). To inhibit RNA primer synthesis we added the RNA polymerase inhibitor, actinomycin D, and then measured Xic1 proteolysis. Our results indicate that at concentrations of actinomycin D that inhibit ~95% of DNA replication in the LSS and the HSS, we observed a marked reduction in Xic1 degradation compared with controls (Fig. 2A). The inhibition of Xic1 degradation by actinomycin D was consistently less in the HSS, perhaps due to increased priming of single-stranded DNA. This result
indicates that Xic1 proteolysis requires RNA priming by Primase and suggests that Xic1 is degraded after the synthesis of RNA primers.

After RNA priming, DNA polymerase α synthesizes a DNA primer that is recognized by RFC to load PCNA onto primed single-stranded DNA (23). Aphidicolin inhibits DNA primer synthesis by DNA polymerase α and prevents the recruitment of RFC and PCNA to DNA. When aphidicolin was added to the LSS or the HSS at concentrations that inhibited ~96% of DNA replication, Xic1 proteolysis was significantly inhibited (Fig. 2B). Furthermore, the addition of a blocking antibody directed against DNA polymerase α (S1K132) (88, 89) also significantly inhibited the degradation of Xic1 in the LSS and the HSS at concentrations that inhibited at least 80% of DNA replication (Fig. 2, C and D). These studies suggest that Xic1 proteolysis requires the activity of DNA polymerase α and indicates that Xic1 proteolysis occurs after the synthesis of DNA primers. These studies also suggest that Xic1 degradation requires the presence of primed single-stranded DNA.

**Xic1 Degradation Requires the Function of RFC**—Following origin unwinding and primer synthesis, RFC recognizes primed single-stranded DNA and loads PCNA at a site of replication initiation (23). To further study the timing of Xic1 proteolysis, we blocked RFC function using three different antibodies against the large subunit of human RFC and then tested whether Xic1 was degraded in the treated extracts (Fig. 3A). At concentrations that blocked ~98% of DNA replication, two monoclonal antibodies directed against the large subunit of RFC significantly inhibited Xic1 proteolysis in the LSS compared with the control antibody (Fig. 3B; Ref. 82). Furthermore, a rabbit antibody directed against the large subunit of RFC also strongly inhibited Xic1 proteolysis in the LSS and moderately inhibited DNA replication and Xic1 proteolysis in the HSS (Fig. 3C). The limited inhibition of DNA replication observed in the HSS with the RFC antibody is most likely due to the RFC-independent DNA replication of linear single-stranded DNA. Linear single-stranded DNA can be replicated by a PCNA-dependent, but RFC-independent, pathway (90). This result indicates that in an RFC-dependent replication system, Xic1 proteolysis requires the function of RFC. These studies also suggest that Xic1 is targeted for degradation during late initiation following RFC function.

**Xic1 Proteolysis Is Dependent upon PCNA Binding**—Our studies suggest that Xic1 may be degraded during DNA polymerase switching and requires the function of RFC and perhaps PCNA. Our previous studies indicated that residues 161–210 of Xic1 were necessary and sufficient for the ubiquitination and degradation of Xic1 and that amino acids 180–183 were critical determinants of Xic1 proteolysis (74, 86). Analysis of this carboxyl-terminal region of Xic1 reveals conserved residues shown to be important for p21CIP1 binding to PCNA (Fig. 4A) (69, 91). Taken together, we wondered whether PCNA might play a critical role in modulating Xic1 ubiquitination and degradation. We first generated a point mutation in Xic1 at amino acid 174 which we predicted would disrupt Xic1 binding to PCNA based on the crystal structure solved for p21CIP1 in association with PCNA (91, 92). We then measured the ability of this and several other Xic1 mutants defective for PCNA binding to be degraded. Our results show a striking correlation between the ability of a Xic1 mutant to bind endogenous PCNA in egg extract and its ability to be degraded in the LSS or the HSS (Fig. 4, B and C; Ref. 74). This result also strongly suggests that the Xic1 mutant, NLS2_180KRKK183 to 180ARA183, was not degraded in previous studies because it was defective for PCNA binding (74). All of the Xic1 mutants tested bound CDK2-cyclin E as efficiently as wild-type Xic1 (data not shown; Ref. 74). Significantly, although the Xic1 I174A mutant efficiently localized to the nucleus and bound to chromatin, it was not ubiquitinated (Fig. 4D).

