The C-terminal Domain of the Xenopus Cyclin-dependent Kinase Inhibitor, p27\textit{Xic}1, Is Both Necessary and Sufficient for Phosphorylation-independent Proteolysis*

Received for publication, June 13, 2005, and in revised form, August 3, 2005 Published, JBC Papers in Press, August 23, 2005, DOI 10.1074/jbc.M506430200

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Cell cycle progression is regulated by cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors. In the frog, \textit{Xenopus laevis}, the CDK inhibitor p27\textit{Xic}1 (Xic1) inhibits DNA synthesis by negatively regulating CDK2-cyclin E. Using the frog egg extract as a model system for the study of Xic1, studies have demonstrated that Xic1 protein levels are regulated by nuclear ubiquitination and proteolysis. To characterize the molecular mechanism that regulates Xic1 turnover, we have identified the minimal sequences of Xic1 that are necessary and sufficient for its nuclear ubiquitination and degradation. Using deletion mutagenesis, our studies indicated that the C-terminal 50 amino acids of Xic1 are critical for its proteolysis beyond a role in nuclear transport. Replacement of the Xic1 C terminus with the SV40 nuclear localization sequence resulted in the nuclear localization of Xic1 but not its ubiquitination or degradation. Our deletion studies also indicated that the CDK2-cyclin binding domain of Xic1 is important for its efficient retention in the nucleus. Further deletion analyses identified at least 3 lysine residues within the Xic1 C terminus that are targeted for specific ubiquitination. Importantly, our studies demonstrated that the Xic1 C-terminal 50 amino acids can serve as a nuclear degradation signal when fused to a stable heterologous nuclear protein. Moreover, a 30-amino-acid region within the C terminus of Xic1 can serve as a nuclear ubiquitination signal. To address the role of phosphorylation on Xic1 turnover, all the potential phosphorylation sites within the C-terminal 50 amino acids of Xic1 were mutated to alanine to prevent possible phosphorylation. This resulted in a Xic1 protein that was nevertheless degraded in a manner similar to wild-type Xic1, suggesting that phosphorylation of Xic1 is not critical for its nuclear ubiquitination or proteolysis.

The progression of the vertebrate cell cycle is positively regulated by cyclin-dependent kinases (CDKs)\(^6\) (1) associated with their cyclin partners and is negatively regulated by CDK inhibitors (reviewed in Refs. 1–4). In mammals, CDK inhibitors of the Cip/Kip family, p21\textit{Cip}1 and p27\textit{Kip}1, are targeted for proteolysis via the ubiquitin-proteasome pathway during the G\(_1\) to S phase transition (5). In the frog, \textit{Xenopus} inhibitor of CDK (p27\textit{Xic}1 or Xic1) shares homology with both mammalian p21\textit{Cip}1 and p27\textit{Kip}1, preferentially inhibits the activity of CDK2-cyclins, and binds all CDK-cyclins and proliferating cell nuclear antigen (PCNA) (6, 7). Xic1 inhibits nuclear DNA synthesis in egg extracts through its binding to CDK2-cyclins and inhibits the replication of single-stranded DNA through its binding to PCNA (6, 7). Xic1 is thought to play a role in establishing G\(_1\) in the somatic cell cycle and has been shown to be important for the differentiation of muscle and neural cells (8–10).

During the initiation of DNA replication, Xic1 is targeted for nuclear- and ubiquitin-dependent proteolysis in \textit{Xenopus} interphase egg extracts (11–14). The ubiquitination and degradation of Xic1 is dependent upon its nuclear localization, which is mediated equally by three nuclear localization sequences (NLSs) located within the Xic1 C terminus and through the binding of CDK2-cyclins (12). Once localized within the nucleus, binding to CDK-cyclins is dispensable for Xic1 proteolysis (12, 14). Additionally, we have shown that amino acids 180–183 of Xic1 serve as a critical determinant of Xic1 degradation, which we postulate functions to bind a critical regulator of Xic1 ubiquitination (12). Further studies have shown that the phosphorylation of Xic1 within six possible CDK consensus (S/T)-P sites is not required for Xic1 turnover (13).

Studies have demonstrated that the targeted proteolysis of mammalian p27\textit{Kip}1 is mediated by the E3 called SCF\textit{skp2} (15–18). The SCF is comprised of an F-box-binding protein called Skp1, a cullin family member called Cul1, a variable F-box protein, and a ring finger protein called Rbx1/Roc1. The ubiquitination of p27\textit{Kip}1 during S phase requires association with CDK2-cyclin E and subsequent phosphorylation on residue Thr-187 for Skp2-mediated targeting to the SCF (19–21). Similarly, the \textit{Saccharomyces cerevisiae} CDK inhibitor, p40\textit{sic}1, is multiply phosphorylated by Cln kinases in G\(_1\) before ubiquitination by the E3, SCF\textit{calc} (22–25). Thus, a common requirement for ubiquitination by the SCF is substrate phosphorylation. Although the E3 of Xic1 has not yet been identified, it is predicted to be the \textit{Xenopus} homolog of mammalian SCF, suggesting that Xic1 may require prior phosphorylation before efficient ubiquitination during DNA replication initiation.

