The regulation of the vertebrate cell cycle is controlled by the function of cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors. The *Xenopus laevis* kinase inhibitor, p27\(^{Xic1}\) (Xic1) is a member of the p21\(^{Cip1}\)/p27\(^{Kip1}\)/p57\(^{Kip2}\) CDK inhibitor family and inhibits CDK2-cyclin E in vitro as well as DNA replication in *Xenopus* egg extracts. Xic1 is targeted for degradation in interphase extracts in a manner dependent on both the ubiquitin conjugating enzyme, Cdc34, and nuclei. Here we show that ubiquitination of Xic1 occurs exclusively in the nucleus and that nuclear localization of Xic1 is necessary for its degradation. We find that Xic1 nuclear localization is independently mediated by binding to CDK2-cyclin E and by nuclear localization sequences within the C terminus of Xic1. Our results also indicate that binding of Xic1 to CDK2-cyclin E is dispensable for Xic1 ubiquitination and degradation. Moreover, we show that amino acids 180–183 of Xic1 are critical determinants of Xic1 degradation. This region of Xic1 may define a motif of Xic1 essential for recognition by the ubiquitin conjugation machinery or for binding an alternate protein required for degradation.

The progression of the vertebrate cell cycle is positively regulated by cyclin-dependent kinases (CDKs)\(^1\) associated with their cyclin partners and is negatively regulated by cyclin-dependent kinase inhibitors (CKIs) (reviewed in Refs. 1–4). The mammalian cell cycle transition between G1 and S phases requires the activity of CDK2-cyclin E (5–7). The function of this kinase is negatively modulated by the CDK2 inhibitor, p27\(^{Kip1}\) in quiescent cells and in G1 phase cells (2, 8–12). Entry into S phase is accompanied by the ubiquitin-dependent proteolysis of p27\(^{Kip1}\) by a protein complex consisting of Cdc34 and SCF\(^{p45Skp2}\) (13–21). The ubiquitin conjugating enzyme Cdc34 (UBC3) was first identified in budding yeast as a protein essential for the G1 to S phase transition (22, 23). The SCF (Skp1, (UBC3) was first identified in budding yeast as a protein essential for the G1 to S phase transition (22, 23). The SCF (Skp1, (UBC3) was first identified in budding yeast as a protein essential for the G1 to S phase transition (22, 23). 

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\(^3\) The abbreviations used are: CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; LSS, low speed supernatant; NLS, nuclear localization sequence; PAGE, polyacrylamide gel electrophoresis; WT, wild type; GST, glutathione S-transferase; PCNA, proliferating cell nuclear antigen.

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EXPERIMENTAL PROCEDURES

Preparation of Xenopus Interphase Egg Extract, Xenopus Demembranated Sperm Chromatin, Histone Ubiquitin, and Methyl-ubiquitin—X. laevis egg interphase extract (low speed supernatant (LSS)) and de-
membrane-bound sperm chromatin were prepared as described previously (32, 35, 36). Mouse ubiquitin was cloned into pQE30 (Qiagen) from a 9.5/10.5 days postcoital mouse embryo library with an N-terminal tag of 6 histidine residues (His₂-ubiquitin). Mouse ubiquitin protein was expressed in E. coli and purified on nickel-nitriiltriocetic acid (Ni-NTA) agarose. The purified protein was then dialyzed and cleaved using factor Xa (Promega) for 10 min. A 20-fold excess of digestion buffer (HBS (10 mM HEPES, pH 7.4, 150 mM NaCl) at 23 °C (31)) was then added at a volume equal to the relative amount of radioactively labeled Xic1-WT or GST-Xic1-WT protein. In general, all [35S]methionine-labeled mutant Xic1 proteins were quantitated by PhosphorImager analysis and added at a volume equal to the relative amount of radioactively labeled Xic1-WT or GST-Xic1-WT protein. In all, generally consistent with a recent study of Xic1 ubiquitination (33), Col-labeled samples were used between final dilutions of 1:15 to 1:72. Chromatin spin down assays were conducted by including 0.1% Nonidet P-40 in NIB (38). The percentage of nuclear transport was calculated as the amount of Xic1 in the nucleus divided by the sum of the amount of Xic1 in the nucleus and the cytosol multiplied by 100%. All the samples were normalized to 100% of the WT (Xic1) transported to the nucleus. For quantitation analyses, each sample was measured at least two or three times, and the standard error of the mean for each sample was calculated and displayed as error bars.