Furstenthal et al. (73) proposed that Xic1 is recruited to chromatin through its interaction with CDK2-cyclin E and that this interaction is critical for its ubiquitination and degradation. However, our studies demonstrate that Xic1 binding to CDK2-cyclin E and to chromatin are
not sufficient for Xic1 ubiquitination. Instead, our studies indicate that Xic1 must bind PCNA to be ubiquitinated and degraded.

**Trimeric PCNA Recruits Xic1 to Chromatin where It Is Targeted for Ubiquitination**—Our studies suggest that PCNA binding to Xic1 is required for Xic1 proteolysis, but they do not exclude the possibility that an alternate protein, which binds to the same COOH-terminal PCNA-binding region of Xic1, may be the critical mediator of Xic1 degradation.

To address this possibility, we depleted the endogenous PCNA from the LSS and the HSS using a GST-Xic1-(161–190) polypeptide containing the PCNA-binding domain (Fig. 5A) (93). Depletion of PCNA prevented Xic1 degradation in the LSS and the HSS, while Xic1 was efficiently degraded in control-depleted extracts (Fig. 5, B and C). Immunoblot analysis confirmed that PCNA was efficiently depleted from extracts, while the concentration of CDK2-cyclin E was not affected (Fig. 5B; data not shown). Importantly, the addition of recombinant PCNA alone was able to fully rescue Xic1 proteolysis in depleted extracts (Fig. 5C). This result demonstrates that although other proteins may bind to Xic1-(161–190) and may be depleted along with PCNA, only the addition of PCNA is required to restore Xic1 proteolysis in depleted extracts. Moreover, because the majority of Xic1 proteolysis was blocked by the removal of PCNA, this implies that PCNA plays a critical role in mediating Xic1 proteolysis in the egg, perhaps through the recruitment of Xic1 to chromatin at a site of initiation.

To test this possibility directly, we generated a mutant of Xenopus PCNA that could not be loaded onto DNA (90) and analyzed the ability of this mutant to mediate Xic1 proteolysis. We based our mutagenesis strategy on studies performed on the highly conserved human PCNA protein, since Xenopus and human PCNA share ~90% amino acid identity. Crystal structure studies demonstrate that human PCNA forms a homotrimeric ring that functions as a sliding clamp encircling a duplex of DNA (91). Mutation of the conserved tyrosine 114 to alanine impairs trimer formation of human PCNA and prevents its RFC-mediated loading onto DNA (90). We generated the Y114A mutation in Xenopus PCNA and tested its ability to rescue Xic1 proteolysis in PCNA-depleted extracts. While the wild-type PCNA fully restored Xic1 proteolysis, the Y114A PCNA mutant did not (Fig. 5C). Binding studies demonstrated that both the wild-type and Y114A PCNA proteins could bind Xic1 efficiently, although the latter was somewhat reduced (Fig. 5D).