To characterize the molecular mechanism, which regulates Xic1 turnover in \textit{Xenopus} interphase egg extracts, we have identified the minimal domain of Xic1 that can efficiently support the nuclear ubiquitination and degradation of Xic1. Additionally, we further addressed whether phosphorylation of Xic1 within this C-terminal domain is a
requirement for nuclei-dependent Xic1 degradation. Our results indicated that the C-terminal 50 amino acids of Xic1 are both necessary and sufficient to target Xic1 for efficient nuclei-dependent proteolysis. This domain of Xic1 (amino acids 161–210) can also serve as a transposable element to target a stable heterologous nuclear protein for degradation, while an abbreviated domain of Xic1-(161–190) can mediate the ubiquitination of a heterologous nuclear protein. Mutation of all possible Xic1 phosphorylation sites within the C-terminal 50 amino acids of Xic1 suggested that the nuclei-dependent turnover of Xic1 in the egg extract does not require phosphorylation within this minimal domain. These studies suggested that Xic1 associates with the ubiquitination machinery and regulators of Xic1 ubiquitination through its C-terminal domain during DNA replication initiation.

**EXPERIMENTAL PROCEDURES**

Preparation of Xenopus Interphase Egg Extract, Nucleoplasmic Extract (NPE), Xenopus Demembranated Sperm Chromatin, and Ubiquitin (Sigma) was methylated as described previously and used at a concentration of 2 μg/ml (27).

Nuclei Spin-down Assay, Degradation Assay, in Vitro Transcription and Translation, and GST Pull-down Assay—These procedures were performed as described previously (12). NPE was prepared as described (26). Ubiquitin (Sigma) was methylated as described previously and used at a final concentration of 2 mg/ml.

Nuclei Spin-down Assay—Immunoprecipitation from the LSS was performed as described previously (12). NPE was prepared as described (26). Ubiquitin (Sigma) was methylated as described previously and used at a final concentration of 2 mg/ml.

Co-immunoprecipitation Assay—Immunoprecipitation from the NPE was performed as follows. 5 μl of NPE was mixed with 1 μl of [35S]methionine-labeled Xic1-WT for 1 h at 23°C. The mixture was incubated with cyclin E antibody, CDK2 antibody, or normal rabbit serum for 30 min at 23°C followed by the addition of 7.5 μl of protein A-Sepharose and further incubation for 1 h at 23°C. The protein A-Sepharose beads were recovered by centrifugation and washed four times with EB (80 mM β-glycerophosphate, pH 7.4, 20 mM EGTA, and 15 mM MgCl2) containing 0.1% Nonidet P-40, 10 mM HEPES (pH 7.4), 150 mM NaCl) at 23°C. The samples were then subjected to SDS-PAGE followed by quantitation by PhosphorImager analysis using ImageQuant software (Amersham Biosciences).

Antibodies, Antibody Production, and Immunoblotting—Full-length Xic1 fused to maltose-binding protein was used as an antigen in rabbits for the production of Xic1 antisem (Covance). Immunoblotting was performed using affinity-purified Xic1 antibodies as described previously (28). CDK2 and cyclin E antibodies were generously provided by P. K. Jackson and M. W. Kirschner.

DNA Constructs—Glutathione S-transferase (GST) was generated by PCR using pGEX-2T as the template and the following PCR primers: forward, 5′-CCATCGATATGGCCCTATATCTAGGTATTTG-3′, and reverse, 5′-GGGCCCTTAAGTGACACTGCTTAAAGGC-3′.

The CTAGACTCTGACTAGTCAATCGAATCTTTTTCTGGGCT-3′. GST-Xic1-WT was generated by PCR using pGEX-2T/Xic1-WT as the template and then cloned into the Clal and EcoRI restriction sites of the pcS2+ vector. The PCRs primers used for GST-Xic1-WT were as follows: GST forward primer as above, and reverse, 5′-CTAGACTCTGACTAGTCAATCGAATCTTTTTCTGGGCT-3′.

Truncation mutants of Xic1 were generated by PCR using pcS2+ / Xic1-WT as the template. PCR products were subsequently cloned into the pcS2+ vector. PCR primers for Xic1 truncation mutants were as follows: forward, (dN25) 5′-CCGAGAATTCACACGAGCAGAGGAGGAGGACCTG-3′; (dN100) 5′-CCGAGAATTCATGACACGCCTGCTCCCTCCCTTGGGTGAC-3′; wild type (WT) 5′-CCGAGAATTCATGACACGCCTGCTCCCTCCCTTGGGTGAC-3′ (dN20) 5′-CTAGACTCTGACTAGTCAATCGAATCTTTTTCTGGGCT-3′; reverse, (WT) 5′-CTAGACTCTGACTAGTCAATCGAATCTTTTTCTGGGCT-3′.

