CKAP2 Is a Spindle-associated Protein Degraded by APC/C-Cdh1 during Mitotic Exit

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We reported here an efficient and generally applicable genomic analysis that uses transcriptional profiling to identify candidate substrates of regulatory enzymes, such as kinases and ubiquitin ligases. We applied this strategy to the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that controls sister chromatid separation and exit from mitosis. We found that a microtubule-associated protein, CKAP2, is a substrate of APC/C and demonstrated that ubiquitination and degradation of CKAP2 in vitro require a KEN-box and is mediated by Cdh1, an activator of APC/C. We showed that the levels of CKAP2 fluctuated across the cell cycle in culture cells, high in mitosis and low during mitotic exit. Overexpression of Cdh1 reduced the levels of CKAP2 in a KEN-box-dependent manner, while knockdown of Cdh1 increased the half-life of CKAP2. CKAP2 associated with centrosomal microtubules in late G2, but only after the separation of the duplicated centrosomes. During mitosis, CKAP2 associated with spindle poles and with spindle microtubules from prophase through anaphase and disappeared from microtubules during cytokinesis. The function of CKAP2 during mitosis does not seem essential, as efficient knockdown of CKAP2 neither altered the cell cycle distribution of the cells, nor generated observable mitotic defects. On the other hand, ectopic expression of either the wild-type or a non-degradable CKAP2 led to a mitotic arrest with monopolar spindles containing highly bundled microtubules. We concluded that CKAP2 is a physiological substrate of APC/C during mitotic exit and that a tight regulation of the CKAP2 protein level is critical for the normal mitotic progression.

Ubiquitin-mediated proteolysis plays key regulatory functions in diverse biological processes, ranging from cell cycle control, cell signaling, transcriptional regulation, immune-response to development (1, 2). The anaphase-promoting complex/cyclosome (APC/C)2 is a key ubiquitin ligase that controls several transitions in the cell cycle (3–5). APC/C-dependent degradation of cyclin A allows cells to progress from prophase to metaphase and the degradation of securin by the APC/C triggers the chromosome separation and anaphase onset. Late in anaphase, the destruction of cyclin B1 leads to the inactivation of the Cdk1 kinase activity and exit from mitosis. The APC/C also recognizes and degrades anillin, TPX2, Aurora A, Aurora B, and Plk1 during cytokinesis, thereby allowing an ordered transition into G1 (6–8). In addition, the APC/C pathway has been linked to the control of DNA replication, and components of the prereplication complexes, such as Cdc6 and Cdt1, and an inhibitor of DNA replication, geminin, have all been shown as substrates of the APC/C (9–11). Thus, APC/C-mediated proteolysis couples the S phase with the completion of mitosis and contributes to mechanisms ensuring that the genome is replicated only once per cell cycle.

The activity of the APC/C is tightly regulated in the cell cycle: The APC/C becomes active from prometaphase until the end of G2. One of the main regulatory mechanisms for the APC/C is through its association with accessory-activating factors, Cdc20/fizzy and Cdh1/fizzy-related. Both Cdc20 and Cdh1 directly bind to and activate the APC/C ligase. Cdc20 associates with the APC/C from prometaphase to anaphase, responsible for the degradation of cyclin A, cyclin B, and securin, whereas Cdh1 maintains the activity of the APC/C from late anaphase through G1, targeting multiple substrates for degradation (12, 13). In addition, phosphorylation of the APC/C by mitotic kinases, such as Plk1, also contributes to its mitotic activation (5). On the other hand, negative regulation of the APC/C by inhibitory proteins, such as Emi1, BubR1, and Mad2, determines the precise temporal activity of the APC/C during mitosis (14–18).

The APC/C recognizes two motifs in its substrates: the destruction box (D-box; RXXXL where X is any amino acid) and the KEN-box (19, 20). Although the exact biochemical mechanism for substrate recognition remains to be characterized, it has been reported that APC/C-Cdc20 recognizes D-box-containing substrates and that APC/C-Cdh1 recognizes both D-box and KEN-box-containing substrates (19).