These studies demonstrate that the binding of Xic1 to PCNA is necessary, but not sufficient, for Xic1 proteolysis. These studies also suggest that beyond the binding of Xic1 to PCNA, Xic1 degradation may be dependent upon the ability of PCNA to be loaded onto DNA by RFC. To study this hypothesis, we examined the chromatin binding of PCNA, Xic1, and RPA in egg extracts during DNA replication initiation. Our studies show that during replication initiation, endogenous RPA associates with chromatin 30–60 min following the addition of sperm chromatin fractionation.
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In the egg, very little Xic1 protein is detected (2 nM), but these levels increase dramatically after the mid-blastula transition when the asynchronous somatic cell cycle begins to be established (Fig. 7B) (data not shown; Ref. 70). We generated extracts from stage 11–12 gastrulating embryos and tested these extracts with the Xic1 COOH-terminal peptide to disrupt the interaction between Xic1 and PCNA. In the peptide-treated LSS and HSS extracts, Xic1 proteolysis was significantly inhibited (Fig. 7A). This result is consistent with a report which demonstrated that the addition of a Xic1-(162–192) fusion protein with a green fluorescent protein inhibits Xic1 degradation in the NPE (71). Additionally, our studies and the studies of You et al. (71) demonstrate that the addition of Xic1 COOH-terminal peptides at ~3 μM does not significantly inhibit DNA replication (data not shown), even though it inhibits Xic1 proteolysis. The inhibition of Xic1 degradation by Xic1 COOH-terminal peptides was fully reversed in the LSS and partially reversed in the HSS upon the addition of recombinant wild-type Xenopus PCNA (data not shown).

In the egg, very little Xic1 protein is detected (~2 nM), but these levels increase dramatically after the mid-blastula transition when the asynchronous somatic cell cycle begins to be established (Fig. 7B) (data not shown; Ref. 70). We generated extracts from stage 11–12 gastrulating embryos and treated these extracts with the Xic1 COOH-terminal peptides. Our results indicate that the proteolysis of Xic1 in stage 11–12 extracts is markedly inhibited by the peptides that disrupt the binding of Xic1 to PCNA (Fig. 7C). In addition, the proteolysis of a Xic1 point mutant defective for PCNA binding (I174A) was reduced in the stage 11–12 extract (Fig. 7D). These studies suggest that the predominant Xic1 degradation pathway present during the Xenopus embryonic and somatic cell cycles is the one mediated by PCNA.

FIGURE 5. Trimeric PCNA is essential for the proteolysis of Xic1. A, Xic1 and PCNA binding assay. Bacterial lysate containing GST and GST-Xic1 WT and mutants proteins were bound to glutathione-Sepharose and then incubated in LSS. Washed beads were eluted and analyzed by SDS-PAGE and immunoblotting using antibody to PCNA. Twenty percent of the input LSS is shown (20% INPUT), and PCNA binding is indicated as a percentage (% BINDING) of the input PCNA for each reaction. The arrow denotes the PCNA protein band. B, immunoblot of depleted extracts using PCNA antibody. Samples were either control-depleted with GST bound to glutathione-Sepharose (CTRL DEPL) or PCNA-depleted with GST-Xic1-(161–190) bound to glutathione-Sepharose (XPCNA DEPL) from LSS (top panel) or HSS (bottom panel) as indicated. Depleted samples were supplemented with XB- (BUFFER), His6-T7-tagged Xenopus WT PCNA (His6-T7-XPCNA WT), or His6-T7-tagged Xenopus Y114A PCNA (His6-T7-XPCNA Y114A) and analyzed by immunoblotting using antibody to PCNA. Arrows denote either endogenous PCNA (XPCNA) or recombinant PCNA (His6-T7-XPCNA). C, Xic1 degradation assay. [35S]Methionine-labeled Xic1 was incubated in control- (CTRL DEPL) or PCNA-depleted (XPCNA DEPL) extracts with (+) or without (−) demembranated sperm chromatin (XSC); 10 ng/μl (LSS, top panel) or M13 single-stranded DNA (ssDNA); 10 ng/μl (ﾙ) (SS, bottom panel) for 0, 1, and 3 h followed by SDS-PAGE. Extracts were supplemented prior to degradation assay with XB- (BUFFER) or recombinant WT (WT PCNA) or Y114A (Y114A PCNA) His6-T7-tagged Xenopus PCNA. The percentage of Xic1 remaining is shown where the 0-h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample (% Xic1 Remaining). DNA replication at the 3-h time point was normalized to 100% of the replication observed with the appropriate control buffer (% DNA Replication). D, Xic1 and PCNA binding assay. Bacterial lysate containing GST or GST-Xic1 WT was bound to glutathione-Sepharose and then incubated in LSS that was either control-depleted with GST bound to glutathione-Sepharose (CTRL DEPL) or PCNA-depleted with GST-Xic1-(161–190) bound to glutathione-Sepharose (XPCNA DEPL) or recombinant WT or Y114A His6-T7-XPCNA. The PCNA-depleted LSS was supplemented before binding to beads with XB- (BUFFER) or recombinant WT or Y114A His6-T7-tagged Xenopus PCNA (WT or Y114A His6-T7-XPCNA). Washed beads were eluted and analyzed by SDS-PAGE and immunoblotting using antibody to PCNA. Ten percent of the input LSS is shown (10% INPUT) and PCNA binding is indicated as a percentage (% BINDING) of the input PCNA. Arrows denote either endogenous PCNA (XPCNA) or recombinant PCNA (His6-T7-XPCNA). The PCNA-depleted LSS used in Fig. 5D is the same depleted LSS used in Fig. 5, B and C, and Fig. 6A.
Xic1 Proteolysis Requires PCNA Loading onto DNA

