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Specificity of the Anaphase Promoting Complex: a single-molecule study

Ying Lu, **Weiping Wang**, and **Marc W. Kirschner**

Department of Systems Biology, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115

Summary

Biological processes require specific enzymatic reactions, paradoxically involving short recognition sequences. As an example, cell-cycle timing depends on a sequence of ubiquitylation events mediated by the Anaphase Promoting Complex (APC) based on short redundant motifs. To understand the origin of specificity we designed single-molecule fluorescence assays that capture transient ubiquitylation reactions. We find that the APC-mediated ubiquitylation involves a highly-processive initial reaction on the substrate, followed by multiple encounters and reactions at a slower rate. The initial ubiquitylation greatly enhances substrate's binding affinity in subsequent reactions, by both increasing the on-rate and decreasing the off-rate. We postulate that these cycles of positive feedback enable high specificity for substrates with short recognition motifs in a complex cellular environment.

Introduction

Regulation of biological processes requires that proteins distinguish the proper binding partners or substrates from non-substrates. Emil Fischer famously viewed the substrate as the key and the enzyme as the lock. There are many proven examples of specificity arising from such complementary binding interactions. However, specificity cannot be attributed completely to differential binding affinity alone, as has been demonstrated by the discrepancy between the error-rate of protein synthesis and the measured binding affinities of the correct and incorrect aminoacyl-tRNAs on mRNA. In a conceptual theory for resolving such discrepancies, called "kinetic proofreading", the energy from ATP or GTP hydrolysis is used to significantly reduce error-rates (1, 2). The kinetic proofreading mechanism has been invoked to explain the high degree of specificity of various biological processes governed by relatively weak binding interactions (3, 4).

The importance of recognition and specificity in protein degradation is increasingly appreciated. During mitosis, cyclinA, cyclinB, securin, polo kinase and UbcH10 are

Corresponding Author: Kirschner MW., marc@hms.harvard.edu, Tel: 617-432-2250.

Supplementary materials
Materials and Methods
Figs. S1 to S38
Captions for Movies S1 to S8
Reference 30–34
Movies S1 to S8

ubiquitylated sequentially by the Anaphase Promoting Complex or APC, and degraded by the proteasome, contributing to an ordered execution of cell-cycle events (5, 6). The precise timing of protein degradation at cell-cycle transitions demands high specificity in APC-substrate interaction. Paradoxically, these interactions are mediated by exceedingly short recognition sequences, such as the D (RXXLXXXX (N)) and KEN (KEN) boxes (where X is any amino acid) (7–9). These recognition sequences are highly redundant, appearing in 69% proteins in the human genome, whereas only 50~100 real substrates for the APC are expected from genome-wide studies (10). This preponderance of "decoy substrates" poses an additional challenge. At a total concentration of approximately 1mM, they should compete for APC binding and reduce the rate of ubiquitylation and degradation of real substrates. Yet, this does not appear to happen. APC substrates, such as securin and cyclinB, can be efficiently degraded in a few minutes in yeast and mammalian cells. Although these small recognition sequences are required, they are not sufficient to confer susceptibility when transferred to other proteins (7). Thus real APC targets must provide other specificity features, even though no additional sequence features are discernible. Therefore, the simple 'Key-Lock' mechanism appears to be inadequate to explain the specificity in APC-mediated ubiquitylation. To explain the required specificity, we looked beyond single binding interactions to the network of reactions that take place in the cellular environment where there are competing substrates and competing reactions.

The APC interacts with substrates transiently and recurrently, conjugating complex and heterogeneous ubiquitin configurations on substrates molecules (11). These features make it difficult using conventional biochemical methods to understand the reaction kinetics and mechanisms. In this study, we have developed single-molecule (SM) fluorescence assays capable of identifying multiple reaction intermediates. Using these assays, we could examine reactions at individual steps to study APC-mediated ubiquitylation in both purified systems and in crude cell extracts. We distinguished different modes of substrate binding, reflected in different kinetics for initial ubiquitin conjugation and subsequent elongation steps. We identified a reaction pathway based on the evolving ubiquitylation status of the substrate that can explain how substrate specificity and reaction efficiency can be achieved in a network of low specificity interactions.

Results

A single-molecule ubiquitylation assay

To observe reactions of single molecules, we used ubiquitin and substrates chemically labeled with different chemical fluorescent dyes (Fig. 1A and B). The labeled proteins were incubated in concentrated extracts of human HeLa cells in the G1 phase of the cell cycle. Endogenous APC was labeled with a fluorescently tagged antibody; the substrates were C-terminally labeled with biotin and tethered with streptavidin to polyethylene glycol and albumin passivated glass slides. Because each ubiquitin molecule was conjugated with a single fluorophore at its N-terminus, the number of conjugated ubiquitins on a substrate molecule could be measured as the total fluorescence intensity. A major problem in developing the SM methods for ubiquitylation in Fig. 1B was the background fluctuation from fluorescently labeled ubiquitin in solution and the nonspecific binding of fluorescent

species to the slide surface. We used combinatorial strategies to increase the signal-to-noise ratios and specificity of the detection (Fig. S1); these allowed us to distinguish the addition and removal of single ubiquitins at a 95% confidence level in the presence of 3uM fluorescent ubiquitin (Methods), near its physiological range of 5 to 10uM. The chemical fluorophores with their high flux of photons, and a relatively long exposure time of 3 seconds afforded by the natural slow reaction rates, along with strong laser excitation allowed us to reduce Poisson noise. Adding 10mM imidazole greatly reduced nonspecific binding of all fluorescent species and lowered the background fluctuation without affecting kinetics of ubiquitylation (Fig. S2). Intensity values of single molecules were calculated by a method of local background subtraction (Fig. S3; S4, Methods).

To distinguish substrate-specific ubiquitylation signals from nonspecific binding of ubiquitylated proteins from cell extracts, we limited our analysis to ubiquitylation events that were coincident with binding of the fluorescently labeled APC to a fluorescently labeled substrate. Because of the sparseness of nonspecific binding events, the chance of background binding of ubiquitin or ubiquitylated proteins coinciding with the labeled APC was less than 10^{-5} (Methods). The accuracy and linearity of the ubiquitylation measurement was assessed and confirmed by photobleaching of preformed ubiquitin chains, a process that randomly inactivates single fluorophores (Fig. 1C; S5; S29–30). The APC antibody labeling was sufficiently stable to allow observation of relatively transient substrate-APC interactions (Fig. 1D). So that each event of ubiquitin conjugation was recorded, we completely depleted endogenous free ubiquitin from extracts by adsorption to beads containing the E2-UbcH10, without affecting ubiquitylated proteins (Fig. S6); the extracts were then supplemented with 3uM alexa647-labeled ubiquitin (Fig. S7).

