Cell

The Processivity of Multiubiquitination by the APC Determines the Order of Substrate Degradation

Michael Rape,[1] Sashank K. Reddy,[1] and Marc W. Kirschner[1,*]
[1] Department of Systems Biology, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA
*Contact: marc@hms.harvard.edu
DOI 10.1016/j.cell.2005.10.032

SUMMARY

The anaphase-promoting complex (APC) coordinates mitosis and G1 by sequentially promoting the degradation of key cell-cycle regulators. Following the degradation of its substrates in G1, the APC catalyzes the autoubiquitination of its E2 UbcH10. This stabilizes cyclin A and allows it to inactivate APC^{Cdh1}. How the APC establishes this complex temporal sequence of ubiquitinations, referred to as substrate ordering, is not understood. Here we show that substrate ordering depends on the relative processivity of substrate multiubiquitination by the APC. Processive substrates obtain ubiquitin chains in a single APC binding event. The multiubiquitination of distributive substrates requires multiple rounds of APC binding, which render it sensitive to lower APC concentrations, competition by processive substrates, and deubiquitination. Consequently, more processive substrates are preferentially multiubiquitinated in vitro and degraded earlier in vivo. The processivity of multiubiquitination is strongly influenced by the D box within the substrate, suggesting that substrate ordering is established by a mechanism intrinsic to APC and its substrates and similar to kinetic proofreading.

INTRODUCTION

The unidirectional progression through the eukaryotic cell cycle is accomplished by the degradation of key cell-cycle regulators. Both the high specificity and the correct timing of these degradation events are crucial for cell-cycle progression. In eukaryotes, protein degradation is in most cases initiated by the attachment of ubiquitin chains, which mark proteins for destruction by the 26S proteasome (reviewed in Pickart, 2001). After its ATP-dependent activation by E1, ubiquitin is transferred to ubiquitin-conjugating enzymes (E2s). Ubiquitin ligases (E3s) then recruit ubiquitin-charged E2s and substrates and promote the formation of an isopeptide bond between ubiquitin and a lysine residue in the substrate. Ubiquitin chains are generated when lysine residues of previously conjugated ubiquitin molecules are used as acceptors. Thus, by recruiting substrates, E3s function as specificity factors for multiubiquitination.

The E3 anaphase-promoting complex/cyclosome (APC) is a central coordinator of cell-cycle progression in mitosis and G1 (reviewed in Peters, 2002). The APC is activated in early mitosis through cyclin B/Cdk1-dependent phosphorylation and the binding of its activator Cdc20. Inhibition of the APC by the spindle-assembly checkpoint ensures that sister-chromatid separation is initiated only after all kinetochores have been correctly attached to the mitotic spindle. During anaphase, Cdc20 is replaced by a homologous activator, Cdh1. By promoting the sequential degradation of key regulators, APC^{Cdh1} orchestrates exit from mitosis and events in G1. The APC is inactivated before entry into S phase by degradation of its E2 UbcH10 and by the E2F-dependent expression of the inhibitors cyclin A and Emi1 (Lukas et al., 1999; Hsu et al., 2002; Rape and Kirschner, 2004).

The correct sequence of APC-mediated ubiquitinations, referred to as substrate ordering, ensures the orderly progression of mitosis and G1. Substrates are initially recognized by Cdc20 and Cdh1, but interactions between substrates and the APC itself may also be important (Burton et al., 2005; Kraft et al., 2005; Yamano et al., 2004). A short stretch of amino acids within substrates, called the D box, promotes recognition by APC^{Cdc20} (Glotzer et al., 1991). APC^{Cdh1} additionally associates with proteins containing a KEN box (Pfleger and Kirschner, 2000). Although most of its substrates contain D boxes, KEN boxes, or both, APC^{Cdh1} further discriminates between them and catalyzes their multiubiquitination in a sequential manner: Cdc20 is ubiquitinated shortly after APC^{Cdh1} activation in anaphase, Plk1 and Aurora A are ubiquitinated later after they have functioned in telophase and cytokinesis, and UbcH10 autoubiquitination is promoted in G1 after these substrates have been degraded.

Additionally, the degradation of UbcH10 stabilizes cyclin A, while APC^{Cdh1} substrates, such as securin or geminin, can still be degraded. How APC^{Cdh1} discriminates between substrates is not understood.

Both models intrinsic and extrinsic to APC could explain substrate ordering. An extrinsic control of APC-substrate interactions could rely on enzymes that modify substrates and increase their affinity toward the APC. This has been described for cullin-based ubiquitin ligases, which depend on previous modifications such as phosphorylation or hydroxylation for substrate binding (Cardozo and Pagano, 2004). Alternatively, the APC might intrinsically control substrate ordering by discriminating between substrates in the absence of modifications.

Here we provide evidence that substrate ordering can be generated by mechanisms intrinsic to APC and its substrates. Specifically, we find that different substrates acquire their ubiquitin chain with varying degrees of processivity and that this correlates with the temporal order of their destruction. While processive substrates can obtain their ubiquitin chain within a single APC binding event, distributive substrates frequently dissociate from APC^{Cdh1} during multiubiquitination. This renders their multiubiquitination susceptible to competition by more processive substrates and to deubiquitination. The differences in processivity are sufficient to recapitulate substrate ordering in vitro, suggesting that the APC can discriminate between substrates in the absence of other regulatory factors.

RESULTS

APC Substrates Differ in the Processivity of Multiubiquitination

The degradation of UbcH10 during G1 stabilizes cyclin A so that it can accumulate before S phase and complete the inactivation of APC^{Cdh1} (Rape and Kirschner, 2004). At the same time, other APC^{Cdh1} substrates, such as securin or geminin, are still degraded. To explore the question of why securin and geminin, but not cyclin A, are degraded in the presence of low UbcH10 levels, we studied in greater detail the kinetics of their APC^{Cdh1}-dependent multiubiquitination. Whereas geminin and securin rapidly obtained full-length ubiquitin chains, the multiubiquitination of cyclin A was notably slower, and ubiquitin chains attached to cyclin A gradually increased in length over time (Figures 1A and 1B). The slow multiubiquitination of cyclin A was not due to the absence of its binding partner Cdk2 since cyclin A purified in an active complex with Cdk2 was ubiquitinated with identical kinetics. The delay in the formation of full-length ubiquitin chains attached to cyclin A was also obvious with recombinant proteins, indicating that it was not caused by some peculiarity of in vitro-translated substrates (Figure 1C). Furthermore, cyclin A was multiubiquitinated with slower kinetics when the E2 UbcH5α was used (see Figures S1A and S1B in the Supplemental Data available with this article online).

The differences in the kinetics of multiubiquitination between cyclin A and securin were amplified when the concentration of APC^{Cdh1} was reduced. Interestingly, at low APC^{Cdh1} concentrations, cyclin A was preferentially monoubiquitinated in the time course of this experiment, indicating that, under these conditions, the elongation of ubiquitin chains attached to cyclin A is unfavorable (Figure 1D). By contrast, securin was modified with full-length ubiquitin chains even at low concentrations of APC^{Cdh1}. Accordingly, the degradation of cyclin A in G1 extracts required higher APC^{Cdh1} concentrations than the degradation of securin. The immunodepletion of APC^{Cdh1} to about 40% of its level in early G1 extracts strongly stabilized cyclin A, while securin was still degraded (Figure 1E). The formation of full-length ubiquitin chains on cyclin A therefore occurs with slower kinetics and requires higher concentrations of APC^{Cdh1} than the respective modification of securin or geminin.

To understand the reason for the slow multiubiquitination of cyclin A compared to geminin or securin, we compared their affinities toward APC^{Cdh1}. Surprisingly, when we measured the kinetics of monoubiquitination using methylubiquitin, we did not find significant differences among cyclin A, geminin, and securin, indicating that all proteins are rapidly recognized by APC^{Cdh1} (Figures 2A and 2B). Additionally, purified cyclin A, geminin, and securin were equally efficient as competitors of the monoubiquitination of radiolabeled cyclin A, indicating that they have comparable K_i values (Figure 2D). This suggests that the slow multiubiquitination of cyclin A by APC^{Cdh1} is not caused by a low affinity of cyclin A toward APC^{Cdh1}. This is consistent with the function of cyclin A as an inhibitor of APC^{Cdh1}, which should bind with high affinity.

Alternatively, differences in the kinetics of multiubiquitination could be caused by differences in processivity. The processivity of multiubiquitination describes the number of ubiquitin molecules that are attached to substrates during a single APC binding event (Carroll and Morgan, 2002). Very processive substrates might obtain full ubiquitin chains in a single binding event, while more distributive substrates would have to associate with the APC multiple times to achieve full-length multiubiquitination. To compare the processivity of multiubiquitination of different APC^{Cdh1} substrates, we prebound radiolabeled geminin and cyclin A to APC^{Cdh1}. We then initiated their ubiquitination by providing ATP and E1 in the presence of excess unlabeled competitor substrate. The competitor inhibits the rebinding of radiolabeled proteins that dissociate from APC^{Cdh1} during the reaction and thereby limits their ubiquitination to the initial APC binding event. Under these conditions, distributive substrates might at best be monoubiquitinated, whereas processive substrates might obtain complete ubiquitin chains. When analyzed in this assay, cyclin A was modified with very few ubiquitin molecules (Figure 2C). By contrast, geminin acquired ubiquitin chains that were almost indistinguishable from reactions performed in the absence of competitor (Figure 2E). Securin likewise obtained longer ubiquitin chains in this assay (see below). APC^{Cdh1} therefore catalyzes the multiubiquitination of securin and geminin processively but that of cyclin A more distributively.