A key question in understanding the function of the APC/C is to identify its substrates and to investigate their physiological function and the biological importance of their degradation. To develop a general approach for identification of APC/C substrates, we analyzed the transcriptional profiles of the known APC/C substrates involved in the cell cycle regulation and found that these substrates had expression profiles that co-varied with those of the known cell cycle regulators in tumor tissues, indicating that these substrates are indeed components of...
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the cell cycle machinery whose expression is under selective pressures for co-variation during tumorigenesis. In addition, for the substrates that function in mitosis and cytokinesis, we found that the majority of them were transcriptionally induced in G2 or in mitosis. Thus, we searched for new candidate mitotic APC/C substrates whose expression co-varied with those of known cell cycle regulators in tumor tissues and whose expression was induced in G2/M in the cell cycle. Physiological substrates were then identified from this list of candidate mitotic regulators using an in vitro ubiquitination assay. In principle, this strategy is generally applicable to the identification of substrates for regulatory enzymes, such as ubiquitin ligases and kinases. Using this method, we have identified 9 new substrates of the APC/C and reported here the characterization of one of the substrates, CKAP2. CKAP2 was initially reported as a cytoskeleton-associated protein that is up-regulated in human gastric adenocarcinomas as well as in various tumor-derived cell lines (21, 22). CKAP2 is a p53 target gene that has been implicated in cell proliferation, genomic stability, and apoptosis, although the mechanism of its function remains largely unknown (23–25).

We showed that CKAP2 is a substrate of the APC/C-Cdh1, both in vitro and in vivo. The levels of CKAP2 fluctuated in the cell cycle, high in mitosis and low as cells exit into G1. Indeed, CKAP2 was degraded during mitotic exit in vivo in a Cdh1-dependent manner, as knockdown of Cdh1 stabilized the CKAP2 protein and increased its half-life. The APC/C-Cdh1 recognized a KEN-box in the N-terminal region of CKAP2, and mutations in this KEN-box abolished ubiquitination in vitro and stabilized the mutant protein in vivo. Furthermore, ectopic expression of CKAP2, either the wild-type or the mutant protein, led to a mitotic arrest with monopolar spindles containing highly bundled microtubules, indicating that the regulation of the CKAP2 protein levels is essential for normal mitotic progression. We concluded that CKAP2 is a physiological substrate of the APC/C whose mitotic degradation regulates its activity, and hence mitotic progression.

MATERIALS AND METHODS

A Genomic Approach to Identify Substrates of the APC/C—To identify candidate G2/M-induced APC/C substrates with expression patterns co-variying with those of known cell cycle regulators in tumor tissues, we used published microarray databases. Whitfield et al. (26) performed genome-wide microarray analysis in HeLa cells across the cell cycle and identified 566 genes transcriptionally induced in G2 or in mitosis. Segal et al. (27) analyzed co-expression profiles of human genes among 1975 published microarrays derived from 22 different types of tumors. Based on statistic analyses of co-expression profiles, they organized human genes into different functional modules, each of which corresponds to a set of genes that act in concert to carry out a specific physiological function. Out of 577 functional modules identified, two modules function in the cell cycle regulation and consist of genes that co-vari (co-upregulate or co-downregulate) with known cell cycle regulators (27). Thus, we analyzed the 566 G2/M-induced genes from Whitfield study (26) for their co-variation with cell cycle genes identified by Segal et al. (27). This allowed us to generate a candidate list of APC/C substrates. The full-length clones for these candidate genes were then purchased from Open Biosystems or requested from individual labs. The gene products were translated in vitro in reticulocyte lysates and assayed for ubiquitination by the APC/C-Cdh1 (8, 13). A detailed list of candidate genes will be reported in a separate study. We focus on the characterization of CKAP2 in this article.

Plasmids and Antibodies—Human CKAP2 gene, a gift from Dr. Joobae Park (Sungkyunkwan University, Suwon, Korea), was subcloned into pCS2-FA and pCS2-eGFP-FA vectors. KEN-box mutation in CKAP2 was generated using the QuickChange site-directed mutagenesis kit (Stratagene). Tagged CKAP2 N-terminal domain (amino acids 1–404) was expressed in Escherichia coli, purified by nickel agarose under denaturing conditions, and used to immunize rabbits for production of antiserum. Anti-CKAP2 antibodies were subsequently purified against the antigen.