Ubiquitylation mediated by the APC at a single-molecule level

Geminin is an APC substrate that functions in control of DNA replication. When geminin was immobilized on a slide as the substrate, we observed a greatly enhanced ubiquitin fluorescence signal, compared to background, that was coincident with APC binding events (Fig. S8, and Video S1; S2). Overall ubiquitylation level was strongly reduced in the presence of the APC inhibitor Emi1, suggesting that APC is the primary E3 for geminin ubiquitylation (Fig. S9). We recorded time traces of APC binding and ubiquitylation on single geminin molecules. In cell extracts the ubiquitylation signal was dynamic, showing features that can be attributed to enzymatic deubiquitylation in the extract, proteasomal degradation of the substrate and a slow rate of photobleaching (half-life=8.6 minutes, Fig. S10). The SM method fully reported on the heterogeneity in the reactions: after the binding of an APC molecule, ubiquitylation on individual geminin molecules occurred with variable rates and reached different numbers of conjugated ubiquitins at the moment of APC disassociation. We present a representative raw trace of these features in Figure 2A/B and 20 more raw traces for each substrate (geminin, the cyclinB-N-terminal domain (cyclinB-NT), and cyclinA in cell extracts; cyclinB-NT, securin, and K64cyclinB-NT in a purified system) in the Supplement (Fig. S31–S36). The SM kinetics of ubiquitylation on geminin and cyclinB-NT were very similar. Each is known to be a processive substrate, where processivity is defined as the average number of ubiquitins conjugated to a substrate

molecule, regardless of its degree or configuration of ubiquitylation, in each round of APC binding (4).

Several observations indicate that the ubiquitin signal on immobilized substrates represents *bona fide* ubiquitylation by the APC, rather than the binding of ubiquitylated APC or ubiquitylation by other E3s. We did not observe a sharp decrease of ubiquitin signal when the APC dissociated from the substrate, suggesting that the APC was not ubiquitylated. Either the APC inhibitor Emi1 or methylation of lysines on substrates, which prevents ubiquitylation, strongly reduced the ubiquitin signal (Fig. S9; S11). In about 22% cases, we observed increased ubiquitin fluorescence in the absence of detectable APC binding. This may have been caused by either APC conjugated to an unmodified (and hence non-fluorescent antibody) or free APC unconjugated to antibody. However we cannot exclude a low level of APC-independent ubiquitylation. Ubiquitylated substrates on the slide were competent to interact with the proteasome (labeled with a fluorescent antibody) in the cell extract, suggesting ubiquitin configurations that are competent for proteasomal degradation had been formed during the reaction (Fig. S12).

In the SM traces, there were always two phases of ubiquitylation. The first was highly processive, soon after the initial binding of the APC, resulting in multiple ubiquityl residues transferred to a substrate molecule within 5 seconds (the time-resolution of the experiment) (Fig. 2A; S31–32). The second phase was much less processive, as the rate of ubiquitin transfer was lower and was interrupted by APC disassociations. Rebinding of APC was also frequently unproductive ("u" in Fig. 2A, B), generating no detectable ubiquitin conjugations. Processive ubiquitylation could reoccur after the substrate had been partially or fully deubiquitylated. To illustrate the biphasic ubiquitylation statistically, we aligned 315 traces of cyclinB molecules ubiquitylated in cell extracts by their initiation points, and plotted the median number of conjugated ubiquitins as a function of time. The processive initial reaction transferred an average of 5 ubiquityl moieties, followed by a slower transfer rate of ~1 ubiquitin/minute (Fig. 2C; S13). The actual transfer rate may be slightly higher due to signal loss by photobleaching (Fig. S10).

To distinguish the kinetics of monoubiquitylation from that of chain elongation, we replaced the endogenous free ubiquitin in cell extract with zero-lysine ubiquitin (Ub0K) to suppress chain formation. In this case 3 rather than 5 ubiquityl moieties were transferred in the initial reaction, suggesting that some short ubiquitin chains were formed during the initial ubiquitylation when wtUb was used (Fig. 2C). In the second (or slower) phase, the rate of ubiquitin conjugation using wtUb was 3 times higher than that using Ub0K, suggesting that chain elongation occurred mainly after the initial processive reaction (Fig. S13). It is unlikely that Ub-chains were transferred *en bloc* (12, 13), because the vast majority of the E2 UbcH10 was charged with a single ubiquitin (Fig. S14).

To generalize this result, we compared histograms of the maximum number of ubiquitins conjugated to individual cyclinB molecules during the experiment using wtUb or Ub0K. For cyclinB, wtUb provided twice the level of ubiquitylation as Ub0K (Fig. 2E), consistent with earlier results by mass spectrometry (11). Kinetics of binding of the APC was substrate-dependent, and strongly affected by substrate ubiquitylation status (see below). On average

4.1 APC-binding cycles were recorded on a cyclinB molecule in a 15-minute experiment, and 3.7 for geminin (Fig. 3A; S15, and Video S3). To understand the contribution of each successive APC-binding cycle to substrate ubiquitylation, we analyzed the median number of ubiquitins, before, within and after, the binding of APC on cyclinB-NT molecules in the cell extract. The first APC-binding cycle conjugated on average 7 ubiquitins, including both the initial processive and the secondary distributive phases (Fig. S16). Subsequent APC-binding cycles conjugated fewer ubiquitins, but were important for chain elongation and for maintaining cyclinB in various ubiquitylated states in the presence of active deubiquitylation. To reduce the complexity due to deubiquitylating and proteasomal activities in the cell extract, we performed the same experiments in a purified system, comprised of purified APC and other recombinant components. As in the cell extract, we found that for the processive substrates, securin and cyclinB-NT, ubiquitylation was biphasic and required multiple encounters with the APC (Fig. 2B; S34–36, and Video S4).

Differences in the processivity of ubiquitylation have been used to explain the order of degradation for APC substrates during the cell cycle (4). Multiple steps in the reaction pathway could contribute to processivity and these can be identified by SM methods. We compared a well-characterized processive substrate cyclinB and a distributive substrate cyclinA for their kinetics of ubiquitylation and APC binding using SM assays. CyclinA-APC interactions were transient, lasting for on average 7s compared to 30s for cyclinB-NT in cell extracts (Fig. 3A). Accordingly, less ubiquitin was transferred to cyclinA in the initial phase or accumulated during the experiment (Fig. 2C; 2E). Nonetheless, ubiquitin transfer was still highly efficient on cyclinA molecules once APC-bound (Fig. 2A; S33). Therefore, the processivity of cyclinA seems to be limited by relatively short APC interaction and also perhaps by cyclinA having fewer ubiquitylatable lysine residues.

The elongation of ubiquitin chains on APC substrates is facilitated by another E2, Ube2S. It has been debated whether UbcH10-mediated ubiquitin nucleation or Ube2S-mediated chain elongation is rate-limiting for ubiquitylation (14–16). Using single-lysine substrate K64cyclinB-NT that can form only a single ubiquitin chain, we dissected the two steps individually. In the absence of Ube2S, K64cyclinB-NT essentially received no more than 3 ubiquityl moieties in an APC reaction (Fig. S17). Ube2S promoted chain elongation in a concentration dependent manner (Fig. S17). In the SM assay with Ube2S at its physiological concentration of 100nM, we found that chain elongation beyond 3 ubiquitins was usually slow and stepwise, compared to the highly processive initial ubiquitylation mediated by UbcH10 (Fig. 2D). We conclude that UbcH10 initiates monoubiquitylation and short chains with high processivity, but fails to form long ubiquitin chains. Further chain elongation by Ube2S is less processive than the initial ubiquitylation by UbcH10, when these E2s are compared at their physiological concentrations.