We subsequently examined whether cyclin A repeatedly dissociates from APC^{Cdh1} at steps following its first

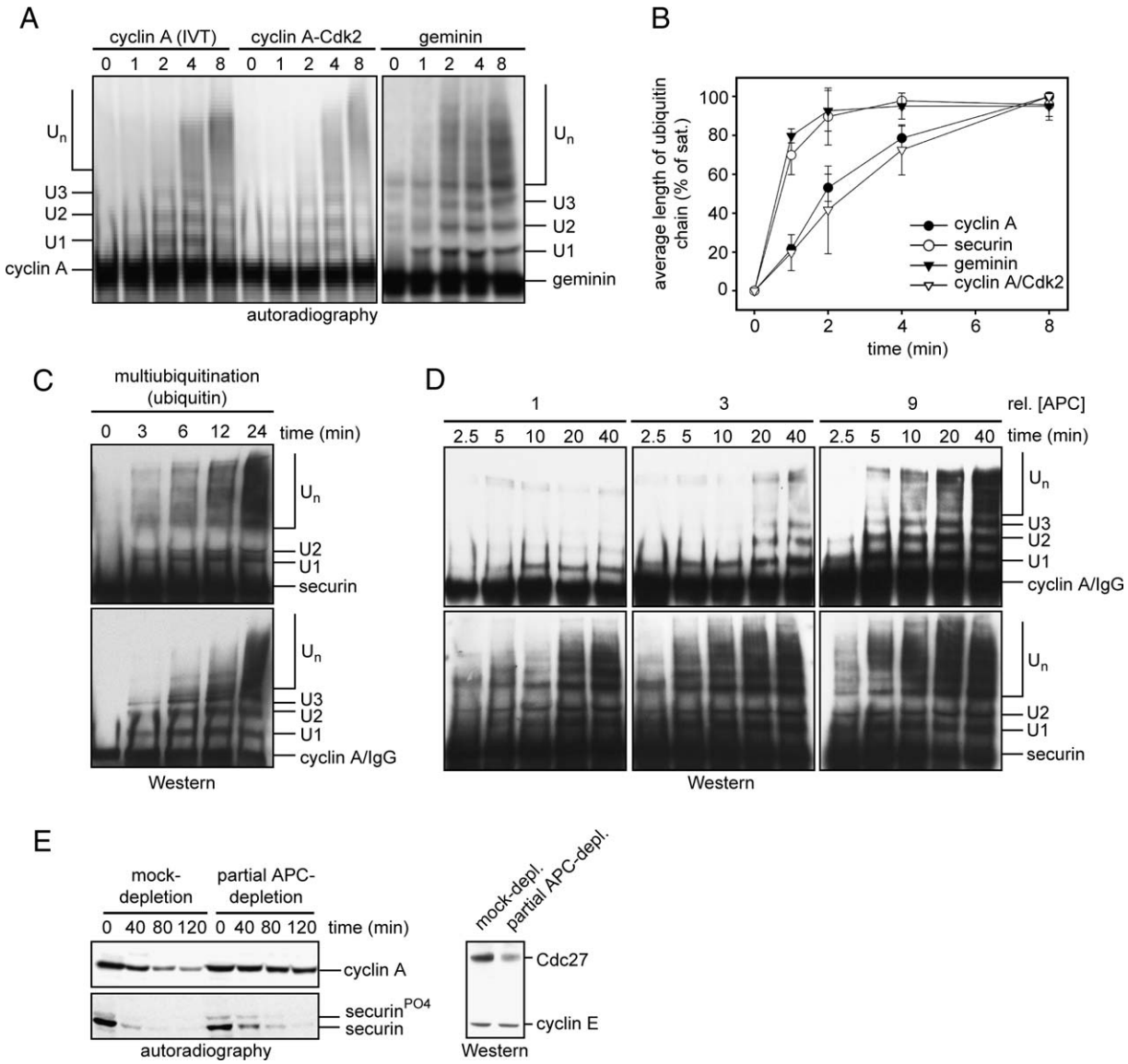


Figure 1. Cyclin A Obtains Ubiquitin Chains with Slow Kinetics

(A) Kinetics of cyclin A and geminin multiubiquitination. ^{35}S-labeled cyclin A and geminin were used after IVT; cyclin A was also purified by binding to Cdk2. The APC^{Cdh1}-dependent multiubiquitination in the presence of ubiquitin was analyzed by autoradiography. U1–U3, mono- or oligoubiquitinated species; U_n, ubiquitin chains.

(B) Quantitative analysis of multiubiquitination. The weighed average length of ubiquitin chains was calculated from intensities as shown in Figure 1A and plotted relative to the length obtained at saturation. Three experiments were used to calculate the standard error.

(C) Kinetics of multiubiquitination of purified securin and cyclin A. Recombinant securin and cyclin A were ubiquitinated by APC^{Cdh1} and analyzed by Western blotting.

(D) Titration of APC^{Cdh1}. Recombinant cyclin A and securin were ubiquitinated in the presence of increasing concentrations of APC^{Cdh1}. The reactions were analyzed by Western blotting.

(E) APC^{Cdh1} was partially depleted from G1 extracts by anti-Cdc27 beads. Depleted and control extracts were then used to measure the degradation of ^{35}S-labeled cyclin A and securin. The reactions were analyzed by autoradiography. The extent of the depletion is shown by Western blotting against Cdc27 and cyclin E.

ubiquitination. We used UBA-domain-containing proteins (UBAs), which preferentially bind ubiquitin chains, but not monoubiquitinated proteins, to capture substrates that have dissociated from APC^{Cdh1} with short ubiquitin chains (Raasi et al., 2004). The UBA domains of hHR23A bind both multi-ubiquitinated cyclin A and geminin once the ubiquitinated proteins have dissociated from APC^{Cdh1}, but not the respective monoubiquitinated proteins (Figure S2A). Significantly, when these UBAs were added to APC^{Cdh1}-dependent ubiquitinations, they reduced the length of ubiquitin chains

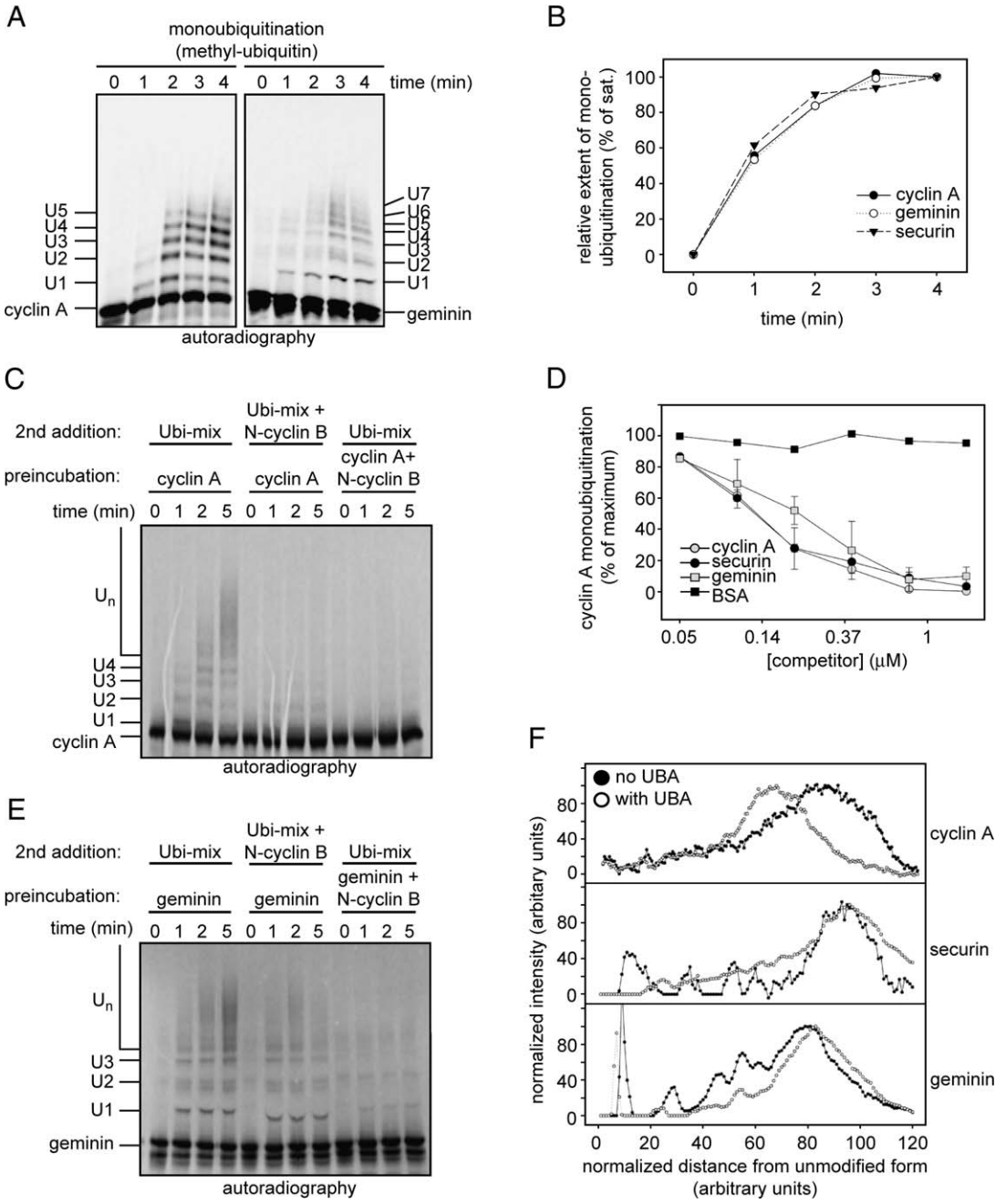


Figure 2. The Multiubiquitination of Cyclin A Is Distributive

(A) Kinetics of APC^{Cdh1}-dependent monoubiquitination. ^{35}S-labeled cyclin A and geminin were ubiquitinated in the presence of methylubiquitin.