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The T175A, T175D, and 9A point mutants of Xic1 were generated by PCR using pGEX-2T/Xic1-WT as the template and then cloned into the Clal and EcoRI restriction sites of the pcS2+ vector. The primers used for GST-Xic1-WT were as follows: GST forward primer as above, and reverse, 5′-CTAGACTCTGACTAGTCAATCGAATCTTTTTCTGGGCT-3′.

15 mM MgCl2) containing 0.1% Nonidet P-40, 10 mM HEPES (pH 7.4), 150 mM NaCl) at 23°C. The samples were then subjected to SDS-PAGE followed by quantitation by PhosphorImager analysis using ImageQuant software (Amersham Biosciences).

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RESULTS

Endogenous Xic1 in the Interphase Egg Extract Is Degraded in a Nuclei-dependent Manner—Previous studies of Xic1 turnover have used the Xenopus interphase egg extract as a model system to study the ubiquitination and degradation of [35S]methionine-labeled exogenously added Xic1 (11–14). To validate the continued use of this model system for the study of Xic1 proteolysis, we generated an antibody to Xic1 to examine the turnover of endogenous Xic1 in the interphase egg extract in the presence of sperm nuclei. Upon the addition of sperm chromatin to the interphase egg extract, endogenous Xic1 was degraded over time in a nuclei-dependent manner with kinetics similar to the degradation of exogenously added [35S]methionine-labeled Xic1 (Figs. 1 and 2B). These results suggested that in the interphase egg extract supplemented with sperm nuclei, both Xic1 present endogenously and Xic1 added exogenously are degraded by a similar mechanism.

Residues within the C Terminus of Xic1 Are Critical for Its Degradation—To characterize the molecular mechanism that regulates Xic1 degradation, we set out to identify the regions of Xic1 that are critical for its nuclei-dependent proteolysis during DNA replication initiation. We reasoned that this knowledge would help us to identify the mediators or regulators that are required for Xic1 proteolysis. To this end, we first performed deletion mutagenesis of Xic1 (Fig. 2A) and found that although deletion of the N-terminal 25 amino acids of Xic1 did not affect its turnover, further deletion of the N-terminal 100 amino acids of Xic1 resulted in a protein that was inherently unstable in the egg extract in the presence or absence of nuclei (Fig. 2, B, top panel and C). This suggests that the N-terminal 25 amino acids of Xic1 are dispensable for its proteolysis, whereas deletion of additional N-terminal amino acids, including the residues required for binding to CDK2-cyclins (amino acids 31–91) (6), results in a mutant of Xic1 that is degraded in an unregulated manner. Deletion of the C-terminal 20 amino acids of Xic1 resulted in a mutant that was degraded in a nuclei-dependent manner but at an efficiency clearly reduced when compared with the wild-type protein, suggesting that the C-terminal 20 amino acids of Xic1 contain residues that are required for efficient Xic1 degradation (Fig. 2B, bottom panel, and 2C). In contrast, deletion of the C-terminal 50 amino acids of Xic1 resulted in a complete stabilization of Xic1 that was not increased by further C-terminal deletions (Fig. 2, B and C). This result suggested that the C-terminal 50 amino acids of Xic1 contain residues that are critical for its nuclei-dependent degradation.

The C-terminal Domain of Xic1 Plays an Essential Role in Xic1 Proteolysis beyond Its Role in Xic1 Nuclear Localization—Our previous studies have shown that Xic1 degradation requires efficient Xic1 nuclear localization, which is independently mediated by binding to CDK2-cyclin E and by nuclear localization sequences within the C terminus (12). To test whether the C terminus of Xic1 functions only to correctly localize Xic1 to the nucleus, where it is ubiquitinated and degraded, we replaced the C-terminal 50 amino acids of Xic1 containing its three nuclear localization sequences with the canonical simian virus 40 (SV40) NLS (Fig. 3A). For these studies and further studies, we utilized a Xic1 protein fused to GST because it facilitated the detection of Xic1 ubiquitinated species by allowing Xic1 to be labeled with [35S]methionine to a higher specific activity (Xic1 contains 2 methionines, whereas GST-Xic1 contains 11 methionines) (12). Our previous studies using a GST-tagged Xic1 demonstrated that it was degraded like the wild-type Xic1, whereas GST alone was stable (12). Additionally, this approach enabled us to determine which sequences of Xic1 could serve as a portable domain to target the normally stable GST for nuclei-dependent proteolysis in the egg extract. Our results showed that although a GST fusion of Xic1 residues 31–210 (GST-Xic1-(31–210)) was efficiently degraded, replacement of the C-terminal 50 amino acids of GST-Xic1-(31–210) with the SV40 NLS resulted in a mutant (GST-Xic1-(31–160)-SV40-NLS) that was not ubiquitinated or degraded, although it was efficiently localized to the nucleus (Fig. 3, B and C). An additional Xic1 mutant bearing the SV40-NLS in place of the C-terminal 50 amino acids and deleted for the N-terminal 91 residues, including the CDK2-cyclin binding domain (GST-Xic1-(92–160)-SV40-NLS), was similarly defective for proteolysis. However, this mutant was not efficiently localized to the nucleus despite its expression of the SV40 NLS, which is sufficient for nuclear transportation (Fig. 3C, lanes 1 and 3). Because Xic1 residues from 33 to 67 have been previously shown to be critical for CDK2-cyclin binding (6), this indicated that the binding of Xic1 to CDK2-cyclins is important for the efficient nuclear retention of Xic1 (Fig. 3C, lanes 3 and 4). Taken together, these results suggested that the C-terminal 50 amino acids of Xic1 play a critical role in the degradation of Xic1 beyond their participation in Xic1 nuclear localization.