Positive feedback in APC-mediated ubiquitylation

APC-substrate interactions are highly dynamic. The kinetics of enzyme-substrate complex formation is commonly used in conventional assays to obtain the reaction rate constants, such as the off-rate, k_{off} by fitting a simple binding model between molecule A and B, described as $[AB](t) \propto 1\text{-}\exp(-k_{off} * t)$ given $K_d \gg [A],[B]$, where [A], [B] and [AB] refer to

the concentrations of A, B and AB respectively; K_d refers to the dissociation constant. Under this condition, the time-scale of the binding curve is dominated by the off-rate, as detailed in Methods. Rate parameters obtained in this way should be consistent with the values calculated from analyzing SM traces; a discrepancy may indicate a time-dependent modulation of the interaction in the population.

In cell extracts the number of APC molecules interacting with a fixed number of substrate molecules increased gradually during the experiment and eventually reached a steady state. This was a common feature for all the processive substrates tested in this study (Video S2, 4, 5, 7). The parameter needed to fit the APC-cyclinB binding curve (Fig. 3B, blue, and video S5) as measured by the number of APC molecules on the slide as a function of time is 0.12min^{-1}. However, this is only 6% the value for the off-rate obtained from analyzing SM traces of APC-cyclinB interaction in the presence of ubiquitylation (Fig. 3A). Therefore, the rate parameter of APC-cyclinB binding curve does not represent the actual off-rate, but the time-scale of an evolving factor that modulates the APC-cyclinB interaction, such as the varying state of ubiquitylation of the substrate, which was suggested by the overall parallel of ubiquitylation with the APC binding kinetics (Fig. S18). Furthermore, low concentrations of free ubiquitin (the cyan curve in Fig. 3B), where no additional ubiquitin was added, led to reduced levels of APC-cyclinB complex formation suggesting that substrate ubiquitylation enhances its affinity with the APC (Fig. 3B, and Video S6). This behavior is not an artifact due to the use of fluorescent ubiquitin, since unlabeled ubiquitin added to a Ub-depleted cell extract restored the APC binding kinetics measured with fluorescent ubiquitin (Fig. 3B, and Video S7). This affinity enhancement was also observed in experiments with purified APC, and with other substrates (Fig. 3C), excluding a major role for unidentified cofactors in the cell extract. It is unlikely that E2s charged with ubiquitin facilitate APC binding to substrates, because we observed a reduced amount of substrate-bound APC when we used a cell extract substituted with Ub0K in which the E2s were still charged with ubiquitin (Fig. 3B; 2C, and Video S8). Because Ub0K cannot form chains, the result shows that ubiquitin chains on the substrate may facilitate but are not absolutely required for enhancement of APC binding. When the elongation factor E2, Ube2S, was added to an *in vitro* ubiquitylation reaction, it increased the number of conjugated ubiquitins on single-lysine K64cyclinB-NT, and resulted in an enhanced binding of the APC (Fig. 3D). These results suggest that the number of conjugated ubiquitins, rather than the presence of specific ubiquitin configurations, determines the binding enhancement of the APC. In conclusion, the apparent discrepancy between SM and ensemble measurements is resolved if we consider the effect of ubiquitylation on increasing the substrate's affinity with the APC; such a binding enhancement occurs with various ubiquitin configurations, including those without chains.

Because of constitutive deubiquitylation and degradation, ubiquitylated substrates usually represent only a small fraction of the total substrate concentration in the cell extract (Fig. S19). Since $K_d=[\text{APC}]*[\text{Substrate}_{\text{total}}]/[\text{Substrate}_{\text{APC bound}}]$, a small increase in bound APC molecules may reflect a large decrease in K_d for ubiquitylated substrates with the APC. We estimated K_d values for the binding of ubiquitylated and unmodified substrate by the SM assay (Methods). Ubiquitylation reduced the K_d of the binding of cyclinB-NT by 60 fold and reduced the K_d for geminin by 40 fold (Fig. 4A). Consistently shown by SM traces, the

interaction of APC with unmodified cyclinB was transient compared to that with ubiquitylated cyclinB, and usually characterized by long intervals between successive binding cycles (Fig. S20). To separate the contribution of on-rate and off-rate to the K_d reduction, we measured these kinetic constants by SM methods on cyclinB and related them to the ubiquitylation status of the same substrate. We used a purified system to prevent interference from the proteasome and deubiquitylating enzymes. Ubiquitylation simultaneously decreased k_{off} and increased k_{on}; together they accounted for the reduction in K_d (Fig. 4B). A similar result was obtained using securin as the substrate (Fig. S22).

To address potential concerns about the SM experimental design affecting enzyme behaviors at solid-liquid interface, we compared the SM results to conventional assays in bulk solution. Excess APC substrates compete for ubiquitylation by the APC and consequently inhibit proteasomal degradation. Our SM measurements indicate that a protein substrate that is non-ubiquitylatable should have a lower affinity for the APC than do ubiquitylated substrates. Lysine-methylation completely blocks ubiquitin conjugation on substrates, while preserving structural stability and the D-boxes on the protein. We used cyclinB-NT, geminin and their methylated forms as competitive inhibitors in degradation assays in cell extracts or as inhibitors of ubiquitylation reactions by purified APC. Indeed, methylated cyclinB and geminin exhibited weaker inhibitory effects in both assays with a low concentration of radioactively labeled cyclinB-NT or geminin as the reporter (Fig. 5A, B). A consistent but smaller effect was observed with securin as the substrate (Fig. S23). We also tested the inhibition of APC by preubiquitylated cyclinB-NT using radioactive cyclinB-NT as the reporter. Preubiquitylated cyclinB-NT was more effective in reducing APC activity in *in vitro* ubiquitylation assays than unmodified cyclinB-NT, with the difference diminishing at lower concentrations (Fig. S24).

To assess directly the effect of ubiquitylation in modulating the interaction with APC, we performed a co-precipitation assay. Ubiquitylated cyclinB-NT interacted more strongly with APC than unmodified cyclinB-NT (Fig. 5C; S25). APC interacted very weakly with substrates, even after substrate ubiquitylation (Fig. 4A). This makes direct binding measurements difficult by conventional methods, which are prone to interference from nonspecific binding (Fig. S25). Therefore, we would not expect the binding and competition experiments to quantitatively reproduce the results of SM measurements; nonetheless, both lines of evidence support a stronger interaction of ubiquitylated substrates with the APC.