(B) Quantitation of APC^{Cdh1}-dependent monoubiquitination. Depicted is the weighed average length of modifications normalized to the modification at saturation.

(C) Cyclin A is a distributive APC^{Cdh1} substrate. ^{35}S-labeled cyclin A was prebound to APC^{Cdh1}. Its multiubiquitination was started by addition of ATP and E1. In the second reaction, an excess of the competitor N-cyclin B was added simultaneously to ATP to limit ubiquitination of cyclin A to the initial binding event. In the third reaction, N-cyclin B was added during prebinding as a negative control. The reactions were analyzed by autoradiography. U1–U4, mono- or oligoubiquitinated cyclin A; U_n, ubiquitin chains.

(D) Cyclin A, geminin, and securin have similar K_i values for the competition of cyclin A monoubiquitination by APC^{Cdh1}. Purified proteins were used as competitors of APC^{Cdh1}-dependent monoubiquitination of ^{35}S-labeled cyclin A. The modification of cyclin A compared to reactions in the absence of competitor was quantitated and plotted against the competitor concentration. The standard error was calculated from three experiments.

(E) Geminin is a processive APC^{Cdh1} substrate. The processivity of APC^{Cdh1}-catalyzed ubiquitination of ^{35}S-labeled geminin was analyzed as before with cyclin A. Full-length ubiquitin chains were formed even when N-cyclin B was added at the start of the ubiquitination.

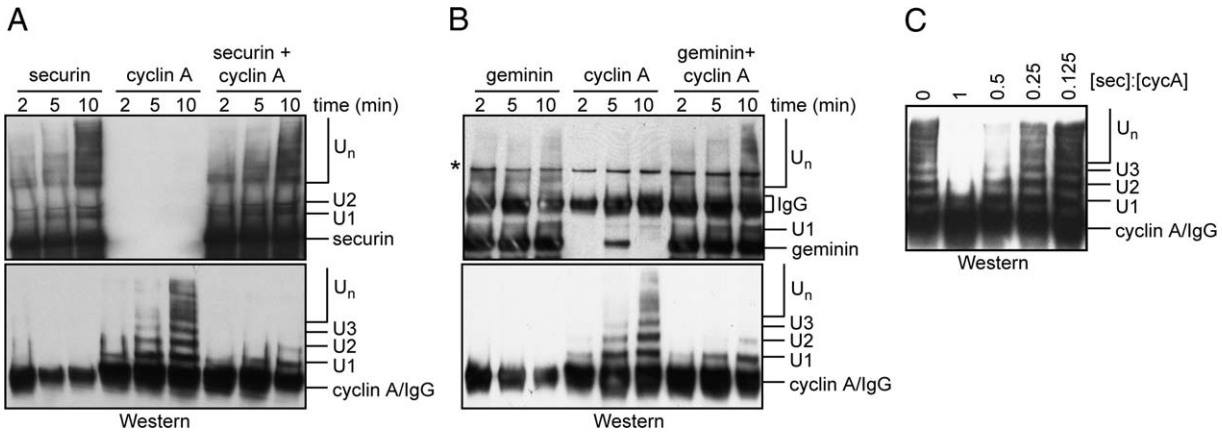


Figure 3. The Preferential Stabilization of Cyclin A Can Be Reconstituted in a Purified System

(A) Securin is preferentially multiubiquitinated by APC^{Cdh1} compared to cyclin A. The multiubiquitination by APC^{Cdh1} of recombinant securin and cyclin A alone or of both proteins simultaneously was analyzed by Western blotting.

(B) Geminin is preferentially multiubiquitinated by APC^{Cdh1} compared to cyclin A. The multiubiquitination of purified geminin and cyclin A was analyzed either alone or after the proteins were combined. The asterisk marks a crossreactive band of the antibody.

(C) The extent of cyclin A multiubiquitination depends on the concentration of competing securin. Cyclin A and decreasing concentrations of securin were combined and incubated with APC^{Cdh1}. The reactions were analyzed by Western blotting.

attached to cyclin A but not to geminin or securin (Figure 2F; Figure S2B). This indicates that cyclin A, but not geminin or securin, dissociated from APC^{Cdh1} with ubiquitin chains of intermediate length. Consistently, UBAs sequestered partially ubiquitinated cyclin A in G1 extracts, whereas they did not affect securin or geminin degradation (Figure S2C). This strongly suggests that cyclin A continuously shuttles on and off the APC^{Cdh1} during its multiubiquitination. The multiubiquitination of cyclin A is therefore much less processive than that of geminin or securin.

Differences in the processivity of multiubiquitination would be mitigated if APC^{Cdh1} preassembled ubiquitin chains on the active site of UbcH10 and transferred them as complete entities onto substrates, as was suggested for HECT E3s (Verdecia et al., 2003). This, however, is unlikely for the RING E3 APC^{Cdh1}. First, the average length of cyclin A-attached ubiquitin chains gradually increases over time, which is not expected if ubiquitin chains are transferred as one entity. Second, preincubation of APC^{Cdh1} with E1, E2, and ubiquitin prior to substrate addition did not accelerate the multiubiquitination of cyclin A, although it should allow the preformation of ubiquitin chains (data not shown). Third, ubiquitin chains linked to the active-site cysteine of UbcH10 in a DTT-sensitive fashion were not observed, presumably because binding of UbcH10 to substrate-free APC leads to autoubiquitination of UbcH10 on lysine residues. Finally, cyclin A is modified in single binding assays with few ubiquitin molecules but not with chains (Figure 2C). Therefore, APC^{Cdh1} elongates substrate-linked chains by adding ubiquitin monomers, allowing it to exploit differences in processivity as a means of discriminating substrates.

Indeed, the differences in the kinetics and processivity of multiubiquitination tightly correlate with the stability of the proteins in G1 when securin and geminin are degraded, but not cyclin A. If these differences are sufficient for APC^{Cdh1} to discriminate between these substrates, we should be able to reconstitute the preferential stabilization of cyclin A in a purified system. Specifically, we predict that more processive substrates would outcompete cyclin A for multiubiquitination since they acquire their ubiquitin chain in fewer APC binding events. To test this notion, we purified cyclin A, securin, and geminin and subjected them to APC^{Cdh1}-dependent multiubiquitination. Importantly, when either securin or geminin was combined with cyclin A in this reaction, the modification of cyclin A was limited to monoubiquitination (Figures 3A and 3B). The high abundance of the monoubiquitinated species shows that cyclin A can associate with APC^{Cdh1} in the presence of more processive competitors but that these competitors inhibit the rebinding required for multiubiquitination. Accordingly, when the concentration of securin is reduced, the length of cyclin A-attached ubiquitin chains gradually increased (Figure 3C). Neither the multiubiquitination of securin nor the multiubiquitination of geminin was inhibited by cyclin A. Similar results were observed when UbcH5α was used as E2 (Figure S1C). Thus, the APC^{Cdh1} can achieve substrate ordering in vitro by preferentially promoting the multiubiquitination of more processive substrates.

(F) Cyclin A shuttles on and off APC^{Cdh1} during its multiubiquitination. The multiubiquitination of ^{35}S-labeled cyclin A, geminin, and securin by APC^{Cdh1} was analyzed in the absence and the presence of UBA domains of hHR23A. The reaction products were resolved by SDS-PAGE and quantitated after autoradiography. The intensities ± UBAs are shown as a function of the distance from the unmodified form.