In Vitro Ubiquitination Assays—Interphase and mitotic extracts were made from Xenopus eggs as described previously (14). Interphase extracts from Xenopus eggs were immunoprecipitated with anti-Cdc27 antibody-protein A beads for 2 h at 4 °C to purify interphase APC/C (iAPC/C) (8, 13). The iAPC/C beads were collected by centrifugation and washed five times in the XB buffer (10 mM HEPES-KOH, pH7.8, 100 mM KCl, 1 mM MgCl2, 0.1 mM CaCl2, 50 mM sucrose) containing 500 mM KCl and 0.5% Nonidet P-40, and two times in the XB buffer. Purified iAPC/C beads were then incubated with in vitro translated Cdh1 or Cdc20 for 1 h at 25 °C and then washed twice with the XB buffer. Ubiquitination reactions were initiated by mixing [35S]–labeled substrate with E1 (50 μg/ml), E2 (50 μg/ml), ubiquitin (1.25 mg/ml), ubiquitin aldehyde (1 μM), and an energy regeneration mix (8, 13). Reactions were preformed at 25 °C and stopped at various times by addition of the SDS sample buffer. Samples from each time point were then analyzed by SDS-PAGE and Phosphorimager.

In Vitro Degradation Assays—Interphase or mitotic Xenopus egg extracts, with or without prior incubation of in vitro translated Cdh1, were incubated with [35S]–labeled substrates in the presence of ubiquitin (1.25 mg/ml), ubiquitin aldehyde (1 μM), and an energy regeneration mix at 30 °C, as described previously (13). Samples from each time point were then analyzed by SDS-PAGE and Phosphorimager.

Cell Culture, siRNAs, and Transfection—HeLa S3 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Invitrogen) and antibiotics. To determine the levels of the CKAP2 protein across the cell cycle, cells were synchronized either at the G1/S boundary by double thymidine treatment or at prometaphase by a thymidine-nocodazole treatment (13).

siRNAs were synthesized by Dharmacon, Inc. Sequences targeted to CKAP2 were (A) 5’-GGATATGTCCTTGCGACTTATTTT-3’, (B) 5’-GGACTACCATGGCAGAAGATT-3’, and (C) 5’-GAGGACAAAGTTGCTTTTATTTT-3’. siRNA against Cdh1 was 5’-GCAAGATGTGTTCCCTATT-3’ and the control siRNA (siGL2) was 5’-CTTAGCGGGAATCTTCCGATT-3’. Short hairpin RNA (shRNA) against Cdh1 was synthesized by in vitro transcription with the following sequence: 5’-GGATTAACGAGAATGGAAGT-3’. Control shRNA
against GFP was 5'-GCAAAGCTGACCCTGAAGTTC-3'. siRNAs were transfected into HeLa cells using DharmaFect 1 (Dharmacon, Inc.). For CKAP2, all three siRNAs gave similar degrees of knockdown and all three gave identical phenotypes, confirming the specificity of the knockdown.

DNA transfection was performed using Effectene (Qiagen) or Lipofectamine 2000 (Invitrogen) as instructed by the manufacturers. In experiments with ectopic expression of CKAP2 and its KEN mutant, the levels of expressed proteins was about 15 folds compared with that of the endogenous CKAP2 protein. To determine the stability of the CKAP2 protein in vivo, transfected cells were arrested at prometaphase by a nocodazole treatment followed by release into fresh media in the presence of 10 μg/ml cycloheximide (8). Cells were harvested, lysed, and subjected to Western blot analysis.

Immunofluorescence—To determine the localization of CKAP2, cells were cultured on poly-lysine-coated glass coverslips overnight and then fixed with −20 °C methanol. After fixation, cells were permeabilized and blocked with PBS-BT (1× PBS, 0.1% Triton X-100, 3% bovine serum albumin) at the room temperature for 30 min. Coverslips were subsequently incubated in primary and secondary antibodies diluted in PBS-BT. Images were acquired with Openlab 5.0 (Improvision) under a Zeiss Axiovert 200M microscope using 100 oil immersion lens. Deconvolved images were obtained using AutoDeblur v9.1 and AutoVisualizer v9.1 (AutoQuant Imaging).