If ubiquitins conjugated to substrates increase affinity for the APC, increased affinity should facilitate further ubiquitylation on the same substrate molecule during subsequent rounds of binding with the APC. To test this expectation, we fused a single ubiquitin to the N-terminus of K48securin to obtain a homogenous population of a monoubiquitylated substrate. Ub-K48securin but not K48securin alone, was ubiquitylated efficiently in the presence of high concentration of competitive inhibitors (Fig. 6A). We also tested the effect of conjugated ubiquitins on subsequent ubiquitylation in a reaction without competitive inhibitors by performing a chase experiment on partially ubiquitylated cyclinB-NT. In the middle of an *in vitro* ubiquitylation reaction on cyclinB-NT, we added excess HA-tagged ubiquitin, and measured the distribution of HA-ubiquitin incorporated onto the substrate by Western Blot. HA-ubiquitin was first incorporated into high molecular-weight conjugates of the total

cyclinB-Ub population. In contrast it was distributed more uniformly if HA-ubiquitin was added before the reaction (Fig. 6B). It is unlikely that incorporation into the high molecular-weight conjugates is due to partially ubiquitylated cyclinB-APC complex at the point of HA-ubiquitin addition, because APC turnover on cyclinB is much faster than the sampling rate (Fig. 3A). Rather, these results suggest that partially ubiquitylated substrates are the preferred targets for subsequent ubiquitylation by the APC. This conclusion, in conjunction with the effect of conjugated ubiquitins to promote binding with the APC, provides evidence of a positive feedback in the APC reaction.

Discussion

Specificity in biology is often thought to be conveyed by complementary interactions between chemical species with the degree of specificity simply reflecting the free energy difference of binding. However, in certain biological systems recognition may require more than complementarity. This may be true of substrate specificity in the ubiquitin system, which is largely determined by E3 enzymes. For one E3 enzyme, APC, substrates are characterized by the requirement for short recognition sequences, which are found throughout the genome on many non-substrates. Studies of the reactions in this system have been stymied by heterogeneities of the products and challenges in measuring the dynamics. To confront these difficulties, we designed single-molecule fluorescence assays for detecting ubiquitin conjugation and E3-substrate interaction. We used multiple strategies, including minimizing fluorescent nonspecific binding and long exposure times to stabilize the local background fluctuation in TIRF microscopy due to our use of near-physiological concentrations of free ubiquitin. We could obtain adequate sensitivity and signal-to-noise ratio to observe directly how the APC promoted polyubiquitylation through multiple encounters with a substrate molecule. Using this approach we examined the mechanism of APC-mediated ubiquitylation reactions in cell extracts and in purified systems, and revealed unusual kinetic features; these features provide a basis for understanding how the APC can function efficiently in a complex cytosolic environment with numerous D/KEN-box containing ‘decoy’ substrates and deubiquitylating enzymes. As discussed below, these experiments suggest a mechanism for how APC can choose substrates among a very large number of proteins with identical recognition sequences.

Substrate molecules are likely to encounter the APC for multiple times before degradation. Although we have not accurately measured the rate of proteasomal degradation in the SM assay, the half-life of cyclinB and geminin is about 30 minutes in bulk assays under our experimental conditions, a time sufficient for multiple encounters to occur. The duration of substrate-APC interactions is very heterogeneous even for molecules with the same number of conjugated ubiquitins, as expected for stochastic processes. Different ubiquitin configurations on the substrate may also contribute to the heterogeneity (11). In both extracts and purified systems, we found an initial highly processive ubiquitylation process, involving both multi-monoubiquitylation and chain elongation. We did not resolve the processive phase into single ubiquitin steps. This does not represent a limitation of the SM detection method *per se* because we were able to observe stepwise addition of single ubiquitins in certain cases, presumably due to heterogeneity in the reaction rates (Fig. S26); rather, it reflected insufficient time resolution due to limitations in the speed of detection.

Our study on a mutant of cyclinB with a single lysine showed a slower ubiquitin transfer rate by the elongation factor Ube2S than the initial ubiquitylation by UbcH10. We expect that Ube2S contributes less to the overall stoichiometry of ubiquitylation in a physiological context where the length of ubiquitin chains is restricted by the action of deubiquitylating enzymes (11). However, this small incremental contribution by Ube2S may be critical in selecting the substrate for degradation, since it solely promotes chain elongation.

Single-molecule kinetic studies demonstrate a highly-processive initial ubiquitylation for APC-substrates. Furthermore, we find that conjugated ubiquitins bias further ubiquitylation by increasing k_{on} and decreasing k_{off} with the APC. In this way, the chemical reactivity of lysine residues (which themselves do not seem to contribute significantly to substrate binding) is converted into enhanced affinity with the APC. This would recursively promote the binding and reaction of the substrate with the APC. The decrease in k_{off} may result from ubiquitin interacting directly with the APC. The APC likely contains weak ubiquitin-interacting regions whose identities have not been fully examined; however, in recent studies, ubiquitin has been found to interact with both RING-domain and HECT-domain of E3 ligases in the E2-ubiquitin-E3 crystal structures (17, 18). Specifically, the RING-domain of APC has been shown to contain separate regions interacting with both donor and receptor ubiquitins (19). Kinetic studies also show that APC-Ube2S complex can interact and track emerging ubiquitin chains on substrates during elongation (20). Modifications of k_{on} have not been typically encountered in biochemistry. In ubiquitylation reactions, an enhanced k_{on} may be a consequence of an increased interaction cross-section between ubiquitylated substrates and the APC, specifically if the latter contains ubiquitin receptors.

The results of our experiments and mathematical modeling suggest a mechanism that would explain the specificity in APC-mediated ubiquitylation. At minimum, specificity should reflect sufficient interaction between enzyme and substrate. The SM measurements indicate that the intrinsic affinity of APC for unmodified substrates is very low, which may reflect the limited size of APC-binding motifs. A low substrate binding affinity with corresponding high off-rates may be required to ensure the functions of APC. Otherwise, the APC would be completely inhibited by the many D or KEN-box containing decoy substrate proteins, the concentration of which reaches about 1mM in the cell. However, low affinity should result in a low reaction rate and specificity for real substrates, which would result in very low reaction specificity. The SM experiments show that when the APC encounters a real target that contains sufficient ubiquitylatable lysine residues (see below), a wave of processive ubiquitylation occurs, and this leads to higher on-rates and lower off-rates for ubiquitylated proteins with the APC, which makes the ubiquitylated protein a ‘preferred’ target in subsequent binding and reaction cycles (Fig. 7A). Such a positive feedback would amplify the initial weak interaction between the APC and substrate. We termed such process, “processive affinity amplification” or “PAA” (Fig. 7A). The dramatic effect of PAA on increasing substrate specificity can be demonstrated using a simple ordinary-differential-equation model simulating substrate-E3 binding, ubiquitylation, deubiquitylation and degradation steps using physiologically appropriate parameters (Fig. 7B).