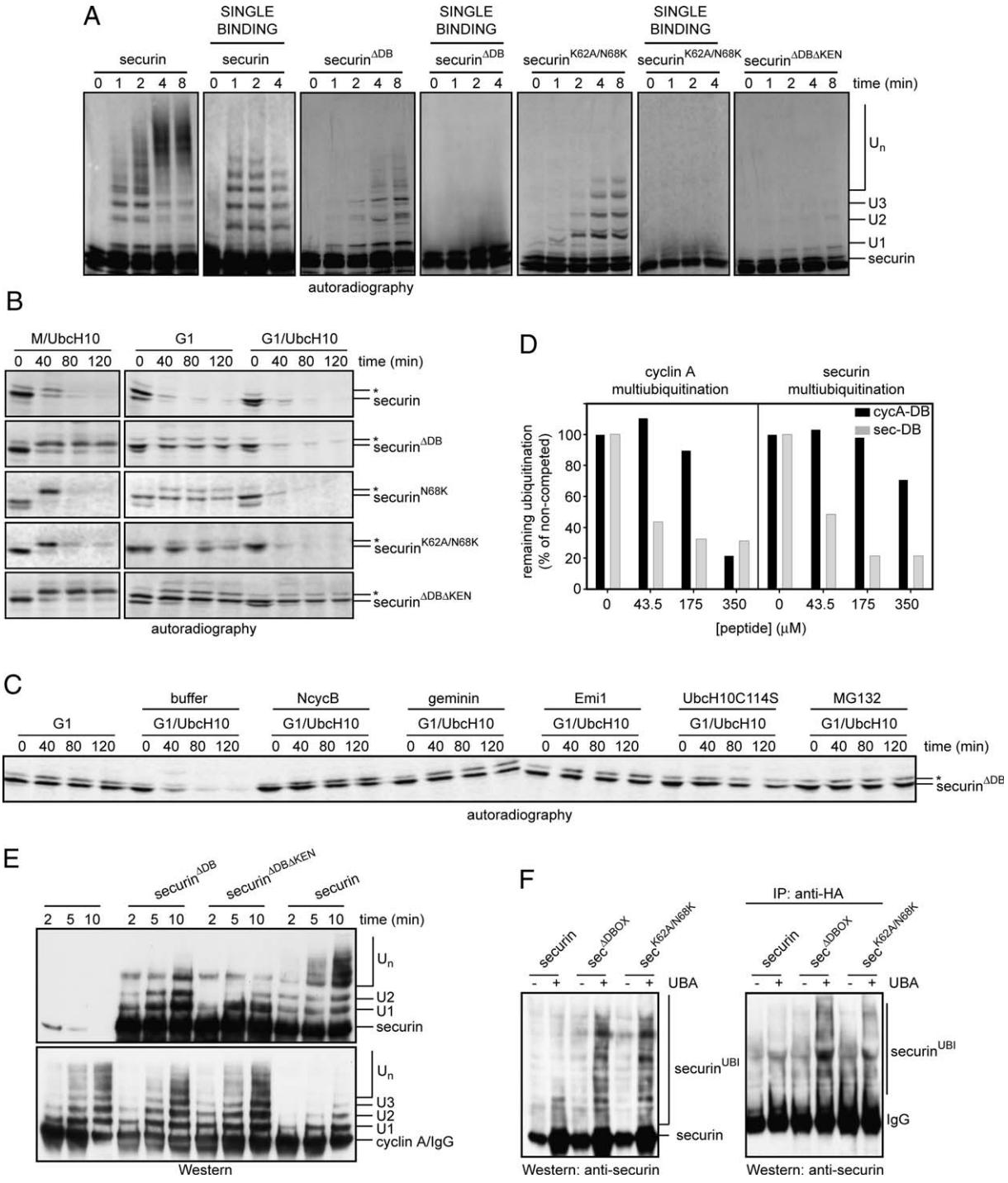


Figure 4. The D Box Regulates Processivity of Multiubiquitination and Substrate Ordering

(A) Kinetics of multiubiquitination of ^{35}S-labeled securin, securin$^{\Delta DB}$, securin$^{K62A/N68K}$, and securin$^{\Delta DB\Delta KEN}$ by APC^{Cdh1}. Time courses of multiubiquitination and single binding assays (where indicated) are shown. The reactions were analyzed by autoradiography. U1–U3, mono- or oligoubiquitinated securing; U_n, ubiquitin chains.

(B) Kinetics of degradation of securin, securin$^{\Delta DB}$, securin^{N68K}, securin$^{K62A/N68K}$, and securin$^{\Delta DB\Delta KEN}$. ^{35}S-labeled proteins were incubated with UbcH10-treated extracts of nocodazole-arrested cells (APC^{Cdc20}-dependent degradation) or with extracts of G1 cells (APC^{Cdh1}-dependent degradation). G1 extracts were supplemented with UbcH10 were indicated. The reactions were analyzed by autoradiography. The asterisk marks phosphorylated securin.

(C) Degradation of securin$^{\Delta DB}$ depends on APC^{Cdh1} and the proteasome. The degradation of securin$^{\Delta DB}$ in G1 extracts was analyzed as before, and purified APC^{Cdh1} substrates N-cyclin B1 and geminin, the APC inhibitor Emi1, inactive E2 UbcH10^{C114S}, or the proteasome inhibitor MG132 was added. UbcH10 was added where indicated.

Recognition of the D Box Is Essential for Processive Multiubiquitination by APC^{Cdh1}

The processivity of multiubiquitination and substrate ordering should be profoundly influenced by sequence motifs that stabilize the interaction of substrates with the APC. Such a motif might be the D box of substrates, which is recognized by both Cdh1 and APC (Burton et al., 2005; Carroll et al., 2005; Kraft et al., 2005; Yamano et al., 2004). Accordingly, cyclin A contains a cryptic D box, which, unlike D boxes of other substrates, does not confer APC-dependent degradation when grafted into other proteins (Klotzbucher et al., 1996).

We first addressed the role of the D box for the processivity of multiubiquitination by mutating the D box of securin (securin$^{\Delta DB}$; R^{61}KAL to A^{61}KAA). Whereas wild-type securin was rapidly multiubiquitinated by APC^{Cdh1}, securin$^{\Delta DB}$ was primarily monoubiquitinated and only slowly obtained short ubiquitin chains (Figure 4A). Importantly, securin$^{\Delta DB}$ was not ubiquitinated in single binding assays, which is indicative of a high dissociation rate from the APC. Consistently, the addition of UBAs shortened ubiquitin chains on securin$^{\Delta DB}$ but not on wild-type securin (data not shown). Expression of UBAs in vivo stabilized ubiquitin chains on securin$^{\Delta DB}$ but not on securin (Figure 4F); these ubiquitinated species could be immunoprecipitated by UBAs. Thus, although securin$^{\Delta DB}$ is still recognized by APC^{Cdh1} via its KEN box, it now acts as a distributive APC substrate. This implies that the D box is required for processive multiubiquitination by APC^{Cdh1}.

More distributive substrates, such as cyclin A, require higher concentrations of active APC^{Cdh1} to be degraded. In extracts of late G1 cells, when the bulk of UbcH10 has been degraded, cyclin A is stable. Importantly, the degradation of securin$^{\Delta DB}$ in G1 extracts is similar to cyclin A. Securin$^{\Delta DB}$ was stable in G1 extracts but was rapidly degraded after the addition of UbcH10 in an APC- and proteasome-dependent fashion (Figures 4B and 4C). Thus, the recognition of the D box by APC^{Cdh1} is required for the processive ubiquitination of securin and its rapid degradation.

To examine whether the D box determines the differences in processivity between cyclin A and securin, we used peptides encompassing the respective D box sequences as competitors of multiubiquitination (Figure 4D; Figure S3B). In APC^{Cdh1}-dependent multiubiquitinations of cyclin A and securin, the D box peptide of securin was a more potent competitor than the D box peptide of cyclin A. Thus, the D box of securin is recognized by APC^{Cdh1} more efficiently than the D box of cyclin A, which correlates with the higher processivity of securin multiubiquitination.

We subsequently introduced two point mutations into securin so that its D box resembled that of cyclin A (securin$^{K62A/N68K}$; Figure S3A). Intriguingly, securin$^{K62A/N68K}$ was slowly ubiquitinated in a distributive manner (Figure 4A). In single binding assays, securin$^{K62A/N68K}$ was modified with very few ubiquitin molecules (Figure 4A). Expression of UBAs in vivo stabilized ubiquitin chains on securin$^{K62A/N68K}$ but not on securin (Figure 4F). Additionally, its degradation in G1 extracts required addition of UbcH10 (Figure 4B). Contrary to securin$^{\Delta DB}$, securin$^{K62A/N68K}$ was still recognized by APC^{Cdc20}, as indicated by its degradation in mitotic extracts supplemented with UbcH10. Thus, by changing its D box to the respective D box sequence of cyclin A, securin is converted into a more distributive substrate. This indicates not only that the D box is necessary for processive multiubiquitination but that its nature can determine the extent of processivity.

These findings allowed us to compare three substrates to evaluate the importance of the D box and processivity for substrate ordering: securin, which contains a potent D box and is multiubiquitinated processively; the more distributive cyclin A, which has a cryptic D box; and securin$^{\Delta DB}$, the mutated D box of which causes its very distributive ubiquitination. As seen before, the simultaneous incubation of securin and cyclin A with APC^{Cdh1} effectively restricts cyclin A ubiquitination to monoubiquitination (Figure 4E). By contrast, securin$^{\Delta DB}$ hardly affects the multiubiquitination of cyclin A. Cyclin A, however, now effectively inhibits the multiubiquitination of securin$^{\Delta DB}$. Thus, the D box of the substrate determines both the processivity of multiubiquitination and substrate ordering in the purified system.

Substrate Ordering Is Not Specifically Regulated by Cdc20 or Cdh1

Substrates are recognized by both Cdh1 and the APC (Burton et al., 2005; Kraft et al., 2005; Yamano et al., 2004). If the latter interaction is crucial for processivity, it should not be important whether Cdc20 or Cdh1 targets a substrate to the APC, which could be tested by comparing the kinetics of multiubiquitination for proteins recognized by both APC^{Cdc20} and APC^{Cdh1}. We purified active APC^{Cdc20} from extracts of cells arrested in mitosis by nocodazole. These extracts contain APC^{Cdc20}, which is inhibited by the spindle-assembly checkpoint and does not promote the degradation of its substrates (Figure 5A). However, addition of UbcH10 activated APC^{Cdc20} and caused the degradation of APC^{Cdc20} substrates in those extracts. Despite the complete degradation of cyclin B1, APC^{Cdh1} was not activated, as indicated by the following observations: endogenous

(D) The D box of securin is recognized more efficiently by the APC^{Cdh1} than the D box of cyclin A. Peptides encompassing the D box of securin and cyclin A were titrated into the APC^{Cdh1}-dependent multiubiquitination of ^{35}S-labeled securin or cyclin A. The reaction products were analyzed by autoradiography and quantitated as a function of peptide concentration.