RESULTS

A Genomic Approach to Efficiently Identify Mitotic Substrates of APC/C—To identify novel mitotic substrates of APC/C, we selected candidate genes based on their gene expression profiles and then assayed their ability to be ubiquitinated by the APC/C in an in vitro reconstituted APC/C assay. Our genomic analysis was based on two predictions. First, we predicted that substrates that solely function in mitosis or cytokinesis are likely transcriptionally induced in G2 or in mitosis prior to their function. On the other hand, we expected that only a subset, but not all, of these G2/M-induced genes acts in mitosis and cytokinesis and therefore needed another criterion to select functionally important ones. Second, we predicted that expression of genes in the core mitosis and cytokinesis machinery tends to co-vary during tumorigenesis, as these regulators are likely to function as one module during tumor proliferation. It has been reported that 566 genes in human genome are induced in G2 or
in mitosis in HeLa cells (26). Thus, we searched in published microarray databases (27, 28) for novel ones among these 566 genes that transcriptionally co-varied (co-induced or co-repressed) in hundreds of tumor tissues with known cell cycle regulators, such as cyclin A, cyclin B, Plk1, Aurora A, Aurora B, and Cdc20.

From these analyses, we generated a list of G2/M-induced genes (the candidate list) whose expression co-varied with those of known cell cycle regulators across hundreds of tumor tissues. Next, we determined whether this list enriched known mitotic substrates of the APC/C. We analyzed 16 known substrates of the APC/C that have been characterized at the beginning of this study. These substrates included cyclin A, cyclin B, Plk1, Aurora A, Aurora B, and Cdc20.

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The CKAP2 Levels Fluctuate in the Cell Cycle—To investigate the regulation of CKAP2 in vivo, we raised and affinity-purified an anti-CKAP2 antibody. Next, we analyzed the protein levels of CKAP2 across the cell cycle and compared the CKAP2 levels with those of known substrates of APC/C. HeLa S3 cells were synchronized at the G1/S boundary by a double thymidine treatment and then released into fresh medium (13). The cell cycle profile of released cells was analyzed by fluorescence-activated cell sorter (FACS), and the mitotic time points were determined by the presence of phospho-histone H3 in the Western blot analysis (Fig. 4A). Total cell lysates were also subject to Western blot analysis for CKAP2 and various mitotic regulators. CKAP2 was present at the G1/S boundary and the protein accumulated to some extent as cells progressed from S and G2 into mitosis (Fig. 4A). Interestingly, the levels of CKAP2 were rapidly decreased as cells exited from mitosis and into G1 (Fig. 4A). The kinetics of the reduction of the CKAP2 protein was very similar to those of cyclin A, securin, cyclin B, and Cdc20, the four known substrates of APC/C in mitosis (4). To further examine the detailed kinetics of mitotic degradation of CKAP2, HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole treatment, followed by a release into fresh media (14). The CKAP2 levels were again down-regulated upon release from the prometaphase arrest. The degradation of CKAP2 occurred with kinetics very similar to that of Cdc20, but slightly slower than that of cyclin B (Fig. 4B). We concluded that CKAP2 is degraded during mitotic exit.

CKAP2 Is a Substrate of APC/C-Cdh1 in Vivo—To confirm that the APC/C-Cdh1 is required for the destruction of CKAP2 in vivo, we inhibited the activity of the APC/C-Cdh1 by RNA interference. HeLa cells were transfected with a Cdh1-specific siRNA (siCdh1) or a control siRNA (siGL2). Transfection of siCdh1, but not siGL2, reduced the Cdh1 protein levels and resulted in an increase in endogenous CKAP2 and anillin, a known APC/C substrate (8) (Fig. 5A). This change in the CKAP2 protein levels was not because of a change in the cell cycle distribution of the transfected cells, as FACS analysis indicated that the knockdown of Cdh1 at the indicated level did not alter the cell cycle profile of transfected cells (Fig. 5A).

A change in the steady-state levels of the CKAP2 protein can be caused by a change either in the rate of protein synthesis and/or in the rate of protein degradation. To demonstrate that this increase in the CKAP2 levels is directly because of the sta-

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**FIGURE 4. The levels of the CKAP2 protein fluctuate in the cell cycle.** A, HeLa S3 cells were synchronized at the G1/S boundary by a double thymidine arrest. Cells were released into fresh media and harvested every hour. The levels of CKAP2, cyclin A, securin, cyclin B, Cdc20, phospho-histone H3 (Ser-10), and p38MAPK (a loading control) were analyzed by Western blotting of total cell lysates. Cell cycle stages were determined by FACS analysis. B, HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole treatment. Cells were released into fresh media and harvested at the indicated time. Cell lysates were Western-blotted with antibodies against CKAP2, securin, cyclin B, Cdc20, phospho-histone H3 (Ser-10), and p38MAPK (a loading control). Cell cycle profile was determined by FACS analysis.