Degradation Assay and Dephosphorylation—[35S]Methionine-labeled Xic1 (0.5 μl) was incubated in LSS (8 μl) containing cycloheximide, an energy regenerating system, and ubiquitin (1.25 mg/ml), or with demembranated sperm chromatin (10 ng/ml) at 23 °C for 0, 1, and 3 h. Aliquots of 1.4 μl were removed from the samples for analysis by SDS-PAGE followed by quantitation by PhosphorImager analysis. The amounts of [35S]methionine-labeled mutant Xic1 proteins were added at a volume normalized to equal the same relative amount of radioactively labeled Xic1-WT protein. Dephosphorylation was performed using calf intestinal phosphatase as described previously (32).

DNA Replication Assay—DNA replication assays were conducted as described previously with the following modifications (32, 36). [35S]Methionine-labeled Xic1 (0.625 μl) was added to LSS (10 μl) containing cycloheximide, an energy regenerating system, ubiquitin (1.25 mg/ml), and demembranated sperm chromatin (10 ng/ml). DNA replication was measured on a 5% acrylamide gel containing rabbit reticulocyte lysate. Each sample was measured three times, and the standard error of the mean for each sample was calculated and displayed as error bars.

RESULTS

Ubiquitination and Degradation of Xic1 Occur in the Nucleus—To begin to understand the requirement for nuclei in Xic1 degradation, we first determined whether Xic1 is transported into nuclei and where ubiquitinated Xic1 is localized. Xenopus interphase egg extracts derived from a low speed centrifugation of eggs (low speed supernatant or LSS) support the formation of transport-competent nuclei as well as a single round of initiation and elongation phases of semi-conservative chromosomal DNA replication (36, 39, 40). Demembranated sperm chromatin was preincubated with LSS, followed by the addition of [35S]methionine-labeled Xic1 and His₂-ubiquitin and separation of the nuclear and cytosolic fractions by centrifugation. Our results show that polyubiquitinated Xic1 is found exclusively in the nuclear fraction, whereas Xic1 in the cytosolic fraction remains unmodified (Fig. 1A). We next asked whether Xic1 degradation also occurs in the nucleus. To do this, we incubated [35S]methionine-labeled Xic1 with demembranated sperm chromatin in LSS and then separated the nuclei and cytosol. The isolated nuclei and cytosol were added back separately to fresh LSS and incubated for 0 or 3 h. The sample containing nuclei in fresh LSS was again centrifuged to separate the nuclear and cytosolic fractions, and all the samples were analyzed by SDS-PAGE and PhosphorImager analysis to quantitate the amount of Xic1 remaining. The results show that the nuclear Xic1 pool is 90% degraded after 3 h (Fig. 1B, lanes 1, 2, 6, and 7), whereas Xic1 in the cytosolic fraction remains stable under these conditions (Fig. 1B, lanes 4, 5, 9, and 10). A slower migrating form of Xic1, which most likely represents a mono-ubiquitinated species, was observed in the nuclear fraction (Fig. 1B, lanes 1, 4, 5, 9, and 10). To eliminate the possibility that Xic1 must be exported to the cytoplasm to be degraded, we tested the degradation of Xic1 in the presence of leptomycin B, a specific inhibitor of CRM1/exportin 1 nuclear export (41–43). We did not observe any inhibitory effect of leptomycin B on the efficiency of Xic1 degradation (data not shown), an observation consistent with a recent study of Xic1 ubiquitination (33). Collectively, these results indicate that Xic1 is predominantly ubiquitinated and degraded in the nucleus.