**FIGURE 6.** Chromatin association of trimeric PCNA parallels the appearance of ubiquitinated Xic1. A, PCNA and RPA chromatin binding in PCNA-depleted LSS during replication initiation. PCNA-depleted LSS was supplemented with Xenopus sperm chromatin and recombinant Hi6-T7-tagged WT Xenopus PCNA (WT, left) or Y114A mutant (Y114A, right). Samples (8 μl) were collected over time as indicated in minutes and subjected to a chromatin spin down assay to isolate the chromatin fractions (CHROM FRAC). The samples were then analyzed by immunoblotting with antibodies against the p70 subunit of RPA (top panel) and PCNA (bottom panel). An aliquot of the total unfractioned sample (0.5 μl) was also analyzed (TOTAL). Arrows denote the migration of either endogenous PCNA (XPCNA), recombinant PCNA (Hi6-XPCNA), or the p70 subunit of RPA, B, CHROM FRACTION: RPA, PCNA, and Xic1 chromatin binding during replication initiation in the LSS. LSS was supplemented with Xenopus sperm chromatin and [35S]methionine-labeled GST-Xic1. The total extract (TOTAL, 0.5 μl), along with chromatin-associated fractions collected at different time points (20 μl), were analyzed by immunoblotting with RPA (top panel) and PCNA (middle panel) antibodies. Arrows denote the p70 subunit of RPA (XPPA-p70) and PCNA (XPXNA). Chromatin-bound Xic1 (5 μl, bottom panel) was visualized by phosphorimager analysis. Arrows denote [35S]methionine-labeled GST-Xic1 (161–210) and GST-Xic1 mono-ubiquitinated at one site (mono-Ub), two sites (di-Ub), or at multiple sites (poly-Ub). Molecular mass markers (M) in kilodaltons are indicated. Right, TOTAL FRACTION: total extract samples (0.5 μl) were also analyzed for total [35S]GST-Xic1 degradation over time. The percentage of Xic1 remaining is shown where the 0-h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample (% Xic1 Remaining). C, Xic1 and PCNA Xic1 chromatin binding in PCNA-depleted LSS. PCNA-depleted LSS was supplemented with Xenopus sperm chromatin, [35S]methionine-labeled GST-Xic1, methyl ubiquitin, and XB (BUFFER) or recombinant Hi6-T7-tagged WT Xenopus PCNA (WT) or Y114A (XPXNA-Y114A) mutant Xenopus PCNA proteins. The total extract (TOTAL, top panel, 0.5 μl) and the chromatin fraction (middle panel, 5 μl) were analyzed by immunoblotting with PCNA antibody. Arrows denote the migration of either endogenous PCNA (XPXNA) or recombinant PCNA (Hi6-T7-XPXNA). The Xic1 chromatin-bound fraction (bottom panel, 5 μl) was also analyzed by phosphorimager analysis. Arrows denote [35S]methionine-labeled GST-Xic1 (161–210) GST-Xic1 and GST-Xic1 mono-ubiquitinated at one site (1-MeUb), two sites (2-MeUb), three sites (3-MeUb), or at multiple sites (*). Molecular weight markers (M) in kilodaltons are indicated.