labeled CKAP2, Cdh1-activated interphase extracts, but not mitotic extracts, efficiently degraded CKAP2 (Fig. 2A). This degradation is APC/C-Cdh1 dependent, as addition of recombinant Emi1 to the Cdh1-treated interphase extracts completely blocked the CKAP2 degradation (Fig. 2C). We concluded that CKAP2 is a specific substrate for APC/C-Cdh1, but not for APC/C-Cdc20.

**Degradation of CKAP2 Is KEN-box-dependent**—We next determined which region of CKAP2 is recognized by APC/C-Cdh1. It has been shown that APC/C-Cdh1 recognizes either a KEN-box (K-E-N) or a Destruction-box (RXL; D-box) motif in substrates (1, 2). CKAP2 contains four D-boxes and one KEN-box (Fig. 3A). To determine the region of CKAP2 essential for its ubiquitination, we constructed N-terminal (CKAP2: 1–404) and C-terminal (CKAP2: 405–683) fragments of CKAP2. In our in vitro ubiquitination assay, only N-terminal CKAP2, not the C-terminal CKAP2, was efficiently ubiquitinated by APC/C-Cdh1 (Fig. 3B). The N-terminal CKAP2 contains 3 D-boxes and one KEN-box, but only the KEN-box is conserved among human, mouse, and chicken CKAP2. Thus, we mutated the KEN motif in the full-length CKAP2 protein to AAA amino acids (CKAP2-KEN). This KEN-box mutant was resistant to ubiquitination by APC/C-Cdh1 and failed to be degraded in Cdh1-activated interphase extracts (Fig. 3, C and D; cf. Fig. 1C). Therefore, we concluded that APC/C-Cdh1 recognizes the KEN-box in CKAP2 for its degradation.

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**FIGURE 3**.  **A**  Detection of CKAP2 in mitotic extracts. B, HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole treatment, followed by a release into fresh media (14). The detailed kinetics of mitotic degradation of CKAP2, HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole treatment, followed by a release into fresh media (14). The CKAP2 levels were again down-regulated upon release from the prometaphase arrest. The degradation of CKAP2 occurred with kinetics very similar to that of Cdc20, but slightly slower than that of cyclin B (Fig. 4B). We concluded that CKAP2 is degraded during mitotic exit.

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A

| Time (h) | siGL2 | siCdh1 | Anillin | CKAP2 | Cdh1 | p38MAPK |
|---------|-------|--------|---------|-------|------|--------|
| 42      |       |        |         | +     |      |        |
| 58      | +     | -      | -       |       |      | +      |

B

| Time (h) | GFP-shRNA | Cdh1-shRNA |
|----------|------------|-------------|
| 0        | Anillin    | CKAP2       |
| 1        |            |              |
| 2        |            |              |
| 4        |            |              |

C

| Time (h) | WT-GFP | KEN-GFP |
|----------|--------|---------|
| G2/M     | HA-Cdh1|         |
| 25       | -      | +       |
| 21       | +      | -       |

To determine the potential role of CKAP2 in mitosis, we reduced the expression of endogenous CKAP2 in HeLa cells by RNAi. Among 12 siRNA sequences analyzed, three (siCKAP2-A, -B, and -C) gave efficient knockdown as analyzed by Western blotting (Fig. 6C). Depletion of the endogenous CKAP2 was also confirmed by immunofluorescence staining (Fig. 6D), which supported the specificity of the CKAP2 localization reported in Fig. 6A. Although we achieved greater than