The Xic1 Primary Sequence Contains Two Potential Nuclear Transportation Regulatory Domains—Because Xic1 appears to be ubiquitinated and degraded in the nucleus, it is important to...
identify the sequences of Xic1 that regulate its nuclear localization. The Xic1 primary sequence contains two regions that are likely to contribute to nuclear localization and/or nuclear retention. These regions are comprised of the CDK-cyclin binding region within the N terminus and several putative NLSs within the C terminus of Xic1 (30). The residues of mammalian p27Kip1 critical for CDK-cyclin binding have been identified by mutagenesis and by examination of the co-crystal structure of human CDK2 and cyclin A with a fragment of p27Kip1 (14, 16, 44). Because Xic1 exhibits significant sequence homology with human p27Kip1 in the CDK-cyclin binding domain, we identified and mutated residues in Xic1 that we predicted would disrupt binding of Xic1 to CDK2 (Xic1<sup>Δ329</sup> and P67A) and to cyclin E (Xic1<sup>Δ163</sup> R33A and L35A) (Fig. 2A). These Xic1 mutants were monitored in vitro translated with <sup>35</sup>S-methionine, added to LSS, and immunoprecipitated with either CDK2 or cyclin E antibodies. The results indicate that both the WT Xic1 and Xic<sup>c</sup> mutant readily form trimeric complexes with endogenous CDK2-cyclin E (Fig. 2B, lanes 1–4), whereas the Xic<sup>k</sup> mutant is decreased in its ability to form a trimeric complex with CDK2-cyclin E (Fig. 2B, lanes 1–4). Interestingly, Xic1<sup>k</sup> protein bands in association with endogenous CDK2-cyclin E were shifted to slower mobility species that could be converted to a faster migrating species of Xic1 by treatment with calf intestinal phosphatase (lanes 5–7).

There are three regions in the C terminus of Xic1 that are rich in arginine and lysine residues (165RRKK, 180KRKK, and 205PRKK), indicating they may be important for nuclear localization of Xic1 through an importin pathway (45). To address how these sequences influence the nuclear localization of Xic1, the residues in each of these three putative NLSs were mutated, resulting in the following mutants: Xic1-NLS1, R166A, K167A, and R168A; Xic1-NLS2 (ARAA), K180A, L182A, and K183A; Xic1-NLS3 (KKKR), K180R, K182R, and K183R; and Xic1-NLS5, R206A, K207A, and K208A (Fig. 2A). Xic1 mutants were also generated that combined all three individual NLS mutations (Xic1-NLS1/2/3) as well as both CDK2-cyclin E binding mutations and all NLS mutations (Xic1<sup>Δk</sup> & NLS1/2/3). These mutants were tested for their ability to bind to endogenous CDK2 and cyclin E in LSS. The results show that Xic1-NLS2 (ARAA) and Xic1-NLS1/2/3 both bind endogenous CDK2

![Image](attachment:image_url)
Nuclear Transport and Degradation of Xenopus \(p27^{Xic1}\)

A Stable Trimeric Complex between Xic1 and CDK2-Cyclin E

Is Not Required for Xic1 Ubiquitination or Degradation—Previous studies have shown that Xic1 degradation is sensitive to the purine analog 6-dimethylaminopurine, an inhibitor of CDKs and DNA replication initiation events (32, 46, 47). This suggests that Xic1 degradation is dependent on CDK activity or initiation events but does not address whether Xic1 must form a stable trimeric complex with CDK2-cyclin E for its ubiquitination and degradation. Studies show that mutations that disrupt the binding of mammalian \(p27^{Kip1}\) to CDK2-cyclin E eliminate ubiquitination of \(p27^{Kip1}\), indicating that a stable trimeric complex between CDK2-cyclin E-\(p27^{Kip1}\) is necessary for ubiquitination and degradation. We studied the ubiquitination and degradation of Xic1 point mutants defective for binding to CDK2-cyclin E (Fig. 2). We used a GST-Xic1 fusion protein for our ubiquitination studies because it facilitated the detection of the ubiquitinated Xic1 species by allowing Xic1 to be labeled to a higher specific activity. Xic1 contains 2 methionines and GST-Xic1 contains 11 methionines. GST-Xic1-WT is localized to both the nucleus and cytosol but is predominantly ubiquitinated in the nucleus, whereas GST-Xic1 is localized predominantly to the cytosol and is not ubiquitinated (Fig. 4A). Equivalent amounts of \([^{35}S]\)methionine-labeled GST-Xic1-WT or GST-Xic1 \(\text{ck}^{\text{K}}\) were added to LSS and nuclei in the presence of methyl-ubiquitin to block polyubiquitination and subsequent degradation and to allow the accumulation of mono-ubiquitinated Xic1 species (37). In the presence of methyl-ubiquitin, both GST-Xic1-WT and GST-Xic1 \(\text{ck}^{\text{K}}\) efficiently accumulate mono-ubiquitinated Xic1 species in the nucleus.
Nuclear Localization is Necessary but not Sufficient for Xic1 degradation. A, [35S]methionine-labeled Xic1 was incubated in LSS with nuclei for 0, 1, and 3 h and was resolved by SDS-PAGE. B, the results of two separate experiments were quantitated by PhosphorImager analysis, and the mean percentage of Xic1 remaining is shown where the 0 h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample.