**FIGURE 7.** Xic1 binding to PCNA is critical for Xic1 proteolysis in the egg and the gastrulating embryo. A, Xic1 degradation assay in the presence of Xic1 peptides containing the PCNA-binding domain. Recombinant GST and GST-Xic1 fusion proteins containing amino acids 161–210 or 161–190 of Xic1 (GST-Xic1-(161–210) and GST-Xic1-(161–190)) were added to the LSS (left panel) or the HSS (right panel) at the indicated concentrations. Extracts were also supplemented with [35S]methionine-labeled Xic1 in the presence (+) or absence (−) of demembranated sperm chromatin (XSC) (10 ng/μl) (LSS) or M13 single-stranded DNA (ssDNA) (10 ng/μl) (HSS) and incubated for 0, 1, and 3 h followed by SDS-PAGE. The arrow indicates the [35S]Xic1 protein band and the percentage of Xic1 remaining is shown where the 0-h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample (% Xic1 Remaining). D, Xic1 degradation assay in stage 11–12 gastrulating extracts. [35S]methionine-labeled Xic1 WT and I174A mutant were analyzed in stage 11–12 gastrulation extracts for 0, 1.5, and 3 h followed by SDS-PAGE. The arrow indicates the [35S]Xic1 protein band, and the percentage of Xic1 remaining is shown where the 0-h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample (% Xic1 Remaining).

**DISCUSSION**

Our studies suggest a model for the proteolysis of Xic1 in the egg extract and in the somatic cell (Fig. 8). We have found that although Xic1 may be recruited to chromatin through an interaction with CDK2-cyclin E and Cdc6 (73, 94), the Cdc6-mediated association of Xic1 to chromatin is not sufficient to trigger Xic1 ubiquitination and the interaction of Xic1 with CDK2-cyclin is dispensable for Xic1 degradation (71, 74). Our findings suggest that Xic1 proteolysis occurs after the formation of the pre-RC, after DNA unwinding, and after the replication requirements for RPA, DNA polymerase α-prime, RFC, and PCNA. Xic1 proteolysis is absolutely dependent upon the presence of DNA and the binding of Xic1 to trimeric PCNA, suggesting that only PCNA that is competent for loading to a site of initiation by RFC can mediate the ubiquitination of Xic1. This also implies that Xic1 is only ubiquitinated when bound to chromatin and suggests that chromatin...
serves to nucleate Xic1 and the Xic1 ubiquitination machinery. Based on studies of mammalian p21Cip1 and PCNA, it is predicted that the binding of Xic1 to PCNA may block the recruitment of DNA polymerase δ to a site of initiation (91). It is still unclear whether the proteolysis of Xic1 is required for the recruitment of DNA polymerase δ or whether processive DNA polymerases may play a role in triggering the ubiquitination of Xic1.