(E) The processivity of multiubiquitination determines substrate ordering. The multiubiquitination by APC^{Cdh1} of recombinant cyclin A alone or together with securin$^{\Delta DB}$, securin$^{\Delta DB\Delta KEN}$, or securin was analyzed by Western blotting. Only securin blocks cyclin A multiubiquitination.

(F) D box mutants of securin behave as distributive substrates in vivo. Securin, securin$^{\Delta DB}$, and securin$^{K62A/N68K}$ were expressed in HeLa cells. Coexpression of the UBA-domain protein ^{HA}hHR23A stabilized ubiquitin chains on the distributive mutations but not on wild-type securin; the ubiquitinated species coimmunoprecipitated with ^{HA}hHR23A (right panel).

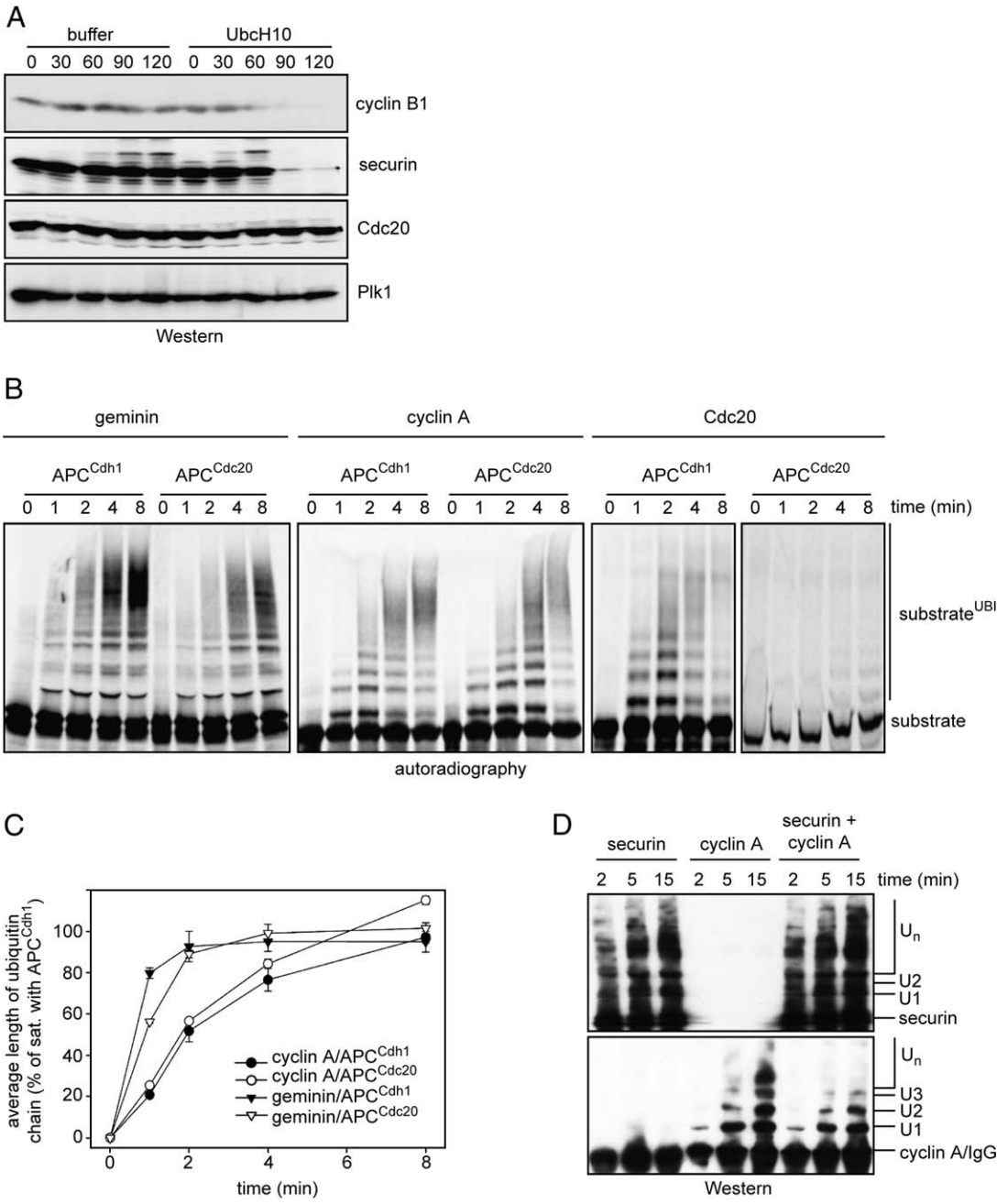


Figure 5. The Cofactor Does Not Determine Processivity of Multiubiquitination and Substrate Ordering

(A) APC^{Cdc20} is activated by addition of UbcH10 to extracts of nocodazole-arrested cells. The degradation of endogenous proteins in extracts after addition of buffer or UbcH10 was monitored by Western blotting. The APC^{Cdc20} substrates cyclin B1 and securin are degraded, whereas the APC^{Cdh1}-specific substrates Cdc20 and Plk1 remain stable.

(B) Kinetics of multiubiquitination of ^{35}S-labeled geminin, cyclin A, and Cdc20 by APC^{Cdc20} and APC^{Cdh1}. The reaction products were analyzed by autoradiography.

(C) Quantitation of the weighed average length of the respective ubiquitin chains on cyclin A and geminin from three experiments as shown in Figure 5B.

(D) APC^{Cdc20} shows the same substrate ordering as APC^{Cdh1}. Recombinant cyclin A or securin was incubated with APC^{Cdc20} either alone or together. The ubiquitination of the proteins was analyzed by Western blotting.

APC^{Cdh1} substrates, such as Cdc20 and Plk1, remained stable in UbcH10-treated mitotic extracts (Figure 5A); exogenously added Cdc20 and Plk1 were stable after incubation of mitotic extracts with UbcH10 (data not shown); and neither Cdc20 nor Plk1 was ubiquitinated by APC purified from UbcH10-treated mitotic extracts (see Figure 5B).

We then compared active APC^{Cdc20} from UbcH10-treated extracts to APC^{Cdh1}. As expected, APC^{Cdc20} multiubiquitinated cyclin A and geminin, whereas it hardly modified the APC^{Cdh1}-specific substrate Cdc20 (Figure 5B). Importantly, for both cyclin A and geminin, no significant differences in the kinetics or in the efficiency of multiubiquitination were observed when APC^{Cdc20} and APC^{Cdh1} were compared (Figure 5C). Additionally, substrate ordering in the purified system was not affected by whether the cofactor Cdc20 or Cdh1 was used (Figure 5D). Consistent with our previous observations with APC^{Cdh1}, the ubiquitination of cyclin A by APC^{Cdc20} was restricted to short ubiquitinations in the presence of the more processive APC-substrate securin. The multiubiquitination of securin was not affected by the simultaneous presence of cyclin A. Thus, the processivity of multiubiquitination and the resulting substrate ordering are independent of whether Cdc20 or Cdh1 recruits the substrate, indicating that interactions between substrate and core APC are important.

Deubiquitination and Distributive Autoubiquitination Delay UbcH10 Degradation

To examine whether differences in processivity are used as a general means of establishing substrate ordering, we turned to UbcH10. Although its function as an E2 requires a high affinity toward the APC, UbcH10 is stabilized until other substrates have been degraded in G1. This is accomplished in part by the inhibition of UbcH10 autoubiquitination by APC substrates (Rape and Kirschner, 2004). However, if its autoubiquitination were processive, UbcH10 would be degraded prematurely before most APC substrates have been destroyed.

As reported previously, the autoubiquitination of UbcH10 is slow, which in this case is indicative of a low k_{cat} (Rape and Kirschner, 2004). Additionally, ubiquitin chains attached to UbcH10 slowly increase in length over time (Figures 6A and 6B). Accordingly, UbcH10 was only monoubiquitinated in single binding assays (Figure 6C), and addition of UBAs to ubiquitination assays profoundly inhibited the elongation of ubiquitin chains on UbcH10 (Figure 6D). Together, these experiments strongly suggest that the autoubiquitination of UbcH10 is highly distributive, which is consistent with its late degradation in vivo.

The slow autoubiquitination of UbcH10 allowed us to examine its fate after dissociating from the APC with short ubiquitin chains. After starting the autoubiquitination reaction, we added purified securin as a competitor. The addition of securin not only inhibited further ubiquitination of UbcH10 but induced a rapid loss of preformed ubiquitin modifications (Figure 6E). The loss of UbcH10 ubiquitination was not due to proteasomal degradation as it occurred in the presence of the proteasome inhibitor MG132 (data not shown). Rather, it was most likely due to deubiquitinating enzymes (DUBs) present in reticulocyte lysate and in somatic-cell extracts. The deubiquitination of UbcH10 could be inhibited by ubiquitin aldehyde and by NEM, which is consistent with the fact that most DUBs are cysteine proteases (Figure 6F; Amerik and Hochstrasser, 2004). Importantly, deubiquitination was not restricted to UbcH10. It was also observed for other APC^{Cdh1} substrates if their reassociation with the APC^{Cdh1} was blocked by an excess of competing substrates and their degradation by the 26S proteasome was impaired (Figure 6F). Thus, if APC substrates dissociate from APC^{Cdh1} before they are recognized by the 26S proteasome, they are prone to deubiquitination.