Xic1 Is Specifically Ubiquitinated on at Least Three Lysine Residues Within the C Terminus—To further define the minimal sequences required for Xic1 ubiquitination and degradation, additional deletion mutagenesis was performed (Fig. 4A). Xic1 mutants were expressed as fusions to GST, and the nuclease localized ubiquitinated Xic1 species were visualized following a nuclei spin-down assay to separate the nuclear and cytoplasmic fractions. Xic1 mutants were also analyzed for nuclei-dependent degradation in the Xenopus interphase egg extract. Our studies indicated that although GST alone was stable, both the GST-Xic1 WT protein and the GST-Xic1-(31–210) proteins were efficiently ubiquitinated and degraded in a nuclei-dependent manner (Figs. 4B, lanes 3 and 5, and 5A, top panel). Moreover, analysis of the GST-Xic1WT by nuclei spin-down assay in the presence of methyl ubiquitin to inhibit polyubiquitination suggested that Xic1 is ubiquitinated on ~3–4 lysine residues (Fig. 4B). Further deletion of Xic1 sequences resulted in Xic1 mutants that could target GST for ubiquitination, but progressive deletions of Xic1 resulted in less and less efficient Xic1 nuclear retention, and thus, reduced Xic1 ubiquitination and degradation. Xic1 mutants GST-Xic1-(92–210), GST-Xic1-(132–210), GST-Xic1-(31–91)(132–210), and GST-Xic1-(92–131)(161–210) were ubiquitinated on ~3–4 lysine residues but at a reduced level due to their less efficient nuclear localization or retention (Fig. 4B). The mutants specifically deleted for residues 31–91 encoding the CDK2-cyclin binding domain of Xic1 were significantly reduced for nuclear localization of Xic1 and were therefore reduced for proteolysis (Fig. 5A). Our results had previously indicated that binding to CDK2-cyclin E was dispensable...
for Xic1 proteolysis but was required for efficient nuclear localization and retention (12). In support of this, our studies showed that mutants deleted for the CDK2-cyclin binding domain were still ubiquitinated. The most extensive N-terminal deletion (1–160) of Xic1 (GST-Xic1-(161–210)) resulted in a mutant that was greatly reduced for nuclear localization and degradation but was still weakly ubiquitinated on 3–4 lysine residues (Fig. 4B, lane 11). These studies suggested that when fused to the cytoplasmic protein, GST, the minimal domain of Xic1 that can support Xic1 ubiquitination and a modest degree of Xic1 degradation is the C-terminal 50 amino acids of Xic1. To more directly test this finding in the absence of the requirement for nuclear transport, we later fused Xic1 to the nuclear protein, CDK8, and studied the minimal sequences required for Xic1 ubiquitination and degradation.

Two Xic1 deletion mutants that were efficiently localized to nuclei, but were not degraded or ubiquitinated, were GST-Xic1-(31–91) and GST-Xic1-(31–91)(161–210) (Fig. 4B, lanes 13 and 15; Fig. 5A). This result indicated that the CDK2-cyclin binding domain (residues 31–91) is not sufficient to support Xic1 ubiquitination or degradation. The GST-Xic1-(31–91)(161–210) mutant was expected to be degraded efficiently because it expressed both the CDK2 binding domain and the C-terminal 50 amino acids of Xic1 that we showed were sufficient for Xic1 turnover, but surprisingly, it was completely stable. We postulated that the direct fusion of the CDK2-cyclin binding domain to the C-terminal domain of Xic1 might have caused some type of steric hindrance for Xic1 binding between CDK2-cyclin E and another mediator of Xic1 degradation that binds to the C-terminal domain. Alternatively, specific interactions with endogenous factors might be needed to trigger Xic1 degradation, and because the CDK2-cyclin binding domain is lost, it would not be sufficient to support Xic1 degradation.

FIGURE 2. The C-terminal domain of Xic1 mediates its nuclei-dependent proteolysis. A, top, schematic of Xic1 showing the CDK binding region and NLS sites. Bottom, schematic of Xic1 deletion (d) mutants with N indicating a deletion from the N terminus, C indicating a deletion from the C terminus, and the number indicating the number of residues deleted. 8, Xic1 degradation assay. In vitro translated [35S]methionine-labeled Xic1 WT or deletion mutants were incubated in the Xenopus interphase egg extract (also called LSS) with (+) or without (−) nuclei for 0, 1, 2, and 3 h and were resolved by SDS-PAGE. The results of two separate experiments were quantitated by PhosphorImager analysis, and the mean percentage of Xic1 remaining is shown (% XIC1 REMAINING), where the 0-h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample. Molecular mass markers (M) are indicated in kilodaltons. C, graph of the quantitated results from panel B presented as the percentage of Xic1 remaining over time.