PAA should also increase the efficiency of the APC-ubiquitylation system, when it is challenged by deubiquitylating enzymes in the cell. Without PAA, these enzymes would

substantially reduce the ubiquitylation and degradation efficiency, especially at high concentrations of substrates (including decoy substrates) (21). APC substrate molecules require multiple encounters with the E3 before they accumulate enough ubiquitins for degradation (Fig. 3A; S15–16). In the presence of a high concentration of substrates and decoy substrates, there will be increased delays between successive binding cycles, long enough for deubiquitylating enzymes to partially or completely remove conjugated ubiquitin; this would drastically increase the time it takes to accumulate sufficient ubiquitins for degradation. Such an inhibitory effect can be demonstrated in the mathematical model as above varying the concentration of total substrates (Fig. 7B). At the concentration of total D or KEN box-containing proteins (about 1mM) in the cell, APC efficiency for any real substrate drops to less than 1% of its maximum. The PAA mechanism, where partially ubiquitylated substrates bind more quickly and with higher affinity than non-ubiquitylated substrates, would increase APC efficiency by focusing the ubiquitylation reactions on a subset of molecules, increasing their complement of conjugated ubiquitin until they are recognized by the proteasome. When we incorporate PAA into the model, the rate of substrate degradation is much less affected by high substrate concentrations (Fig. 7B). Such ‘focused’ reaction kinetics can also be demonstrated experimentally. In an APC ubiquitylation reaction the average number of conjugated ubiquitins was more than that obtained in a single encounter assay (using inhibitory concentrations of competitor to prevent substrate-APC reinteraction), suggesting that ubiquitylated species have multiple encounters with the APC. But this was also the case when only a small fraction (5 to 10%) of substrates had reacted (Fig. 6C; S27). In this case it is unlikely that the small fraction has multiple encounters and reactions with the APC, while the vast majority of substrates does not, unless the APC ‘focuses’ on the small subset by the process of affinity amplification. A similar ODE model that incorporates the PAA mechanism to simulate this experiment faithfully reproduces the time evolution of the average conjugated ubiquitins, as well as that of each ubiquitylated species (Fig. S28).

Information for recognition by the APC must ultimately be encoded somewhere in the sequence or structure of the substrate molecules. The D and KEN-boxes provide the initial interaction with the APC. These motifs are not equally accessible by the APC on all proteins, but because their sequences contain argininyl or lysyl side chains, they are likely to be exposed on a protein surface or in disordered regions (22). Besides the D/KEN-boxes, sufficient lysine residues must be present on substrates to initiate the PAA. Indeed, we found 64% more lysine residues within 60 residues of the D-box of published APC substrates, compared to random D-box containing proteins in the human proteome (23) (Fig. 7C). Lysine residues are not equally ubiquitylatable. Ubiquitylatability must certainly be affected by the distance and orientation of these residues to the ubiquitin thioester bond on E2 molecules, and also affected by pKa of lysyl side chains, which would be affected by neighboring amino acids (24). There are no known sequence motifs for optimized lysine locations, and we might not expect to find them. Therefore, it appears that what determines substrate specificity in APC reactions, after the complementarity achieved by the simple recognition sequences, is the reactivity of lysine residues, likely near the D and KEN boxes. Ubiquitylatable lysine residues *alone* may not be sufficient to promote high specificity and efficiency for APC substrates in the absence of PAA. The ubiquitin transfer rate on APC

substrates dropped to ~1 ubiquitin/minute on average after the initial phase of reaction (Fig. 2C). Without PAA supporting a stronger interaction with the APC, the very low affinity provided by D/KEN boxes with the E3 should not be able to sustain further ubiquitylation for proteasomal degradation in the presence of active deubiquitylation in the cell.

Finding an optimal tradeoff of specificity and efficiency is likely to be a central requirement of all biological systems and therefore, to be under strong selection. It has long been known that mutations in ribosome and DNA polymerase that increase specificity in protein synthesis and in DNA replication decrease the efficiency (or rate) of these processes, whereas mutations that decrease specificity actually increase efficiency (25, 26). Finding the right substrate is basically a search procedure where high on-rates and weak affinity translate into the speed of the search. If the affinity were strong, APC would spend too much time interrogating related sequences on a large number of decoy substrates in the cell. Results from the SM measurements and mathematical modeling explain how the APC may achieve high specificity without sacrificing too much efficiency. The PAA mechanism only increases the affinity of the APC with real substrates, but not with decoy substrates. The resultant stronger binding of the APC with ubiquitylated substrates will not slow down degradation by interfering with proteasome recognition, because even at sub-uM K_d, the APC still turns over quickly enough on substrates, compared to the rate of degradation. Consistent with PAA, non-ubiquitylatable cyclinB with lysine residues mutated to arginine when overexpressed does not cause a cell-cycle block in *S. pombe* (27). This is expected because its interaction with the APC should remain weak, whereas overexpression of a wt-cyclinB, which can be ubiquitylated, inhibits cell cycle progression. Interaction between APC and K11-linked ubiquitin chains can also be detected *in vitro* (28); however most substrate-E3 interactions are not stable enough to be detected in the cell. Besides the positive feedback on reaction rates, deubiquitylation and multiple encounters with the APC are essential components in PAA, similar to the requirements in the kinetic proofreading model (1).