The results on the timing of Xic1 proteolysis are somewhat controversial. Furstenthal et al. (73) report that Xic1 is degraded following pre-RC formation but before DNA polymerase α activity. Their studies are based on the detection of ubiquitinated Xic1 associated with chromatin in the presence of 40 μg/ml aphidicolin (73). We also find that in extracts containing chromosomes such as the LSS, some ubiquitinated Xic1 can be observed in the chromatin fraction at aphidicolin concentrations below 50–100 μg/μl, while in different extracts, aphidicolin concentrations that block Xic1 degradation may vary by 2–3-fold (data not shown). In contrast, we do not observe this variation in the HSS. You et al. (71) show that Xic1 proteolysis occurs during late initiation following Cdc45 recruitment but before RPA or DNA polymerase α function. These studies are based on the observation that Xic1 is proteolyzed in RPA-depleted extracts and in the presence of 1 mg/ml SJK132 IgG directed against DNA polymerase α, although the kinetics of Xic1 degradation are noticeably slowed upon both treatments (71). Similar to You et al. (71), we also observe that the kinetics of Xic1 degradation are slowed in the presence of 1 mg/ml SJK132 IgG (data not shown). However, our studies show that at higher concentrations of SJK132 between 2 and 3 mg/ml, Xic1 degradation is inhibited. Because we study Xic1 degradation in both the LSS and the HSS, we believe that our findings provide a more reliable examination of the timing of Xic1 proteolysis.

Our finding that the ubiquitination and degradation of Xic1 occurs during DNA polymerase switching is unexpected, since it has been predicted that Xic1 targets CDK2-cyclin E prior to DNA unwinding. Furstenthal et al. (73) have reported that Xic1 bound to CDK2-cyclin E can be recruited to chromatin through the association of cyclin E with Cdc6, although high levels of Xic1 block the association of CDK2-cyclin E to chromatin via Cdc6. The RXL motifs of Cdc6 and Xic1 both bind to the MRAIL domain of cyclin E, indicating that the binding of Cdc6 and Xic1 to cyclin E is mutually exclusive (94). Therefore, Xic1 recruited to chromatin by a CDK2-cyclin E-Cdc6 interaction would be associated to chromatin only through its interaction with CDK2, and it is unknown whether this interaction is sufficient to fully inhibit kinase activity. Consequently, the degradation of Xic1 associated with CDK2-cyclin E and Cdc6 on chromatin prior to DNA unwinding may be dispensable. Alternatively, during the somatic cell cycle when Xic1 is more highly expressed, Xic1 proteolysis may be required prior to DNA unwinding in a PCNA-independent manner as well as during DNA polymerase switching by a PCNA-dependent manner.

Based on our findings of the timing and mechanism of Xic1 ubiquitination and degradation, we propose that during the somatic cell cycle, the PCNA-dependent degradation of Xic1 during DNA polymerase switching may activate CDK2-cyclin A and/or PCNA. Consistent with this model, we have observed in the LSS that the addition of recombinant wild-type Xic1 to 60 μM, a concentration that more closely mimics the Xic1 concentration in a somatic cell, delayed the entry into S phase, while the addition of the non-degradable I174A Xic1 mutant blocked S phase entry, suggesting that the PCNA-dependent proteolysis of Xic1 is important for the onset of elongation. The degradation of Xic1 by this mechanism would couple the activation of a CDK2-cyclin with the start of processive DNA replication and would ensure that any process that halts S phase before DNA polymerase switching would result in the stabilization of Xic1. In Xenopus, cyclin A2 most closely resembles human cyclin A in sequence and expression, although the function of cyclin A2 is not well characterized (95).

Although we believe our studies in the embryonic and gastrulation stage extracts suggest that Xic1 binding to PCNA is important for Xic1 turnover, it is still unclear exactly how Xic1 proteolysis is regulated during the somatic cell cycle. Our studies and the studies of other groups have primarily used egg extracts and exogenously added Xic1 to characterize the regulation of Xic1 turnover (71–74, 83). Notably, we have observed that endogenous Xic1 is efficiently degraded in the LSS in the presence of nuclei, demonstrating that the proteolysis of Xic1 is not just an artifact of exogenously added Xic1 (86). The requirement for added nuclei at a ratio of ~3200–5000 sperm to 1 μl of cytoplasm may indicate that the degradation of endogenous Xic1 is not normally observed in the embryo until around the start of the mid-blastula transition (96). Studies have demonstrated that Xic1 plays an important role in the differentiation of neural and muscle cells, and it is possible that during differentiation, Xic1 protein levels may be modulated by protein stability (97–100). Future studies that examine endogenous Xic1 turnover in the somatic cell and during cellular differentiation will be required to fully understand the function and regulation of Xic1 and its targets.