FIGURE 3. The Xic1 C terminus is required for its proteolysis beyond its role in nuclear localization. A, top, schematic of Xic1 showing the CDK binding region and NLS sites. Bottom, schematic of Xic1 mutants with the numbers indicating the first and last amino acid residues, and SV40-NLS indicating the SV40 NLS sequence. B, Xic1 degradation assay. In vitro translated [35S]methionine-labeled WT Xic1 or Xic1 wild type or mutants fused to GST were incubated in the LSS with (+) or without (−) nuclei for 0, 1, and 3 h and were resolved by SDS-PAGE. The percentage of protein remaining was quantitated by PhosphorImager analysis and is indicated (% PROTEIN REMAINING), where the 0-h time point for each sample was normalized to 100% of the protein remaining. Molecular mass markers (M) are indicated in kilodaltons. C, Xic1 nuclei spin-down assay. In vitro translated [35S]methionine-labeled GST-Xic1 was incubated in LSS with sperm nuclei and methyl-ubiquitin and then separated into nuclear (N) and cytosolic (C) fractions by centrifugation. The samples were resolved by SDS-PAGE and analyzed by PhosphorImager analysis. The solid arrow indicates the GST-Xic1(31–160)SV40NLS protein band, and the broken arrow indicates the GST-Xic1(92–160)SV40NLS protein band.
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FIGURE 4. Xic1 is ubiquitinated at 3–4 lysine residues within its C terminus. A, top, schematic of Xic1 showing the CDK binding region and NLS sites. Bottom, schematic of Xic1 deletion mutants with the numbers indicating the first, last, or intervening amino acid residues. B, Xic1 nuclei spin-down assay. In vitro translated [35S]methionine-labeled GST or GST-Xic1 WT or mutants were incubated in LSS with sperm nuclei and methyl-ubiquitin and then separated into nuclear (N) and cytosolic (C) fractions by centrifugation. The samples were resolved by SDS-PAGE and analyzed by PhosphorImager analysis. The unmodified GST-Xic1 WT protein band (GST-Xic1) is indicated by an arrow, whereas the carets indicate the unmodified protein bands for the GST-Xic1 mutants. Monoubiquitinated (1-MeUb), diubiquitinated (2-MeUb), and tri-ubiquitinated (3-MeUb) Xic1 protein bands are indicated by arrows, whereas polyubiquitinated Xic1 is indicated by the asterisk and bracket. Molecular mass markers (M) are indicated in kilodaltons.

intervening Xic1 sequences may have been required for Xic1 ubiquitination. To test these possibilities, we engineered two additional mutants of Xic1 (GST-Xic1-(31–131)(161–210) and GST-Xic1-(31–91)(132–210)), which contain mutually exclusive intervening sequences that separate the CDK2-cyclin binding domain from the C-terminal 50 amino acids of Xic1. When tested for ubiquitination and degradation, both of these mutants were efficiently ubiquitinated and degraded (Fig. 4B, lanes 17 and 19). This result suggested that specific intervening sequences are not required for Xic1 ubiquitination and degradation but that any sequence that separates the CDK2-cyclin and C-terminal domains will support the nuclei-dependent turnover of Xic1. To test whether CDK2-cyclin E can bind to the GST-Xic1-(31–91)(161–210) mutant, a binding assay was performed using interphase egg extract, antibodies to cyclin E, and the CDK2 antibody (Fig. 5A). This result suggested that specific intervening sequences are not required for Xic1 ubiquitination and degradation but that any sequence that separates the CDK2-cyclin and C-terminal domains will support the nuclei-dependent turnover of Xic1. To test whether CDK2-cyclin E can bind to the GST-Xic1-(31–91)(161–210) mutant, a binding assay was performed using interphase egg extract, antibodies to cyclin E, and the CDK2 antibody (Fig. 5A). This result suggested that specific intervening sequences are not required for Xic1 ubiquitination and degradation but that any sequence that separates the CDK2-cyclin and C-terminal domains will support the nuclei-dependent turnover of Xic1. To test whether CDK2-cyclin E can bind to the GST-Xic1-(31–91)(161–210) mutant, a binding assay was performed using interphase egg extract, antibodies to cyclin E, and the CDK2 antibody (Fig. 5A). This result suggested that specific intervening sequences are not required for Xic1 ubiquitination and degradation but that any sequence that separates the CDK2-cyclin and C-terminal domains will support the nuclei-dependent turnover of Xic1. To test whether CDK2-cyclin E can bind to the GST-Xic1-(31–91)(161–210) mutant, a binding assay was performed using interphase egg extract, antibodies to cyclin E, and the CDK2 antibody (Fig. 5A). This result suggested that specific intervening sequences are not required for Xic1 ubiquitination and degradation but that any sequence that separates the CDK2-cyclin and C-terminal domains will support the nuclei-dependent turnover of Xic1.
ferred sites of Xic1 ubiquitination, whereas residues Lys-138, Lys-143, Lys-146, Lys-152, Lys-156, Lys-207, and Lys-208 appear to serve as alternative sites of Xic1 ubiquitination.