3 G. Fang, unpublished data.
95% reduction of the CKAP2 protein in HeLa cells (Fig. 6C; data not shown), no change in the cell cycle profile was observed between siCKAP2 cells and control cells as revealed by the FACS analysis (Fig. 6E). Furthermore, mitosis in knockdown cells was carefully assayed by examining the spindle structure on fixed mitotic cells as well as by analyzing time-lapse movies on the kinetics of mitotic chromosome congression and segregation in HeLa cells stably expressing GFP-Histone H2B. Cytokinesis in knockdown cells were also extensively investigated by time-lapse DIC imaging of HeLa cells undergoing division. In all assays examined, no defect was observed for mitosis and cytokinesis in CKAP2 knockdown cells (data not shown). In
addition, the stability and the dynamics of the mitotic spindle in CKAP2-knockdown cells were assayed by analyzing microtubule depolymerization and repolymerization kinetics in the nocodazole-treated and released mitotic cells, respectively. Again, no difference was observed when compared with the control knockdown cells (data not shown). Recently, CKAP2 has been implicated in the transition through the restrict point in G1, as reduction of its expression in human foreskin fibroblasts leads to a reduction of phospho-pRb and an accumulation of p27 (25). However, in the transformed HeLa cells we analyzed, efficient knockdown of CKAP2 did not affect the levels of the p27 protein (Fig. 6F), suggesting a difference between transformed versus primary cells. We concluded that at the level of knockdown we achieved (>95%) CKAP2 does not seem to play an essential role in cell cycle progression and that the function of CKAP2 on the mitotic spindle is likely redundant to other spindle-associated proteins.

Ectopic Expression of CKAP2 Leads to Defects in the Mitotic Spindle—We attempted to address the physiological importance of CKAP2 degradation during mitotic exit. Our initial plan was to establish stable cell lines expressing the CKAP2 or CKAP2-KEN transgene at levels similar to that of the endogenous protein and then examine the effect of expression of the nondegradable variant on cell cycle progression. However, the CKAP transgene cannot be stably expressed; out of 24 stable CKAP2 clones analyzed, not a single clone expressed the CKAP2 transgene at a level above 5% of the endogenous protein (data not shown), indicating that a tight regulation of the CKAP2 level is essential for cell proliferation.

Next, we ectopically expressed GFP, CKAP2-GFP, or CKAP2-KEN-GFP in HeLa cells in transient transfection experiments. To determine phenotypes directly derived from CKAP2 expression, but not indirect or secondary phenotypes, we analyzed cells within their first two cell cycles post-transfection (collected at 38-h post-transfection). Based on FACS analysis of GFP-positive cells, ectopic expression of CKAP2 increased the G2/M populations (30, 28, and 20% for CKAP2-lysate of GFP-positive cells, ectopic expression of CKAP2 expression (collected at 38-h post-transfection). Based on FACS analysis of GFP-positive cells, ectopic expression of CKAP2 increased the G2/M populations (30, 28, and 20% for CKAP2-GFP, CKAP2-KEN-GFP, and GFP-expressing cells, respectively). To investigate whether this increase in G2/M cells is due to a mitotic arrest, mitotic index was determined by counting the percentage of phosphorylated histone H3 cells among the GFP-positive cells. We found that CKAP2-GFP, CKAP2-KEN-GFP, and GFP-expressing cells have a mitotic index of 11.8% (n = 441 cells), 11.9% (n = 486 cells), and 4.9% (n = 490 cells), respectively, indicating a mitotic arrest or delay upon expression of CKAP2.

Morphological analysis of fixed cells indicated that expression of CKAP2, independent of the wild-type protein or the KEN-box variant, accumulated cells at early prometaphase with the monopolar spindle containing highly bundled microtubules (Fig. 7A). In fact, the majority of the CKAP2-GFP- and CKAP2-KEN-GFP-expressing mitotic cells (64 and 58%, respectively) were arrested with the monopolar spindle, whereas no such cells were observed in GFP-transfected cells (Fig. 7D). Time-lapse imaging indicated that cells with the monopolar spindle were stably arrested at prometaphase for greater than 10 h, the duration of our experiments (data not shown). Immunofluorescence staining of fixed cells with anti-γ-tubulin and anti-centrin antibodies indicated that centrosomes were duplicated in cells with the monopolar spindle and that the γ-tubulin signals existed as a pair of dots without separation at the pole of the monopolar spindle (data not shown). A population of transfected mitotic cells (19, 23, and 28% for CKAP2-GFP, CKAP-KEN-GFP, and GFP-expressing cells, respectively) did reach the metaphase with bipolar spindles at the time of our analysis (Fig. 7, B–D). However, among cells with bipolar spindles, greater than three-fourths of CKAP2-GFP- and CKAP-KEN-GFP-expressing metaphase cells had asymmetric bipolar spindles containing highly bundled microtubules, whereas only one-tenth of GFP-transfected metaphase cells had asymmetric bipolar spindles and none with highly bundled spindle (Fig. 7, B and D). Thus, the predominant phenotype of the ectopic expression of CKAP2, either the wild-type protein or the KEN variant, was defects in the mitotic spindle structure with the formation of the highly bundled monopolar spindle or bundled asymmetric bipolar spindle. These phenotypes, which occurred at prometaphase and metaphase, cell cycle stages prior to the degradation of CKAP2, precluded our attempt to analyze the physiological importance of the CKAP2 destruction during mitotic exit.