(Nuclear localization is necessary, but not sufficient for Xic1 degradation. A, [35S]methionine-labeled Xic1 was incubated in LSS with nuclei for 0, 1, and 3 h and was resolved by SDS-PAGE. B, the results of two separate experiments were quantitated by PhosphorImager analysis, and the mean percentage of Xic1 remaining is shown where the 0 h time point of Xic1 was normalized to 100% of Xic1 remaining for each sample.

(FIG. 5). Based on the molecular mass of ubiquitin (~8 kDa) and the estimated molecular masses of the stabilized bands in Fig. 4B, ubiquitination appears to occur at ~3–5 independent sites on Xic1. The amount of ubiquitinated GST-Xic1ck observed in the nucleus was reduced by ~2–3-fold compared with the GST-Xic1 WT, correlating with the ~2–3-fold reduction in nuclear transport proficiency observed for Xic1ck compared with Xic1 WT (Fig. 3, lanes 2 and 8). Thus, the inability of Xic1ck to bind CDK2-cyclin E reduces the efficiency of its nuclear transport by ~2-fold but does not prevent the efficient ubiquitination of Xic1ck once it is localized to the nucleus.

To fully understand how Xic1 binding to CDK2-cyclin E modulates its turnover, the c, k, and ck− mutants were analyzed for degradation in LSS containing nuclei. Both the Xic1-WT and Xic1k, which form stable trimeric complexes with CDK2-cyclin E, are efficiently degraded (Fig. 4C, lanes 1–8). The Xic1ck mutant, which was reduced for CDK2-cyclin E binding, is still degraded effectively (Fig. 4C, lanes 9–12). The Xic1ck− mutant, which was totally defective for CDK2-cyclin E binding, is also degraded with only slightly reduced efficiency (Fig. 4C, lanes 13–16). The slight reduction in degradation efficiency of Xic1ck− can be attributed to its reduced nuclear transport proficiency. Degradation of all the mutants required the presence of nuclei (Fig. 4C). These results suggest that binding of Xic1 to CDK2-cyclin E or the formation of a stable trimeric complex is dispensable for nuclei-dependent degradation of Xic1.

Nuclear Localization of Xic1 Is Necessary but Not Sufficient for Degradation—We next asked how the C-terminal NLS sites contribute to the degradation of Xic1 by examining the turnover of the Xic1 NLS mutants. To accomplish this, Xic1 single, double, and triple NLS mutants were tested for Xic1 degradation, and the results are shown in Fig. 5A. The data from two separate experiments were averaged and are shown graphically in Fig. 5B. The results show that greater than 85% of Xic1 WT is degraded after 3 h (Fig. 5, A, lanes 1–3, and B). Xic1-NLS1, Xic1-NLS3, and Xic1-NLS1/3 are also degraded efficiently to levels comparable with the Xic1 WT (Fig. 5, A, lanes 4–6, 10–12, and 16–18, and B). Interestingly, Xic1-NLS2 (ARAA) exhibits a striking defect in degradation with only ~15% of the Xic1-NLS2 (ARAA) degraded after 3 h. This indicates that even though Xic1-NLS2 is transported to the nucleus as efficiently as WT Xic1 (Fig. 3), it is not appreciably degraded (Fig. 5, A, lanes 7–9, and B). As expected, the degradation of the Xic1ck−&NLS1/2/3 mutant that is not efficiently transported to the nucleus as well as other NLS mutant combinations containing NLS2 (ARAA) are not degraded (data not shown and Fig. 5, A, lanes 13–24, and B). The destruction of the degradable NLS mutants occurred in a nuclei-dependent manner (data not shown). These results indicate that Xic1 degradation involves at least two functional requirements, one being nuclear transport and the other involving at least one nuclear event that is defective in the Xic1-NLS2 mutant. Therefore, we conclude that nuclear transport is necessary but not sufficient for Xic1 degradation.