*Residues 161–190 Are Sufficient to Support Nuclei-dependent Ubiquitination of Xic1*—To eliminate the influence of nuclear localization on Xic1 ubiquitination and proteolysis, we created fusions between Xic1 and the nuclear protein, human CDK8 (hCDK8) (29), and tested these proteins for ubiquitination and degradation. Although human CDK8 is a nuclear protein, it was not retained efficiently in nuclei when assayed by nuclear spin-down in *Xenopus* egg extracts, and it was not degraded (Fig. 6, A and B). In contrast, when hCDK8 was fused to Xic1 amino acids 161–210, this fusion protein was efficiently localized to nuclei and was readily ubiquitinated and degraded (Fig. 6, A and B). These studies supported that Xic1 residues 161–210 appear to be the minimal domain of Xic1 that can support its efficient nuclei-dependent proteolysis. Further deletion analyses indicated that Xic1 residues 161–190 could support the ubiquitination of hCDK8, suggesting that this was the minimal domain of Xic1 that could support its nuclei-dependent ubiquitination but that this domain of Xic1 was not sufficient to support its degradation (Fig. 6, A and B). A similar region of Xic1-(162–192) was identified by You et al. (14) as sufficient for the ubiquitination, but not the proteolysis, of a Xic1 fusion protein with GFP in the NPE. We postulated that the efficient proteolysis of Xic1 requires the ubiquitination of other lysine residues within amino acids 191–210. We believe that the C-terminal 20 amino acids of Xic1 provide at least one lysine ubiquitination site but is not essential for the recruitment of the Xic1 ubiquitination machinery.

**Binding to CDK2-Cyclin Is Not Essential for the Ubiquitination or Degradation of Xic1**—Our previous studies suggest that the trimeric complex of Xic1-CDK2-cyclin E is not essential for the proteolysis of Xic1 (12). To further study the requirement of CDK2-cyclin binding for Xic1 ubiquitination and degradation, we tested the Xic1 deletion mutants for their ability to bind CDK2 in the interphase egg extract we used for degradation and ubiquitination studies, as well as in NPE, which contains a significantly higher concentration of CDK2-cyclin E. Our results indicated that Xic1 binding to CDK2-cyclins is not a requirement for Xic1 ubiquitination or degradation (Figs. 5B and 6C). In particular, the CDK2-cyclin binding domains of Xic1 mutants GST-Xic1-(92–210), GST-Xic1-(132–210), and hCDK8-Xic1-(161–210) are completely deleted, yet they still support efficient Xic1 ubiquitination and degradation (Figs. 5 and 6). These studies re-emphasized that although the interaction between Xic1 and CDK2-cyclin E is important for the nuclear retention of Xic1, it is not essential for its ubiquitination and proteolysis once Xic1 is localized in the nucleus.

**Phosphorylation of Xic1 Does Not Appear to be Essential for Its Proteolysis**—Although the ubiquitination machinery for Xic1 has not yet been identified, it is postulated that Xic1 is targeted for ubiquitination by the E3 called SCF. This hypothesis is based on previous studies suggesting that the degradation of Xic1 is dependent on Cdc34 function and on studies that indicate that both ubiquitinated Xic1 and SCF components, Cul1 and Skp1, are localized to chromatin during DNA replication in egg extracts (11, 13). In the nucleus, it is unclear whether chromatin-associated Xic1 must be phosphorylated before Xic1 is targeted for ubiquitination. Additionally, the mammalian orthologs of Xic1, p27Kip1 and p21Cip1, have been shown to be ubiquitinated by the SCF (15–18, 30). Studies have indicated that the phosphorylation of SCF substrates is a common requirement for efficient ubiquitination by the SCF (31). Because Xic1 is a putative substrate of the SCF, we wanted to address whether phosphorylation may be a prerequisite for the nuclei-dependent ubiquitination of Xic1 during DNA replication initiation in the egg extract. We first tried to determine whether nuclearly localized Xic1 was targeted for phosphorylation during the initiation of DNA replication in the egg extract. However, our attempts to visualize a phosphorylated Xic1 species in replicating nuclei were not reproducibly successful, perhaps because at any particular time during DNA replication initiation, only a small fraction of the total Xic1 may be transiently phosphorylated.
phosphorylated in nuclei just prior to its ubiquitination and degradation. Because we were unable to reproducibly directly detect a phosphorylated Xic1 species in replicating interphase nuclei, we instead took a more brute force alternative approach and mutated all the possible phosphorylation sites within the Xic1 C-terminal 50 amino acids and then tested this Xic1 mutant for its ability to be degraded.