We also noted that 7–9% of CKAP2-GFP- and CKAP2-KEN-GFP-expressing mitotic cells underwent anaphase and cytokinesis at the time of our analysis, but without obvious defects in cytokinesis (Fig. 7D). Furthermore, no increase in bi-nucleated cells was observed even at 72-h post-transfection (data not shown), indicating that ectopic expression of CKAP2 did not directly interfere with cytokinesis. Quantitative Western blot analysis indicated that the levels of ectopically expressed CKAP2-GFP in our experiments were about 15-fold of that of the endogenous CKAP2 (Fig. 7E).

It has recently been reported that CKAP2 is involved in growth control in human foreskin fibroblasts, as constitutive expression of CKAP2 increased pRb phosphorylation and decreased the level of p27 (25). However, when analyzed in our transient transfection experiments, ectopic expression of CKAP2-GFP or CKAP2-KEN-GFP did not alter the levels of the p27 protein in HeLa cells (Fig. 7F), suggesting that the degradation of CKAP2 is unlikely to be directly involved in the control of proliferation in our system.

DISCUSSION

A Genomic Approach to Identify Substrates of a Ubiquitin Ligase—Key biological transitions are usually controlled by regulatory enzymes, such as kinases and ubiquitin ligases and identification of substrates for these enzymes is essential for understanding the regulatory mechanisms. However, substrate identification has traditionally proved to be a challenging task due to the weak and transient nature of substrate-enzyme interactions. In this report, we demonstrated a generally applicable approach for the identification of substrates based on the function of the regulatory enzyme under study.

In the case of the APC/C, a ubiquitin ligase acting in the cell cycle regulation, we expected that the majority of its substrates are also involved in the cell cycle control. Thus, we searched for candidate cell cycle substrates based on their transcriptional
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Through the approach described above, we identified CKAP2 as a candidate substrate of the APC/C. The following evidence indicated that CKAP2 is indeed a physiological substrate of the APC/C. First, the full-length CKAP2 was efficiently ubiquitinated by APC/C-Cdh1 in vitro and degraded in Xenopus extracts in a Cdh1-dependent manner (Figs. 1 and 2). Second, Western blotting experiments showed that, similar to cyclin B, securin and Cdc20, the levels of CKAP2 fluctuated in the cell cycle, high in mitosis and low as cells exit into G1 (Fig. 4). This is consistent with a recent report showing that the levels of CKAP2 are low in serum-starved cells as we were able to identify 9 new mitotic substrates of APC/C by a single person in three months (see “Results”).

This strategy can be easily applied to other ubiquitin ligases and kinases functioning in different cellular processes, such as in S phase, in DNA damage responses or in apoptosis. Indeed, this is a generally applicable approach to identify key regulatory proteins important for many biological processes, independent of knowledge on any known enzymatic components in these processes. For example, we have used a similar strategy to identify novel mitotic and cytokinesis regulators (33, 34). We first generated a candidate list of novel cell cycle regulators based on their co-variation with known cell cycle regulators in tumor tissues and then defined their functional specificity to mitosis and cytokinesis based on their induction in G2 and in mitosis. These candidate genes were then analyzed for their cellular localizations and their loss-of-function phenotypes. Through this approach, we have previously identified the hepatitis up-regulated protein (HURP) and Cep55 as candidate mitotic regulators (33, 34). We further showed that HURP is a microtubule-associated protein localized to the chromatin-proximal spindle and that HURP is required for the high fidelity of chromosome separation (33). Similarly, we demonstrated that Cep55 is a midbody matrix component required for cell abscission at the terminal stage of cytokinesis (34). Thus, our transcriptional profiling-based method reported here is a powerful genomic approach to identify novel molecular players in different cellular processes.