Xic1-NLS2 Mutation Does Not Inhibit DNA Replication in the Xic1 Degradation Assay—One trivial explanation for the stability of Xic1-NLS2 (ARAA) is that this nondegradable mutant inhibits the events of DNA replication initiation in the degradation assay, events that appear to be required for Xic1 degradation (32). To test this possibility, we measured DNA replication and Xic1 degradation in parallel, using the same preparation of LSS and the conditions we use for Xic1 degradation. Xic1-WT and Xic1-NLS2 (ARAA) were [35S]methionine-labeled and were added to Xic1 degradation and DNA replication assays. Under the degradation assay conditions, we found that the added amount of Xic1-WT and Xic1-NLS2 (ARAA) did not appreciably inhibit DNA replication compared with a control sample containing unprogrammed rabbit reticulocyte lysate sample was normalized to 100%.

FIG. 6. Xic1-NLS2 (ARAA) does not inhibit DNA replication under conditions used for Xic1 degradation. Semi-conservative DNA replication was measured in LSS using demembranated sperm chromatin as a template and [α-32P]dATP. The replication assay was conducted under degradation assay conditions with either unprogrammed rabbit reticulocyte lysate, [35S]methionine-labeled Xic1-WT, or Xic1-NLS2 (ARAA). The results are the mean of three experiments, and the error was calculated as the standard error of the mean. DNA replication for the control rabbit reticulocyte lysate sample was normalized to 100%.

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replication initiation by Xic1-NLS2 (ARAA) in the degradation assay.

Basic Lysine Residues within NLS2 Are Essential for Xic1 Degradation, but Do Not Comprise the Only Critical Ubiquitination Sites of Xic1—It is possible that the region of Xic1-NLS2 (ARAA) may comprise the only critical lysine residues that are ubiquitinated in Xic1 or, alternatively, that this region of Xic1 is important for binding a cofactor protein required for its degradation. This putative cofactor might be a component of the ubiquitin conjugation machinery or a regulator required for post-translational modification of Xic1 prior to ubiquitination. To test these possibilities, we engineered an additional Xic1 mutant to carry conservative arginine mutations at the NLS2 residues (K180R, K182R, and K183R). This mutant would prevent ubiquitination at NLS2 residues but should not disrupt the interaction of Xic1 with a regulator that is dependent upon the basic residues of NLS2 for binding. The Xic1-NLS2 (RRRR) mutant localizes to the nucleus as efficiently as Xic1-WT (Fig. 3, lane 5). Equivalent amounts of [35S]methionine-labeled GST-Xic1 proteins were incubated with LSS in the presence of nuclei and methyl-ubiquitin. The results show that GST-Xic1-WT, GST-Xic1-NLS1, GST-Xic1-NLS3, and GST-Xic1-NLS2 (RRRR) are all efficiently mono-ubiquitinated on 3–5 independent sites in the nucleus (Fig. 7, lanes 1, 2, 4, and 5). In contrast, GST-Xic1-NLS2 (ARAA) and GST-Xic1-NLS1/2/3 are not appreciably ubiquitinated (Fig. 7, lanes 3 and 6). None of the GST-Xic1 derivatives was appreciably ubiquitinated in the cytosol (Fig. 7, lanes 7–12). There was little difference in the ubiquitination pattern of a specific NLS mutant when tested as a GST or a non-GST fusion protein (data not shown). These results indicate that although Xic1-NLS2 (ARAA) is not efficiently ubiquitinated, when the NLS2 lysine residues are converted to arginine residues, NLS2 (RRRR) is now efficiently ubiquitinated. NLS2 (RRRR), like NLS2 (ARAA) cannot be ubiquitinated at lysine residues 180, 182, or 183, indicating that NLS2 (RRRR) must be ubiquitinated at alternative lysine residues. Because NLS2 (RRRR) can be ubiquitinated, this demonstrates that lysines 180, 182, and 183 are not the only critical lysine residues that may be ubiquitinated in Xic1.