Our studies indicated that Xic1 residues 161–210 were sufficient to support efficient Xic1 proteolysis, further suggesting that any Xic1 phosphorylation required for ubiquitination should be contained within this region. An analysis of Xic1 residues 161–210 revealed 10 possible phosphorylation sites within this region (shown in bold): 161-(T)(S)(T)QRRKKREI I(T)(T)PDYFPK RKKILSAKPD A(T)KGVHLLCP LEQ(T)PRKKIR-210. We eliminated five of these sites as possible phosphorylation sites (shown above in parentheses) based on the following criteria. We have shown that Xic1 (161–190) is ubiquitinated in the LSS (12, 14), and You et al. (14) demonstrated that Xic1-(162–192) was ubiquitinated in the NPE, suggesting that any required phosphorylation sites should fall within residues 162–190 (this eliminated residues 161, 192, and 204). Additionally, Furstenthal et al. (13) demonstrated that phosphorylation at (S/T)-P CDK consensus sites was not required for Xic1 degradation, and we demonstrated that phosphorylation of Thr-163 was not required for Xic1 degradation (data not shown) (this eliminated residues 163, 172, and 204). This suggested that Xic1 may be phosphorylated at five possible critical sites: Ser-162, Thr-171, Thr-175, Tyr-177, and Ser-186 (shown above in bold and underlined).

We individually mutated these five potential phosphorylation sites and then analyzed these Xic1 mutants for degradation. Our results indicated that the individual S162A, T171A, Y177F, and S186A mutants were degraded at a level similar to wild-type Xic1 (data not shown). In contrast, the T175A mutant exhibited an extended half-life (>3 h) when compared with the wild-type Xic1 (1–2.5 h) (Fig. 7, A and B). We reasoned that the defect in Xic1-T175A degradation could be due to several reasons. First, Xic1 may require phosphorylation at this site for efficient degradation, or second, a mutation at this residue may inhibit binding of an important mediator of Xic1 degradation. To test the first possibility, we mutated residue 175 to aspartic acid (T175D) to mimic constitutive phosphorylation, reasoning that if phosphorylation of this residue was required for efficient Xic1 degradation, then substituting a negatively charged amino acid at this site should promote the degradation of Xic1-T175D when compared with Xic1-T175A. Because there was little change in the half-life of Xic1-T175D when compared with Xic1-T175A (Fig. 7B), this suggested that phosphorylation of Xic1 at residue 175 was not critical for Xic1 degradation. To examine the second possibility, we tested the ability of Xic1-T175A and Xic1-T175D to associate with known Xic1-binding proteins, cyclin E and PCNA. Although the T175A and T175D mutants bound cyclin E at levels similar to wild-type Xic1, the mutants were significantly reduced for PCNA binding, similar to another Xic1 mutant (I174A) defective for PCNA binding (Fig. 7C). This suggested that the defect in Xic1-T175A proteolysis is most likely due to its inability to bind PCNA and that PCNA binding may be important for Xic1 proteolysis, which is addressed in the accompanying publication (35).
To address whether phosphorylation is required on a combination of the 10 possible Xic1 phosphorylation sites within the C-terminal 50 amino acids, we also mutated every site simultaneously, except for residue 175, and tested this mutant (9A) for degradation. When compared with the half-life of wild-type Xic1 (~1.2 h), the Xic1–9A mutant was somewhat delayed (~1.7 h) for degradation (Fig. 7D) but nevertheless was still proteolyzed in a nuclei-dependent manner. To exclude the requirement for phosphorylation within Xic1 N-terminal residues (1–160), the Xic1 C-terminal 50 amino acids of the mutant 9A were fused to hCDK8, and the degradation of this mutant (hCDK8-Xic1-(161–210)9A) was compared with wild-type hCDK8-Xic1-(161–210) (Fig. 7E). Our results indicated that the hCDK8-Xic1-(161–210)9A mutant was degraded similar to the wild-type hCDK8-Xic1-(161–210), suggesting that phosphorylation of Xic1 is not essential for its ubiquitination and degradation in the interphase egg extract. Further studies are required to address the role of phosphorylation on Xic1 degradation during the somatic cell cycle.

DISCUSSION

Endogenous Xic1 Is Degraded in a Nuclei-dependent Manner—Previous studies of targeted Xic1 proteolysis have taken advantage of the Xenopus cell-free egg extract as a model system for the study of Xic1 ubiquitination and degradation. The interphase egg extract (LSS) has proven useful for the study of Xic1 since it fully supports DNA replication as well as the nuclei-dependent ubiquitination and degradation of exogenously added Xic1 (11). However, the egg extract contains only low concentrations of Xic1 (~2 nM) (7), and it is unclear whether endogenous Xic1 is targeted for degradation by a similar mechanism as has been described for exogenously added in vitro translated Xic1, which we add to a final concentration of ~15 nM. Our current studies indicated that upon the addition of sperm nuclei to a final concentration of ~3200 nuclei/μl, similar to the concentration of nuclei at the midblastula transition (~5000 nuclei/μl), the endogenous Xic1 is readily degraded. This suggested that both exogenously added Xic1 and endogenously present Xic1 are targeted for degradation by a similar mechanism.