In the PAA model, APC achieves high substrate specificity with short binding motifs, while maintaining reaction efficiency. Similarly, recognition motifs of many important enzymes, such as the Cyclin-Dependent Kinases (CDK) and the Polo Kinase, are short. For these and many other kinases phosphorylation often occurs on multiple sites. Phosphorylation, like ubiquitylation, is opposed by hydrolases that reduce the efficiency of the process. In the case of CDK, when associated with the Cks protein, the complex has a higher affinity for substrates that have been phosphorylated by CDK. Thus the initial phosphorylation could facilitate additional phosphorylation, essentially creating a positive feedback like PAA (29). Iterative cycles of binding and reversible modification, found in many systems, may be a common contrivance in biology to increase the specificity of a process, without too much sacrifice in speed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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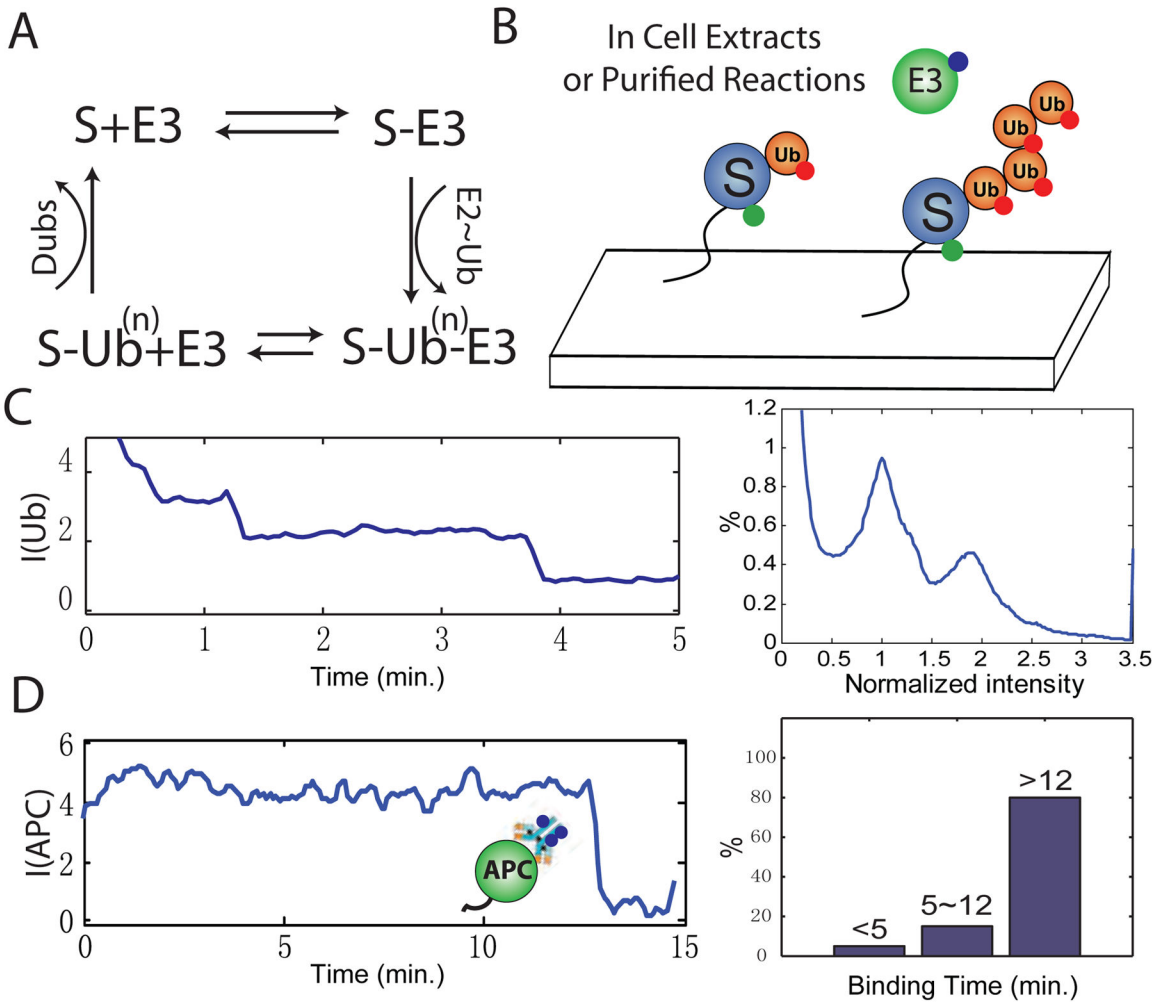

Figure 1. The single-molecule ubiquitylation assay

A, major reaction pathways in the experiments. S: substrates; Dubs: deubiquitylating enzymes. B, schematics of the experimental setup. Substrates(S), ubiquitin and the APC were fluorescently labeled. Substrates were also C-terminally biotinylated and immobilized on slides. Substrate-coated slides were incubated with functional cell extracts or purified APC reaction components. The kinetics of ubiquitylation and APC-substrate interaction were monitored on a TIRF microscope. C, a representative trace of photobleaching a fluorescent polyubiquitin chain synthesized in an E2-25K reaction (more traces in Fig. S29–30). Right: Pairwise intensity difference distribution of 168 photobleaching traces. The position of the first peak has been normalized to “1”. The photobleaching experiment was performed in the absence of free ubiquitin. D, biotinylated, purified APC molecules on the slide were incubated with fluorescent anti-APC4 antibody to analyze the stability of antibody-APC interaction. Left: a representative trace, right: histogram of apparent interaction durations.

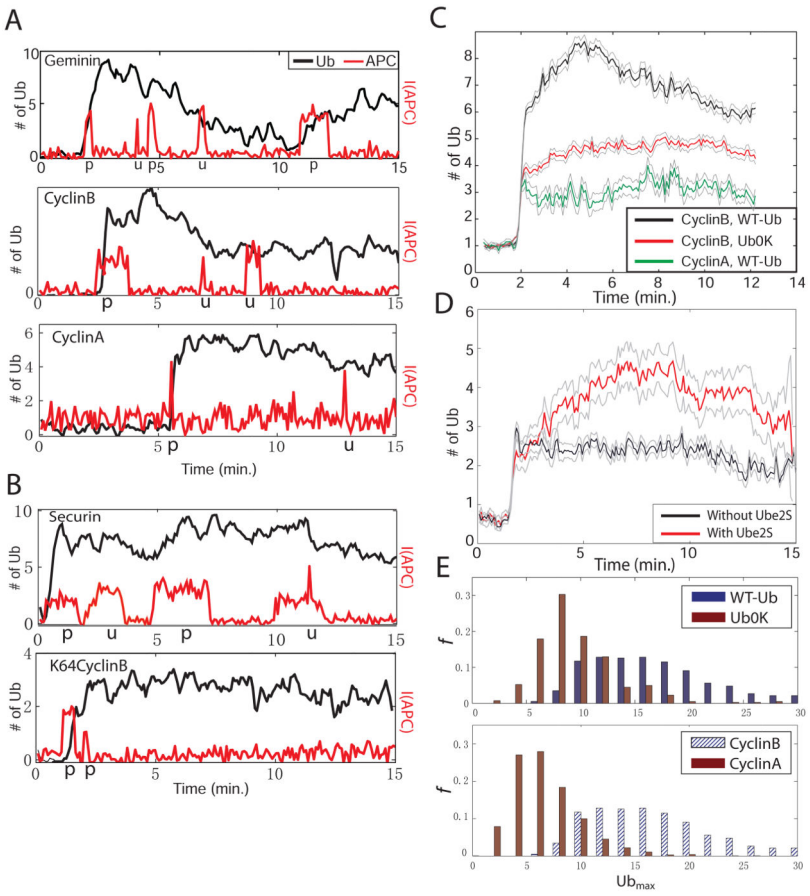

Figure 2. Kinetics of APC-mediated ubiquitylation in cell extracts and in purified reaction systems

A, representative traces of ubiquitylation and APC binding on geminin, cyclinB-NT and cyclinA molecules in Hela cell G1 extracts. Ubiquitylation signal (black) has been converted to the number of ubiquitin molecules as in the Y-axis. The APC traces (red) are plotted in arbitrary units. More examples are shown in the supplement. ‘p’: productive binding; ‘u’: unproductive binding. B, as in (A), but using wt-securin and single-lysine K64cyclinB-NT in purified APC ubiquitylation reactions. C, 315 traces of ubiquitylation on individual cyclinB-NT molecules in cell extracts with either wt-ub (black) or zero-lysine-ub (Ub0K; red), or cyclinA with wt-ub (green) were aligned by the ubiquitylation initiation point (arbitrarily set at t=2) of each substrate molecule. The median number of ubiquitins on a substrate molecule is plotted vs. time. Grey traces show the boundary of standard deviation of the mean. D, as in C, but using single-lysine K64cyclinB-NT with or without 100nM Ube2S in purified APC reactions. E, histogram of the maximum number of ubiquitins on each substrate molecule during a 15-minute experiment in the cell extract. Upper, on cyclinB with either wt-ub or zero-lysine-ub; Lower, on either cyclinB or cyclinA with wt-ub.