This indicates that differences in processivity could be further modulated to establish the correct timing of the degradation of APC substrates. With respect to UbcH10, its distributive autoubiquitination and the possible deubiquitination should make its degradation very sensitive to inhibition by low concentrations of APC^{Cdh1} substrates. We tested this by directly comparing the inhibitory effect of APC substrates on the APC^{Cdh1}-dependent ubiquitination of UbcH10 and geminin. As predicted, low concentrations of purified securin dramatically inhibited UbcH10 autoubiquitination and led to the accumulation of mono- and diubiquitinated UbcH10 (Figure 6G). By contrast, geminin ubiquitination was hardly affected at all competitor concentrations tested. Thus, the distributive autoubiquitination of UbcH10 together with possible deubiquitination upon dissociation suffice to establish a striking sensitivity of UbcH10 degradation to competition by other APC substrates. Differences in processivity therefore contribute to both the correct timing of UbcH10 autoubiquitination and cyclin A stabilization in G1.

APC^{Cdh1} Multiubiquitinates Late Mitotic Substrates with Decreasing Processivity

We finally investigated whether the ubiquitination reactions catalyzed by APC^{Cdh1} in late mitosis are regulated by mechanisms similar to those in G1. We compared the kinetics of the APC^{Cdh1}-catalyzed ubiquitination of Cdc20, Plk1, and Aurora A, which are degraded in that order during mitotic exit (Lindon and Pines, 2004). Interestingly, the kinetics of multiubiquitination were tightly correlated with the relative timing of degradation in the cell cycle. The multiubiquitination of Cdc20, which is the first APC^{Cdh1}-specific substrate to be degraded, was faster than that of Plk1 (Figures 7A, 7B, and 7D). Aurora A, which is degraded later than Cdc20 and Plk1, was ubiquitinated with much slower kinetics (Figures 7C and 7D). When UBAs were added to multiubiquitination reactions, they increasingly inhibited the ubiquitin-chain elongation of Plk1 and Aurora A, suggesting that the respective multiubiquitination reactions are more distributive (Figure 7E; Figure S4). Importantly, both Plk1 and Aurora A were degraded with slow kinetics in G1 extracts, but increasing k_{cat} by addition of UbcH10 and inhibiting deubiquitination by addition of ubiquitin aldehyde converted them into unstable proteins (Figure 7F). Together, these results indicate that differences in the relative processivity of multiubiquitination by APC^{Cdh1} and deubiquitinating enzymes are decisive factors in establishing substrate ordering in late mitosis and G1.

DISCUSSION

The sequential ubiquitination of substrates by the APC is critical for the proper timing of cell-cycle events. A rough ordering of substrates is achieved by the sequential activation of

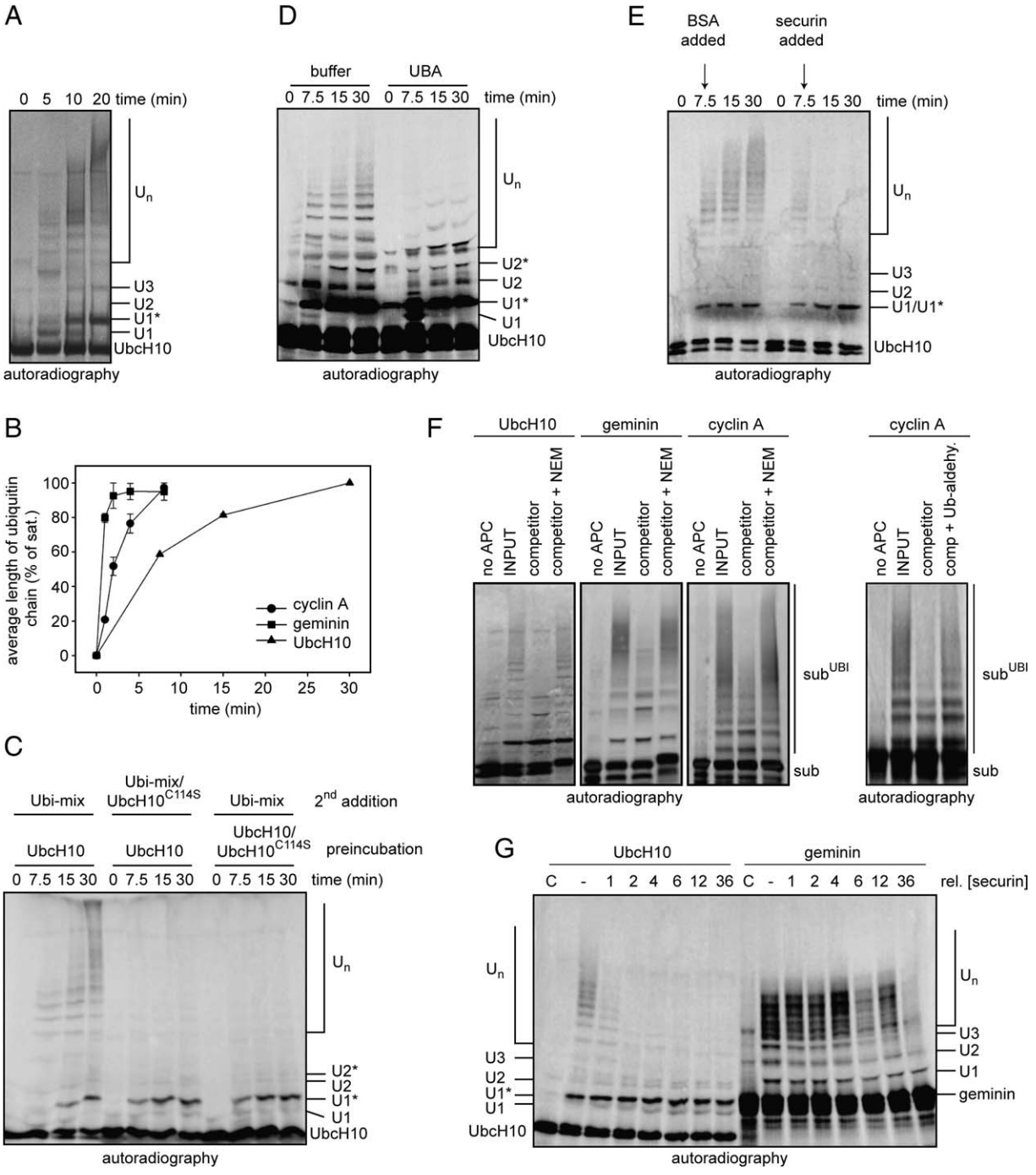


Figure 6. UbcH10 Autoubiquitination Is Distributive and Sensitive to Deubiquitination

(A) Kinetics of autoubiquitination of ^{35}S-labeled UbcH10 by APC^{Cdh1}. Reaction products were analyzed by autoradiography. U1*, monoubiquitinated UbcH10, which is formed independently of APC.

(B) Kinetics of ubiquitin-chain formation on geminin, cyclin A, and UbcH10 from three experiments.

(C) Single binding assay with UbcH10. ^{35}S-labeled UbcH10 was preincubated with APC^{Cdh1} in the presence or absence of excess unlabeled UbcH10^{C114S}. Where indicated, UbcH10^{C114S} was added simultaneously to the start of the ubiquitination to inhibit rebinding of dissociated UbcH10.

(D) UBAs inhibit the elongation of ubiquitin chains on UbcH10 by APC^{Cdh1}. The reaction products were analyzed as before.

(E) Addition of APC substrates during the autoubiquitination of UbcH10 leads to deubiquitination of UbcH10. 7.5 minutes after UbcH10 autoubiquitination was started, either BSA or recombinant securin was added for a further 30 min. The reaction products were analyzed as before.

(F) Substrates are deubiquitinated in reticulocyte lysate if their reassociation with APC^{Cdh1} is inhibited. The APC^{Cdh1}-dependent ubiquitination of UbcH10, geminin, or cyclin A was started. At 15 min, stop buffer (INPUT), N-cyclin B (which inhibits reassociation of dissociated proteins), or N-cyclin B and NEM (to

APC^{Cdc20} and APC^{Cdh1} (reviewed in Peters, 2002). APC^{Cdc20} binds to D box-containing proteins, whereas the subsequently activated APC^{Cdh1} additionally associates with KEN box-containing proteins. However, APC^{Cdh1} ubiquitinates Cdc20, Plk1, Aurora A, and UbcH10 sequentially and can in time stabilize cyclin A. Despite its importance for understanding the cell cycle, the mechanism by which APC^{Cdh1} discriminates between these substrates has remained elusive.

The results of this study support a model in which APC^{Cdh1} establishes substrate ordering based on relative differences in the processivity of multiubiquitination of the various substrates. Very processive substrates, such as securin or geminin, obtain their multiubiquitin chain in a single binding event, while more distributive substrates, such as cyclin A or UbcH10, continuously shuttle on and off the APC (Figure 7G). Differences in processivity can originate from the catalytic rate of multiubiquitination, as might be the case for Plk1, Aurora A, and UbcH10, or from the rate of dissociation from the APC, which may account for the distributive nature of cyclin A multiubiquitination. Irrespective of how processivity is determined, we find a striking correlation between the processivity of multiubiquitination and the relative timing of degradation in the cell cycle: The more processive the multiubiquitination of an APC substrate, the earlier it is degraded relative to the other substrates. The only notable exception, cyclin A, will be discussed below. The timing of degradation of an APC^{Cdh1} substrate may thus be determined by the processivity of its multiubiquitination relative to other substrates.