It is possible that some of our experimental conditions in the LSS activated a DNA replication or DNA damage checkpoint that inhibited

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4 L.-C. Chuang and P. R. Yew, unpublished observation.
the proteolysis of Xic1 through an ATR (ATM and Rad3-related kinase) or ATM (ataxia telangiectasia mutated) and Chk1/Chk2 pathway (101–106). One possibility is that ATR phosphorylation of Xic1 mediates its stability during a checkpoint. Xic1 contains one consensustr ATR site at threonine 163, but ourfindings demonstratethat mutation of this residue has no effect on Xic1 proteolysis in the absence or presence of a checkpoint. Alternatively, Chk1/Chk2 phosphorylation by ATR/ATM could result in the inhibition of CDK2-cyclin activation (105–109), therebypotentially inhibiting Xic1 proteolysis in the LSS. However, all of our studies were also performed in the HSS where CDK2 activity is dispensable for DNA replication and Xic1 degradation (87). Thus, our studies using the HSS are not sensitive to the effects of ATR/ATM checkpoint pathways that target CDK2-cyclin activity. Furthermore, the studies of the Xic1 T174A mutant defective for PCNA binding and the inhibition of Xic1 proteolysis through the addition of Xic1 peptides that disrupt the Xic1-PCNA interaction were not performed under checkpoint conditions, since DNA replication was not inhibited. Therefore, we believe our findings support the model that Xic1 proteolysis occurs during DNA polymerase switching through the interaction of Xic1 with PCNA on chromatin.

Because the E3 SCF components, Cull1 and p19Skp1, have been shown to be associated with chromatin (73), it is predicted that Xic1 is targeted for ubiquitination by a chromatin-bound SCF complex when associated at a site of initiation. However, to date, the machinery responsible for Xic1 ubiquitination, including its putative F-box protein, have yet to be identified. Additionally, although immunodepletion of Cdc34 blocks Xic1 degradation in the LSS with sperm chromatin (72), surprisingly, immunodepletion of Cdc34 from the HSS does not inhibit Xic1 degradation in the presence of single-stranded DNA.6 This indicates that a Cdc34 requirement for DNA replication initiation exists and occurs before the timing of Xic1 degradation, implying that Cdc34 either is not the E2 for Xic1 or is not the only E2 for Xic1. It is also unclear whether the recruitment of Xic1 to a site of initiation by PCNA is sufficient to trigger Xic1 ubiquitination or whether additional signals may be required. If Xic1 is truly targeted for ubiquitination by a SCF complex, then it is predicted that Xic1 phosphorylation may play a role in the binding of Xic1 to its cognate F-box protein. However, our previous studies suggest that phosphorylation of Xic1 is not critical for its ubiquitination or degradation in the egg extract (86). During a somatic cell cycle when Xic1 is more highly expressed, the phosphorylation of Xic1 may play an important role in regulating the stability of Xic1. Studies have demonstrated that CDK2-cyclin A associates with SCF proteins, p45Skp2 and p19Skp1, in a manner that is mutually exclusive of its binding to p21Cip1 (110, 111). Interestingly, CDK2 has been shown to bind directly to PCNA suggesting the possibility that PCNA may directly recruit to chromatin both a CDK inhibitor and a CDK2-cyclin bound to Xic1 Proteolysis Requires PCNA Loading onto DNA

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5 L.-C. Chuang, A. Conley, and P. R. Yew, unpublished observation.
6 P. R. Yew, unpublished observation.
7 L.-C. Chuang, Z. Guo, and P. R. Yew, unpublished observation.
Proliferating Cell Nuclear Antigen Recruits Cyclin-dependent Kinase Inhibitor Xic1 to DNA and Couples Its Proteolysis to DNA Polymerase Switching

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