To confirm and extend the ubiquitination results in Fig. 7, the GST-Xic1 fusion proteins were tested for degradation. The results indicate that Xic1-WT and GST-Xic1-WT are efficiently degraded, although GST alone is not (Fig. 8A). GST-Xic1-NLS3, GST-Xic1-NLS1, and GST-Xic1-NLS2 (RRRR) are all degraded, whereas GST-Xic1-NLS2 (ARAA) and GST-Xic1-NLS1/2/3 are not degraded (Fig. 8A). Similarly, as non-GST fusion proteins, Xic1-NLS2 (RRRR) is degraded, whereas Xic1-NLS2 (ARAA) is not appreciably degraded (Fig. 8B). Taken together, these results suggest that the basic lysine residues of Xic1 amino acids 180–183 are essential for the efficient ubiquitination and degradation of Xic1. We postulate that this region of Xic1 is critical for binding a mediator of Xic1 degradation.

DISCUSSION

Regulation of Xic1 Nuclear Transport and Additional Events Required for Xic1 Nuclear Degradation—Our work indicates that Xic1 degradation is dependent upon its transport to the nucleus, a requirement mediated through CDK2-cyclin E binding and three C-terminal NLS sites of Xic1. It is unclear from our studies whether Xic1 binding to CDK2-cyclin E influences its active nuclear transport, nuclear retention, or both. Our results also suggest that the binding of Xic1 to CDK2-cyclin E and importin may be sufficient for its efficient nuclear localization. However, because we do not demonstrate a direct interaction between the C terminus of Xic1 and importin, it is possible that an alternative and as yet unidentified protein binds to the Xic1 C terminus and mediates its nuclear import.
Although nuclear transport is necessary, it is not sufficient for Xic1 degradation. There is at least one other nuclear event required for Xic1 degradation that is defective in the Xic1 mutant NLS2 (K180A, K182A, K183A). The ubiquitination and degradation defects of Xic1-NLS2 (ARAA) are not attributable to defective nuclear import or defective CDK2-cyclin E binding. Conservative arginine substitutions at lysine residues 180, 182, and 183 do not prevent ubiquitination of Xic1, indicating that these residues do not comprise the only possible ubiquitination sites of Xic1. However, because the degradation of Xic1-NLS2 (RRRR) is reduced 2-fold compared with the Xic1-WT, this may indicate that the lysine residues at amino acids 180–183 are important sites of Xic1 ubiquitination and that alternative lysine residues are not used quite as efficiently. Nonetheless, despite the ubiquitination of alternative sites in NLS2 (RRRR), these sites are not ubiquitinated in NLS2 (ARAA). This indicates that the basic residues of Xic1 (amino acids 180–183) more importantly define a region that is important for binding a protein required for efficient nuclear ubiquitination and degradation. This putative regulator is likely to be either a component of the ubiquitination machinery such as an F-box protein or alternatively, a regulator that must modify Xic1 prior to ubiquitination such as a kinase. Substrates of SCF are thought to require specific phosphorylation to bind their designated F-box proteins (reviewed in Ref. 48). The binding of Xic1 to an F-box protein is predicted because it has been shown that Xic1 is degraded in a Cdc34-dependent manner, implying that it will be degraded in a SCF- and F-box-dependent manner (32). However, to date, a requirement for SCF has not been demonstrated, nor has a Xic1 F-box protein been identified and shown to be required for Xic1 degradation. Xic1 has also been shown to bind to PCNA within unspecified C-terminal residues between Xic1 amino acids 97 to 210 (30). Based on weak homology to the PCNA binding regions of p21Cip1 and p53, we predict that Xic1 may bind to PCNA through amino acids flanking the NLS2 sequence (49, 50). How NLS2 may influence the binding of Xic1 to PCNA is currently under investigation.