The C-terminal Domain of Xic1 Mediates Its Nuclei-dependent Proteolysis—The characterization of Xic1 degradation has provided unique insights into the proteolysis of a CDK inhibitor during the temporal events of DNA replication initiation, but the molecular mechanism of Xic1 proteolysis is still unknown. We reasoned that the identification of the minimal domain of Xic1 that is sufficient to mediate its ubiquitination and degradation could help to define the critical sequences of Xic1 that contact the Xic1 ubiquitination machinery or other regulators of Xic1 proteolysis. Our results demonstrated that the C-terminal 50 amino acids (161–210) of Xic1 are both necessary and sufficient to mediate the efficient nuclei-dependent ubiquitination and proteolysis of Xic1 fused to the stable heterologous nuclear protein, human CDK8. Furthermore, amino acids 161–190 were sufficient to support the nuclei-dependent ubiquitination of Xic1. These results suggested that amino acids 161–190 of Xic1 define a functional motif sufficient to mediate the ubiquitination of Xic1 in nuclei. Our data also indicated that the ubiquitination of 3–4 critical lysine residues within the Xic1 C-terminal 50 amino acids functions to trigger Xic1 for proteolysis by the 26 S proteasome. The C-terminal domain of Xic1 also appeared to function as a portable degradation signal since it can trigger the nuclei-dependent ubiquitination and degradation of the stable proteins, GST and human CDK8.

Previously, we described a mutant called Xic1-NLS2 (180ARAa) mutated at 3 lysine residues within amino acids 180–183 (12). This mutant was efficiently localized to nuclei but was not degraded. When the lysine residues were instead mutated to arginine, this Xic1-NLS2 mutant (180RRRR) was degraded, suggesting that these residues did not serve as essential ubiquitination sites. We postulated that these residues of Xic1 served as a functional domain to trigger Xic1 degradation by recruiting an essential mediator of Xic1 ubiquitination. Our current studies identified 30 amino acids (161–190) of Xic1 that are sufficient to support the ubiquitination of Xic1, and as expected, this region of Xic1 includes the region we previously identified by the Xic1-NLS2 mutant (180–183). Taken together, our results suggested that Xic1 amino acids 161–210 serve to bind essential mediators of Xic1 ubiquitination, including putative positive regulators and the Xic1 ubiquitination machinery, resulting in the ubiquitination of Xic1 on 3–4 lysine residues, and ultimately, the degradation of Xic1.

CDK2-Cyclin E Promotes the Nuclear Retention of Xic1—Previous studies showed that a point mutant of Xic1 (Xic1-CK−) unable to bind CDK2-cyclin E in egg extracts was still degraded, suggesting that the formation of a trimeric complex between CDK2-cyclin E and Xic1 was not essential for the ubiquitination and degradation of Xic1 in nuclei (12, 14). Our current results indicated that binding to CDK2-cyclin E promotes the efficient nuclear retention of Xic1, but once localized in the nucleus, CDK2-cyclin E binding is not required for Xic1 ubiquitination and degradation. We showed that Xic1 mutants bearing complete deletions of the CDK2-cyclin binding domain (31–91) are still ubiquitinated and degraded when localized to nuclei.

A Novel Mechanism Triggers the Proteolysis of Xic1—Our studies suggested that the mechanism that degrades Xic1 in the egg extract is unique among metazoan CDK inhibitors. Xic1 shares sequence homology with both p27Kip1 and p21Cip1 but may in fact be a primordial CDK inhibitor in the frog. However, unlike mammalian p27Kip1, once Xic1 is localized to nuclei, it does not require binding to CDK2-cyclin E for ubiquitination. Additionally, Xic1 phosphorylation did not appear to be essential for its ubiquitination in the egg extract. Also, unlike p21Cip1, which is degraded in a proteasome-dependent and lysine-independent manner (33–35), lysine ubiquitination within the Xic1 C terminus appeared to be important for the proteolysis of Xic1. The key to the regulation of Xic1 ubiquitination in the egg extract appeared to lie within a 30-amino-acid domain within the Xic1 C terminus important for PCNA binding, suggesting that PCNA may play an important role in regulating Xic1 ubiquitination and degradation, a hypothesis we address in the accompanying publication (35). A requirement for PCNA in the turnover of p21Cip1 has not been described. The apparent differences observed between mammalian and frog mechanisms of CDK inhibitor proteolysis could be due to differences in the regulation of CDK inhibitors in embryonic versus somatic cells. Future studies will be needed to fully understand how CDK inhibitors are regulated in metazoans.

Acknowledgments—We are grateful to T. G. Boyer and to all the past and present members of the Yew laboratory for helpful discussions; T. G. Boyer for pBluescript/hCDK8; P. K. Jackson and M. W. Kirschner for anti-CDK2 and anti-cyclin E antibodies; J. Walter for the detailed NPE protocol, and Carlos R. Herrera and Michael Parker for excellent technical assistance.

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The C-terminal Domain of the *Xenopus* Cyclin-dependent Kinase Inhibitor, p27\textit{Xic1}, Is Both Necessary and Sufficient for Phosphorylation-independent Proteolysis

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*J. Biol. Chem.* 2005, 280:35290-35298.
doi: 10.1074/jbc.M506430200 originally published online August 23, 2005

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