Importantly, processivity differences allow the APC to generate substrate ordering without prior substrate modification. Distributive APC substrates dissociate frequently from the APC during their multiubiquitination. The probability of quickly rebinding and escaping deubiquitination is determined by the availability of free APC^{Cdh1}, which in turn depends on the total concentration of remaining substrates. Thus, distributive substrates will be multiubiquitinated efficiently only after more processive substrates have already been degraded. This implies that substrate ordering by the APC is self-organizing or "substrate controlled," which may have facilitated its conservation throughout evolution (Georgi et al., 2002).

The regulation of an E3 based on differences in processivity introduces a discrimination step subsequent to the initial recognition of substrates. In case of the APC, this permits the stabilization of substrates that also fulfill important functions on the APC. It is exemplified by the APC-specific E2 UbcH10 and by cyclin A, which, in conjunction with Cdk2, inhibits APC^{Cdh1} at the G1/S transition. Both proteins bind the APC with high affinity, but, due to the distributive nature of their ubiquitinations, they are multiubiquitinated only under specific conditions: UbcH10 undergoes efficient autoubiquitination only after other APC substrates have been degraded, while cyclin A is efficiently multiubiquitinated only in the presence of high E2 concentrations. Thus, catalyzing the multiubiquitination of its substrates with different processivity substantially expands the functional repertoire of the APC.

Regulation of Substrate Ordering by Kinetic Proofreading

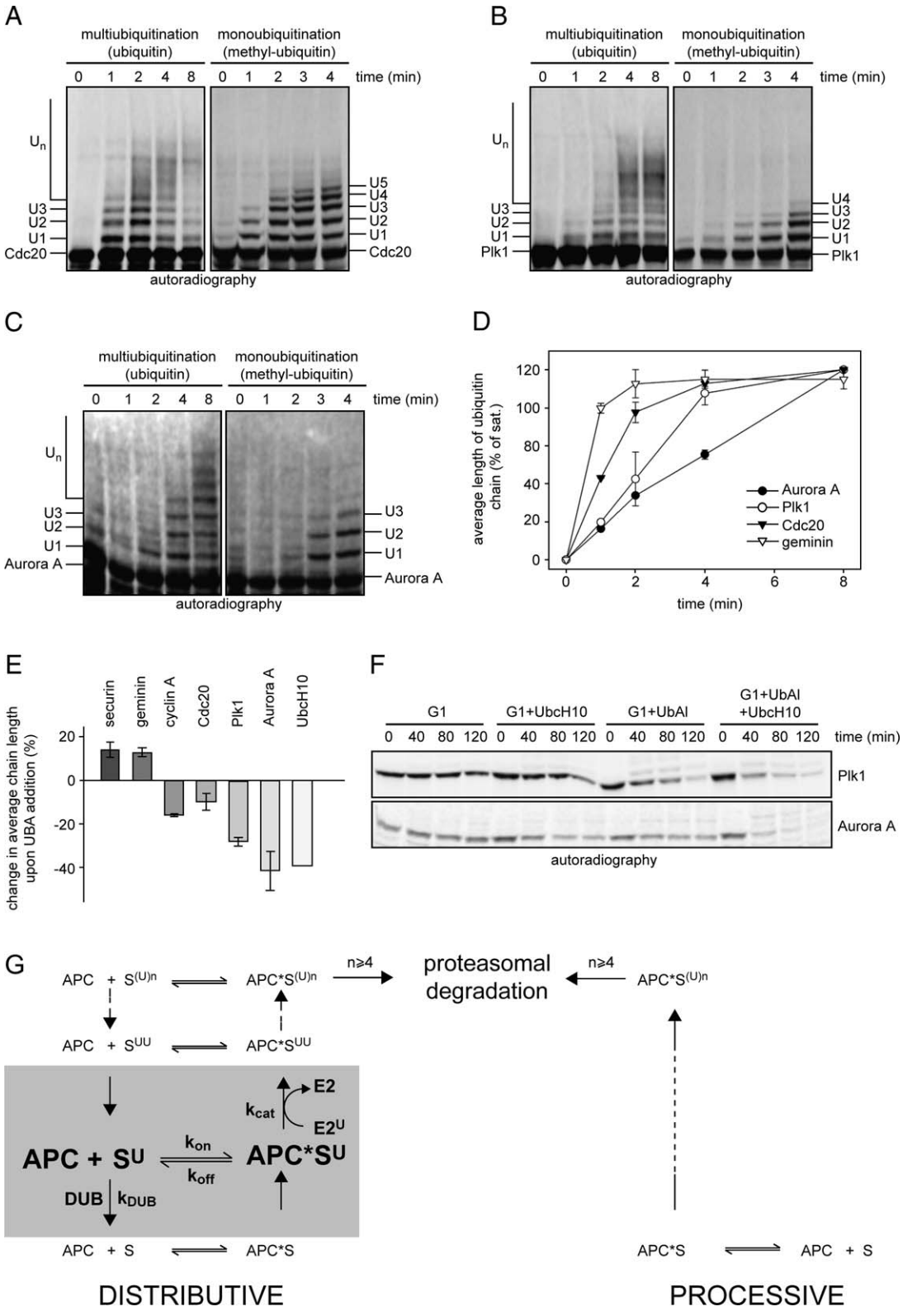
The proposed regulation of substrate ordering by the APC is highly reminiscent of the concept of kinetic proofreading, which was introduced to explain the high accuracy of translation and DNA replication (Hopfield, 1974). During kinetic proofreading, a substrate undergoes a series of modifications, with only the fully modified state being able to produce a signal. If the series of modifications is interrupted before completion, the substrate reverts to its basal state. The repetition of modifications or a series of required reactions is used to amplify small differences in binding affinities or rate constants. During translation, 100-fold differences in the binding affinities of cognate versus noncognate aminoacyl*tRNA*EF-Tu complexes to the ribosome are amplified into a more than 10,000-fold higher probability of incorporation of the correct amino acid into the polypeptide chain. This increase in fidelity is paid for by GTP hydrolysis, which effectively makes dissociation reactions irreversible. Multiubiquitination by APC^{Cdh1} involves the sequential elongation of ubiquitin chains attached to a substrate, and only proteins modified with a tetraubiquitin chain are recognized by the 26S proteasome (Thrower et al., 2000). If substrates dissociate from the APC before they are multiubiquitinated, they can be converted into the basal state by deubiquitination. The more distributive the multiubiquitination of a substrate, the more likely it will undergo proofreading before reaching the tetraubiquitinated state. By exploiting differences in processivity, APC^{Cdh1} could thus amplify differences in affinity or even discriminate between substrates of nearly identical affinities, such as cyclin A and geminin.

In contrast to kinetic proofreading in translation, the dissociation of substrates from the APC does not automatically lead to reversal of all modifications. After dissociation, substrates could either be captured by DUBs or rebind the APC. It is not known whether DUBs act processively and remove the complete ubiquitin chain in a single binding event or whether they compete with the APC at multiple stages. It has also not been clarified whether specific DUBs control the deubiquitination of specific APC substrates and whether these DUBs are regulated in the cell cycle. Irrespective of this potential specificity, DUBs are likely to have profound effects on the timing of degradation by amplifying small differences in the processivity of ubiquitination.

If the DUB activity is constant, the degradation of distributive APC substrates is determined by the availability of active APC. Increasing local APC concentrations might promote

additionally inhibit deubiquitinating enzymes) were added, and the reactions were incubated for 30 min. In the right panel, ubiquitin aldehyde was added instead of NEM.

(G) UbcH10 autoubiquitination is hypersensitive to substrate competition. Increasing concentrations of recombinant securin were used to compete for APC^{Cdh1}-dependent ubiquitination of UbcH10 or geminin. The reaction products were analyzed as before.


A
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time (min)
Aurora A
Plk1
Cdc20
geminin
E
change in average chain length upon UBA addition (%)
securin
geminin
cyclin A
Cdc20
Plk1
Aurora A
UbcH10
20
0
-20
-40
F
G1
G1+UbcH10
G1+UbAl
G1+UbAl +UbcH10
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APC*S(U)n
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APC*SUU
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the ubiquitination of a substrate in a specific compartment, while DUBs would stabilize it elsewhere in the cell. This could be relevant for cyclin B1, which is first degraded at the spindle poles, where active APC accumulates during mitosis (Clute and Pines, 1999; Kraft et al., 2003).

Implications for the Regulation of the APC

A critical feature determining the processivity of multiubiquitination is the stability of the substrate-APC interaction, which, as our experiments indicate, might depend on both Cdh1 and core APC subunits. Although cyclin A and securin$^{\Delta DB}$ are recognized by Cdh1, they are multiubiquitinated distributively. Cyclin A can even be purified from cells in a stable complex with Cdh1, indicating that its binding by Cdh1 is not sufficient for processive multiubiquitination (Sorensen et al., 2001). Accordingly, the processivity of multiubiquitination is independent of whether Cdc20 or Cdh1 targets the substrates to the APC, suggesting that an interaction of substrates with subunits of the core APC is necessary for processive multiubiquitination. These subunits presumably constitute the D box binding activity demonstrated for *Xenopus* APC (Yamano et al., 2004). They might include the APC10 subunit, which is required for the stable binding and processive ubiquitination of various yeast APC substrates (Passmore et al., 2003; Carroll and Morgan, 2002; Carroll et al., 2005). We propose that substrates are recognized and delivered to the APC by the activators Cdc20 and Cdh1. The D box of the substrate will then be transferred to the core D box receptor on the APC, and the stability of this interaction will in part determine the processivity of the ubiquitination reaction. Contribution of the D box to the stability of the Cdh1-substrate interaction might further enhance the discrimination between APC^{Cdh1} substrates. Consistent with this notion, the distributive substrate securin$^{\Delta DB}$ has a mutated D box; cyclin A possesses a cryptic D box, which does not convey APC-dependent degradation when transferred into a heterologous protein; and a bona fide D box has yet to be defined for the distributive autoubiquitination of UbcH10.