**Binding of Xic1 to CDK2-Cyclin E Is Dispensable for Nuclear Degradation**—We find that although Xic1 binding to CDK2-cyclin E is required for efficient nuclear transport or nuclear retention of Xic1, it is not required for Xic1 degradation. The Xic1<sup>ctk</sup> mutant that retains no measurable binding to CDK2 or cyclin E, is still ubiquitinated and degraded efficiently in the nucleus. This finding also implies that the phosphorylation of Xic1 by CDK2-cyclin E may not be required for its nuclear degradation. Our results conflict somewhat with a recent study suggesting that an association with CDK2-cyclin E is required for Xic1 destruction (33). This study demonstrates that immunodepletion of cyclin E inhibits Xic1 destruction (33). Xic1 degradation has previously been shown to be sensitive to the CDK inhibitor, 6-dimethylaminoquinine, which inhibits DNA replication initiation (32, 46, 47). In light of our current results, it is likely that Xic1 degradation is dependent on initiation events mediated by CDK2-cyclin E but is not directly dependent on CDK2-cyclin E binding or phosphorylation. In vivo, it is unclear whether Xic1 is a physiological substrate of CDK2-cyclin E or how phosphorylation of Xic1 by CDK2-cyclin E may regulate its function.

**Proteolysis of Vertebrate CKIs: Similarities and Differences**—Although a stable trimeric complex of CDK2-cyclin E with mammalian p27<sup>kip1</sup> has been shown to be required for its ubiquitination and degradation, our results indicate that a stable complex of Xic1 with CDK2-cyclin E is not essential for the degradation of Xic1 (14, 16). Additionally, the degradation of p27<sup>kip1</sup> exhibits a strict dependence on CDK2-cyclin E phosphorylation of Thr<sup>187</sup>, whereas phosphorylation of Thr<sup>204</sup> (the residue equivalent to Thr<sup>187</sup> of p27<sup>kip1</sup>) is not required for Xic1 degradation (14, 16, 20). Two individual mutations of Xic1 at this residue, T204A and T204E, are both degraded in a nuclei-dependent manner with the exact kinetics as the Xic1-WT. This is surprising because Thr<sup>187</sup> of p27<sup>kip1</sup> and Thr<sup>204</sup> of Xic1 are located within highly conserved C-terminal sequences termed the QT domain. Moreover, although both Xic1 and Kix1 are efficiently degraded in a nuclei-dependent manner in Xenopus interphase extracts, neither human nor mouse p27<sup>kip1</sup> are degraded in Xenopus extracts with or without nuclei. Considering the high homology of the vertebrate CKIs, why then does Xic1 appear to be degraded so differently compared with p27<sup>kip1</sup>? The differences observed to date may be explained by the structural differences that exist between the vertebrate CKI molecules, their possible functional differences or experimental differences. It is likely that Xic1 possesses additional functions compared with p27<sup>kip1</sup> as suggested by the homology of the C-terminal Xic1 and p21<sup>Cip1</sup> sequences, as well as the observed binding of Xic1 to PCNA (30, 31). The unique features of Xic1 may result in altered regulation by protein turnover for the Xenopus CKI compared with the mammalian CKI. Additionally, Xic1 may be degraded along more than one pathway as suggested by Swanson et al. (33), one dependent upon and one independent of phosphorylation by CDK2-cyclin E. These alternate degradation pathways may be dictated by the binding status of Xic1 to CDK2-cyclin E. p21<sup>Cip1</sup> has been shown to be degraded by the proteasome only in the nucleus, suggesting that the degradation of Xic1 may resemble the degradation of p21<sup>Cip1</sup>, although p21<sup>Cip1</sup> degradation does not require ubiquitination (34). With regard to the methodology used in mammalian versus Xenopus degradation experiments, one chief difference exists that may potentially account for some discrepancies observed between p27<sup>kip1</sup> and Xic1 degradation. The Xenopus extract measures S phase events that occur within early embryonic cell cycles, whereas mammalian extracts measure S phase events that occur largely in differentiated somatic cells. Further studies will be required to clarify the differences observed in vertebrate CKI degradation and to fully understand the mechanisms regulating the events of DNA replication initiation.

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