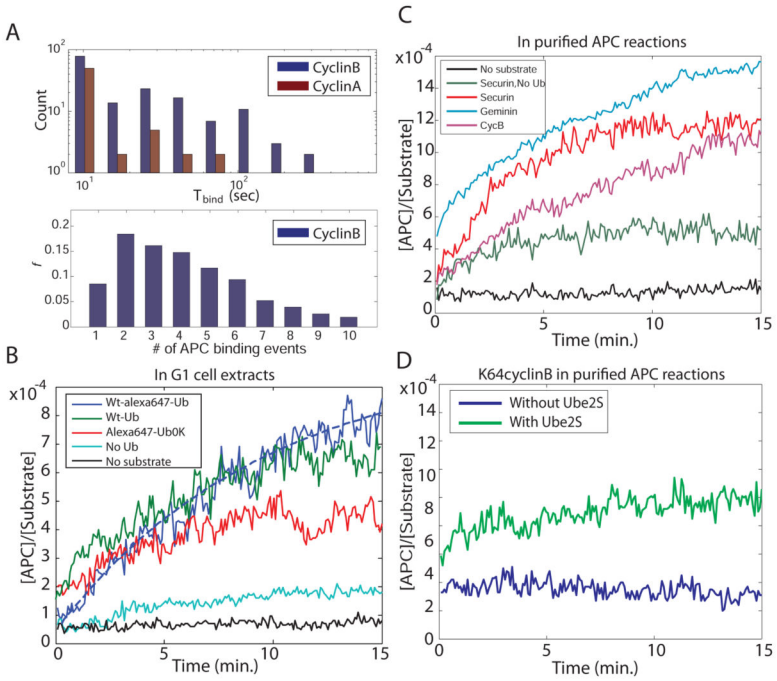

Figure 3. Kinetics of APC-substrate interaction

A, upper: Histogram of the duration of APC-substrate interaction in the cell extract, plotted on a log-log scale. Only productive binding events have been considered. Lower: Histogram of the number of APC binding events on cyclinB-NT molecules in the cell extract during a 15-minute experiment. B, time traces of the fraction of cyclinB-NT molecules bound by the APC in HeLa cell G1 extracts under the indicated conditions. About 2×10^5 cyclinB-NT molecules were immobilized as the substrate. The dashed line represents a curve fitted using a simple exponential mode described in the text. C, as in B, but performed in a purified APC ubiquitylation system with wt-alexa647-ub. Results of cyclinB-NT, geminin and securin are shown. The same analysis but using k64cyclinB-NT as the substrate in purified APC reactions, with or without 100nM E2 Ube2S, is shown in D.

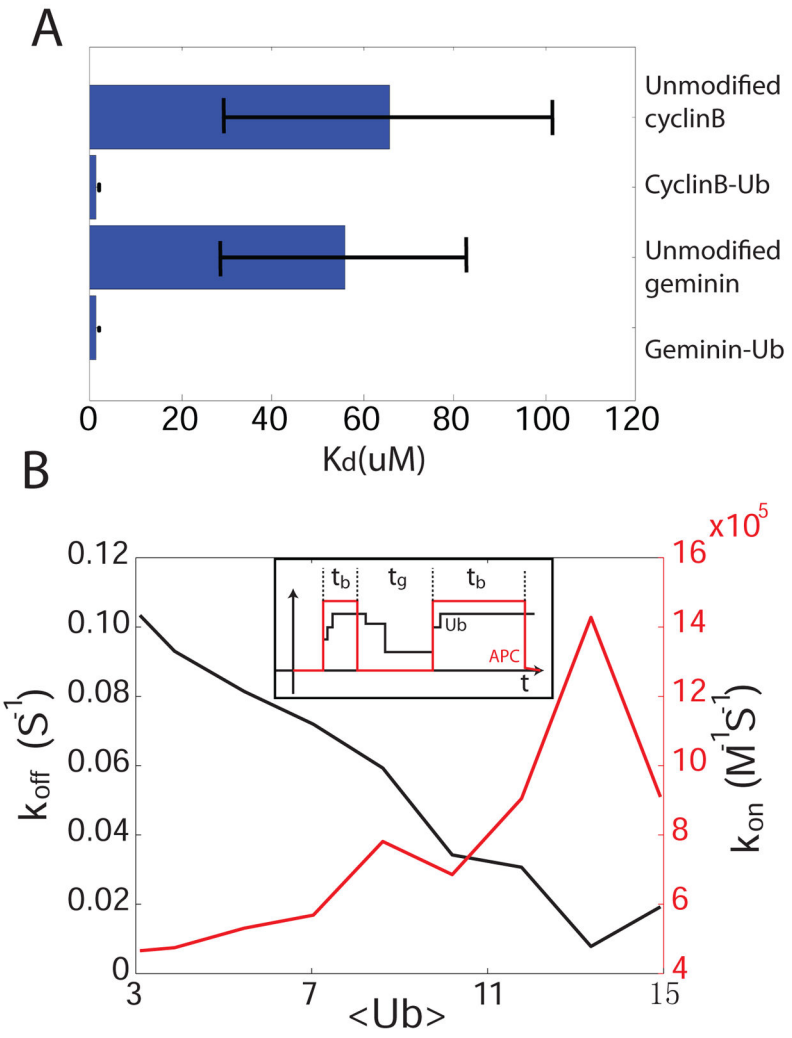

Figure 4. Ubiquitylation on a substrate enhances its affinity with the APC

A, the average K_d values for cyclinB-NT, geminin and their ubiquitylated forms with the APC in cell extracts. Error-bars represent 4 experiments. B, APC binding rate constants as a function of ubiquitylation on cyclinB-NT in a SM experiment using purified APC components. ~2300 cyclinB-NT molecules were analyzed. Inset: a schematic diagram explaining the calculation of binding rate constants. k_{off} was calculated from APC binding time t_b distribution, and k_{on} was calculated from binding gap time t_g. The distribution of t_b and t_g is shown in Fig. S21.