The only notable exception to the tight correlation between the processivity of multiubiquitination and the timing of degradation is cyclin A. The ubiquitination of cyclin A occurs in a distributive fashion and should be restricted to monoubiquitination in the presence of processive substrates. Contrary to this expectation, cyclin A is degraded as one of the first proteins during mitosis when processive APC substrates are still present (Geley et al., 2001). However, at this time, the spindle-assembly checkpoint interferes with the APC^{Cdc20}-dependent ubiquitination of most substrates, but not of cyclin A. The function of the spindle checkpoint is required for cyclin A degradation before processive substrates such as securin (Geley et al., 2001). Based on these previous results and on our studies, we propose that cyclin A possesses an additional, checkpoint-independent APC-recognition motif. By circumventing the inhibition of the spindle-assembly checkpoint, cyclin A could escape substrate ordering based on differences in processivity and therefore will be degraded early in mitosis.

The experiments described here have provided general kinetic features of APC-dependent multiubiquitination. They have used purified and extract systems, and the general features are congruent with in vivo results. While these experiments established the importance of processivity or deubiquitinating enzymes, we could not at this time obtain the detailed kinetic features that would enable us to model the reactions. These features include the on and off rates of all substrates at various stages of ubiquitination, the on and off rates of the E2s, and the nature and specificities of deubiquitinating enzymes. Further complicating the analysis for the present are the multiple ubiquitination sites on the substrates and the heterogeneous nature of APC^{Cdh1} preparations. Although challenging, experiments addressing these issues are likely to deepen our insight into the mechanisms governing progression through mitosis and G1 and therefore should be a major focus of subsequent studies.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies

UbcH10 and UbcH10^{C114S} were in pCS2$^{ProA/TEV}$ for IVT or in pET28 for purification. Securin, geminin, Cdc20, Plk1, Aurora A, cyclin A, and the N terminus of cyclin B1 (NcycB) were all in pCS2 for IVT or in pET28-TEV for

Figure 7. The Processivity of Multiubiquitination of APC^{Cdh1} Substrates in Late Mitosis Correlates with the Relative Timing of Their Degradation in the Cell Cycle

(A) The kinetics of ubiquitination of ^{35}S-labeled Cdc20 by APC^{Cdh1}. Both its multiubiquitination with ubiquitin and its monoubiquitination with methylubiquitin were analyzed. The reaction products were visualized by autoradiography. U1–U5, mono- or oligoubiquitinated species; U$_n$, multiubiquitinated Cdc20.

(B) The same analysis as in (A) using ^{35}S-labeled Plk1.

(C) The same analysis as in (A) using ^{35}S-labeled Aurora A.

(D) Quantitation of the weighed average length of ubiquitin chains in APC^{Cdh1}-dependent multiubiquitinations from three experiments each.

(E) The effect of UBAs on the length of ubiquitin chains attached to various APC^{Cdh1} substrates. The change in the average length upon UBA addition is shown. The standard error was calculated from three experiments. Securin and geminin have apparently longer ubiquitin chains because UBAs inhibit deubiquitination.

(F) Degradation assays in G1 extracts of ^{35}S-labeled Aurora A and Plk1. Both proteins are degraded after addition of UbcH10 and the DUB inhibitor ubiquitin aldehyde.

(G) Model of substrate multiubiquitination by the APC. The APC ubiquitinates substrates (S) with different degrees of processivity. Ubiquitin is delivered to the APC by charged E2 complexes (E2^{U}). Processive substrates (right panel) do not dissociate from the APC during multiubiquitination. By contrast, more distributive substrates could dissociate from the APC at any stage during multiubiquitination (left panel). Upon dissociation, they could either rebind the APC or be deubiquitinated by deubiquitinating enzymes (DUB). The presence of more processive substrates will inhibit rebinding and favor deubiquitination. Thus, the more distributive the multiubiquitination of a substrate, the less likely it will acquire a tetraubiquitin chain sufficient for proteasomal degradation and the later it will be degraded.

purification. Mutant securin$^{\Delta DB}$ (RKAL to AKAL) and securin$^{\Delta DB\Delta KEN}$ (KEN to AAA, from Olaf Stemmann) were cloned into pCS2 and pET28-TEV. securin^{N68K} and securin$^{K62A/N68K}$ were cloned into pCS2. pGEX-hHR23A and pGEX-UBA1-2 (from Cecile Pickart; Raasi et al., 2004) were cloned into pET28. The Zn binding domain of Emi1 was in pET28. All antibodies were from Santa Cruz except the anti-securin-antibody (MBL).

Tissue Culture

HeLa S3 cells were arrested with 2 mM thymidine medium for 24 hr. After release into DMEM/FBS for 6 hr, cells were arrested in prometaphase by addition of nocodazole (0.1 μg/ml) for 11 hr. G1 cells were obtained by releasing nocodazole-arrested cells into DMEM/FBS for 3 hr.

Extract Preparation and In Vitro Degradation

Extracts of synchronized cells were prepared and analyzed for APC activity as described (Rape and Kirschner, 2004).

Peptides and Proteins

The D box peptides of cyclin A (VQQPRTRAALAVLKSGNPRGLA) and securin (ALPKATRKALGTVNRATEKSV) were from Sigma Genosys. ^{His}UbcH10, ^{His}UbcH10^{C114S}, His-tagged Zn binding domains of Emi1, ^{His}hHR23A, ^{His}NcycB, ^{His}securin, ^{His}securin$^{\Delta DB}$, ^{His}securin$^{\Delta DB\Delta KEN}$, ^{His}geminin, and ^{His}cyclinA were purified from BL21(DE3) pRIL cells (Stratagene) by binding to NiNTA agarose (QIAGEN) according to the manufacturer's manual. Proteins were dialyzed against PBS and treated with TEV for 16 hr at 4ºC. Gst and Gst-UBA1-2 encompassing the two UBA domains of hHR23A were purified from BL21(BE3) pRIL cells using glutathione Sepharose according to the manufacturer's manual (Novagen).

Ubiquitination Assays

Ten micrograms of anti-Cdc27 antibodies was coupled to ProG agarose (Roche) for 1 hr at 4ºC. 1.4 milliliters of HeLa S3 extract was cleared at 10,000 rpm and incubated with Cdc27 beads for 4 hr at 4ºC. The Cdc27 beads were washed three times with SB + 0.05% Tween 20 and twice with SB (25 mM HEPES [pH 7.5], 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 1× complete protease inhibitors [Roche], 15 mM creatine phosphate, 2 mM ATP). The beads were resuspended in 40 μl SB. 17.5 micromolar E1 (Boston Biochem), 100 nM UbcH10, 20 mM ATP, 1.5 mg/ml ubiquitin, 10 mM DTT, and 1 U creatine phosphokinase in UBAB buffer (25 mM Tris/HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) were added to 4 μl beads. Substrates were prepared by IVT (Promega) in the presence of [^{35}S]methionine. Ubiquitinations were started at room temperature by addition of substrate, stopped with gel loading buffer, and resolved by 5%–15% SDS-PAGE and autoradiography. To analyze dissociation from the APC, 100 nM Gst-UBA1-2 or 100 nM ^{His}hHR23A was added where indicated. For single binding assays, ^{35}S-labeled substrates were preincubated with APC in UBAB buffer in three replicates on ice for 45 min and, after addition of UbcH10, for a further 15 min. Their ubiquitination was started by addition of ATP and E1. In a replicate, 2 μM NcycB was added simultaneously to ATP and E1 to inhibit rebinding. In another replicate, 2 μM NcycB was added during preincubation as negative control.

Quantitation

Ubiquitination assays were resolved by SDS-PAGE. The intensities of the autoradiograph were determined using Quantity One (Bio-Rad). The intensities were plotted as a function of distance from the unmodified forms, and from this data the weighed average length of ubiquitin chains was calculated.

Substrate-Ordering Assay

To investigate substrate ordering by APC^{Cdh1} in vitro, we purified APC^{Cdh1} and recombinant cyclin A, geminin, and securin. Two micromolar recombinant substrates were subjected to multiubiquitination alone or in combination with each other. Ubiquitinations were analyzed by 8%–16% SDS-PAGE and Western blotting using specific antibodies.

Deubiquitination Assay

^{35}S-labeled substrates were ubiquitinated with APC^{Cdh1} for 15 min at room temperature in the presence of MG132. The rebinding of dissociated substrates to APC^{Cdh1} was inhibited by addition of 2 μM NcycB. The reactions were incubated for a further 30 min and analyzed by SDS-PAGE and autoradiography. Where indicated, 2 mM NEM or ubiquitin aldehyde was added to inhibit deubiquitination.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/cgi/content/full/124/1/89/DC1/.

ACKNOWLEDGMENTS

We thank Julia Schaletzky and members of the Kirschner lab for many helpful discussions and J. Schaletzky, Michael Springer, and Paul Jorgensen for comments on the manuscript. We are grateful to Olaf Stemmann and Cecile Pickart for providing essential reagents. M.R. is a Human Frontiers Science Program Long-Term Fellow. S.K.R. was funded by the Medical Scientist Training Program. This work was funded by grants from the National Institutes of Health to M.W.K.

Received: July 27, 2005
Revised: September 25, 2005
Accepted: October 21, 2005
Published: January 12, 2006

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