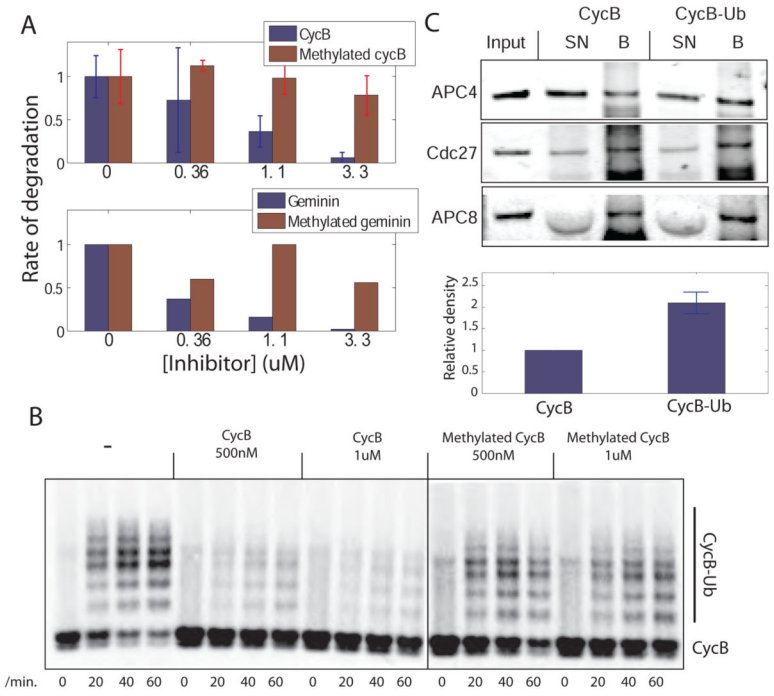

Figure 5. Substrate ubiquitylation affects APC binding kinetics in bulk assays

A, the rate of radioactive geminin degradation in HeLa cell G1 extracts, with indicated concentrations of unlabeled cyclinB-NT, geminin or their methylated forms as a competitive inhibitor. The first set of data is an experimental replicate with no inhibitor. B, radioactive cyclinB-NT was ubiquitylated by purified APC in the presence of unlabeled cyclinB-NT or methylated cyclinB-NT as a competitive inhibitor. C, assay for the effect of substrate ubiquitylation on binding with the APC. 2uM cyclinB-NT was incubated with purified APC reaction components (CycB-Ub) or without E2 (CycB), and was allowed to bind to beads. The amount of APC on beads was quantified by Westernblot, shown below. Error-bars represent the standard deviation of quantification from 3 blots. SN: supernatant, B: beads-bound

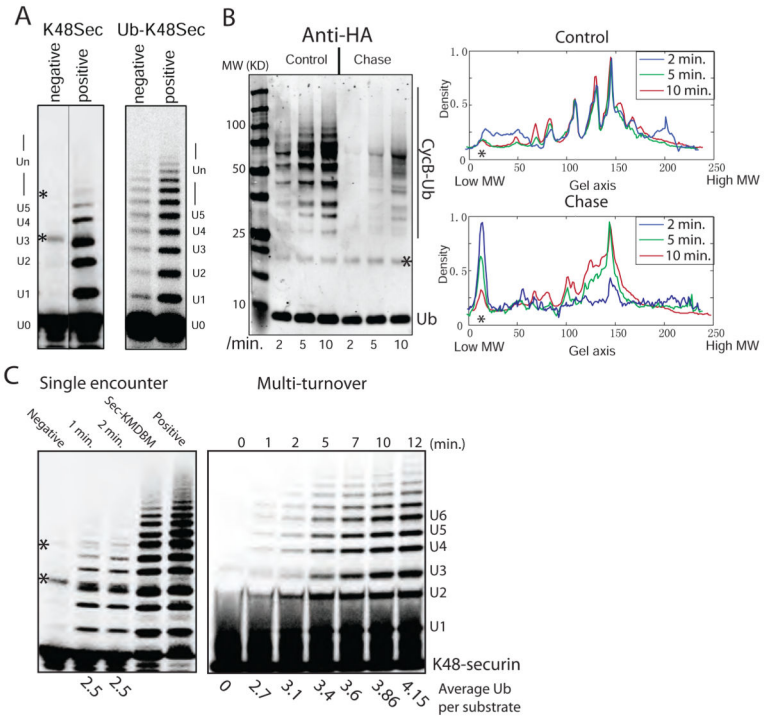

Figure 6. APC preferentially reacts with ubiquitylated substrates

A, the effect of N-terminal ubiquitin on the selectivity of ubiquitylation. Radioactive K48securin or K48securin with an N-terminal ubiquitin fusion (Ub-K48Sec) was used as the substrate in an APC ubiquitylation reaction in the presence (negative) or absence (positive) of 50uM unlabeled wt-securin as a competitive inhibitor. B, a chase experiment to determine the preference of addition of ubiquitin to substrates. 200nM cyclinB-NT was subjected to ubiquitylation by purified APC. After 5 minutes when ~40% cyclinB-NT had been ubiquitylated, excess HA-ubiquitin was added to the reaction (Chase). In Control, HA-ubiquitin was added at the beginning. Samples were taken at indicated time points and analyzed by Westernblot with anti-HA antibody. Line profiles of corresponding lanes at each time point are compared in the right panels. '*' indicates a nonspecific band. C, The degree of ubiquitin conjugation in single and multiple encounter assays. Radioactive single-lysine K48securin was used as the substrate in an APC ubiquitylation reaction including 100nM E2 Ube2S. In the single-encounter assay (left panel), 50uM unlabeled wt-securin was added to prevent rebinding of K48securin to the APC, and sampled after 1 and 2 minutes. Negative: the competitive inhibitor was added together with labeled substrate. Sec-KMDBM: using 50uM wt-securin whose D-box and KEN-box had been mutated as a competitive inhibitor. Positive: no competitive inhibitor was added. The multi-turnover assay (right panel) is a standard APC ubiquitylation reaction with no competitive inhibitor. The average number of conjugated ubiquitins on each substrate molecule was quantified using a Phosphorimager.

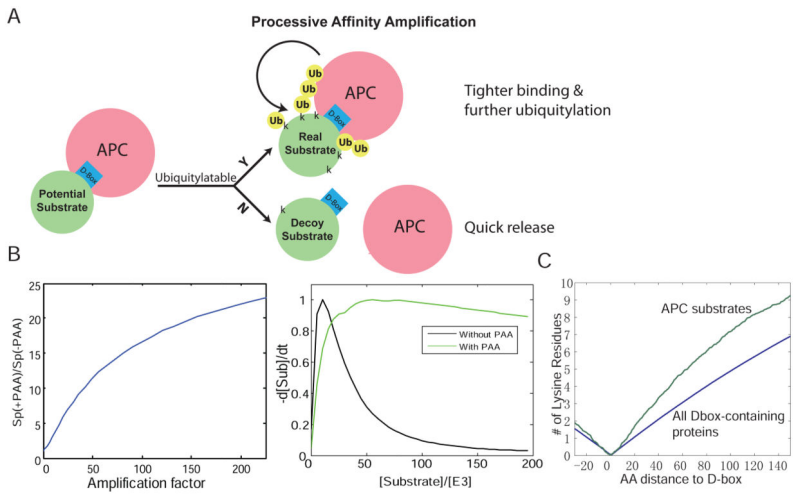

Figure 7. Processive affinity amplification enhances the specificity of APC

A, a schematic diagram showing how the PAA mechanism could enhance the specificity of APC in cytosolic environments. See the text for details. B, left, the ratio of substrate specificity of the APC in the presence and absence of PAA as a function of the amplification factor, calculated from an ODE model (Methods). Sp: specificity, defined as the ratio of concentration of APC interacting with the real substrates and APC interacting with decoy substrates. The amplification factor represents the maximum fold of K_d reduction due to PAA. Right, the maximum rate of substrate degradation as a function of total substrate concentration, calculated from the ODE model with or without the PAA mechanism. C, the average number of lysine residues within a certain distance (x-axis) next to the D-box is plotted for 74 published APC substrates from multiple species whose D-box has been identified, and for all D-box containing proteins in human proteome as a comparison.