**FIGURE 7.** Ectopic expression of CKAP2-GFP accumulates cells at prometaphase with monopolar spindles. A–D, effects of ectopic expression of CKAP2 on mitotic progression. HeLa cells were transfected with CKAP2-GFP, CKAP2-KEN-GFP, and GFP and then fixed and immunostained at 38-h post-transfection. Shown in A–C are maximum projections from deconvolved z-stacks of CKAP2-GFP (A and B) and control (C) transfected cells stained for GFP (A and B)/CKAP2 (C) (green), β-tubulin (red), and DNA (blue). Overexpression of CKAP2-GFP generated the monopolar spindle containing highly bundled microtubules (A), as well as the narrow, asymmetric bipolar spindle (B). Bars in A, B, and C, 5 μm. Shown in D is a statistical analysis of CKAP2-expressing mitotic cells. GFP (n = 213 mitotic cells), CKAP2-GFP (n = 243 mitotic cells), or CKAP2-KEN-GFP (n = 247 mitotic cells) transfected mitotic cells were quantified and categorized into cells with a monopolar spindle, with an asymmetric bipolar spindle, with a symmetric metaphase bipolar spindle, and with normal prometaphase and anaphase spindle structure. E, quantitative Western analysis on the levels of ectopically expressed CKAP2-GFP. Lysates from control transfected (lane 1), CKAP2-GFP-transfected (lanes 2 and 4), and CKAP2-KEN-GFP-transfected (lanes 3 and 5) cells were analyzed by Western blot (WB) with antibodies against CKAP2 and p38MAPK. The amounts of cell lysates loaded in lanes 2 and 3 and in lanes 4 and 5 were equal to 25 and 40% of that in lane 1, respectively. F, effect of ectopic expression of CKAP2 on the p27 levels. HeLa cells were transfected with CKAP2-GFP (lane 1), CKAP2-KEN-GFP (lane 2) or GFP (lane 3). Cells were collected at 38-h post-transfection, and the levels of p27 and p38MAPK (a loading control) were determined by Western blot analysis of total cell lysates.
to an increase in CKAP2 and this increase was directly due to the stabilization of the CKAP2 protein during mitotic exit (Fig. 5). Fourth, overexpression of Cdh1 resulted in a decrease in the wild-type CKAP2 levels (Fig. 5). Finally, APC/C specifically recognized a KEN-box in the N-terminal region of CKAP2 and mutation of this KEN-box prevented ubiquitination in vitro and led to a stabilization of the mutant protein in vivo (Figs. 3 and 5). Thus, we concluded that CKAP2 is degraded by the APC/C-Cdh1 pathway during mitotic exit.

Physiological Function of CKAP2 in the Cell Cycle—CKAP2 has recently been implicated in growth control, aneuploidy, and apoptosis (24, 25). For example, CKAP2 has been proposed to be involved in the proliferation of human foreskin fibroblasts, as knockdown of CKAP2 reduces pRb phosphorylation and increases p27 expression in these cells, whereas constitutive expression of CKAP2 has the opposite effects (25). However, we found that at least in HeLa cells, neither knockdown nor ectopic expression of CKAP2 affected the levels of the p27 protein (Figs. 6 and 7). This discrepancy is likely due to a difference between transformed and primary cell lines, or between different tissue types, suggesting that CKAP2 may have cell line- or tissue-specific functions.

It has been previously reported that ectopic expression of CKAP2 in a p53-negative cell line, such as HCT116, leads to failed cytokinesis, suggesting that down-regulation of CKAP2 by proteolysis may be critical for cell division (24). We found that in the p53-negative HeLa cells, ectopic expression of CKAP2 did not lead to failed cytokinesis (Fig. 7). We also performed our experiments with ectopic expression of CKAP2-GFP and CKAP2-KEN-GFP in the p53-positive HCT116 cells and observed a phenotype similar to that found for HeLa cells (data not shown). Furthermore, we did not detect any increase in failed cytokinesis in HCT116 cells expressing either the wild-type or the KEN-box mutant of CKAP2 (data not shown). This discrepancy with the previous report (24) may be due to the different expression levels or due to different experimental design. As we are interested in the functional importance of the degradation of the CKAP2 protein at its physiological levels during mitotic exit, we controlled our ectopically expressed proteins at relatively low levels. Our data in both HeLa cells and HCT116 cells suggested that down-regulation of CKAP2 is not absolutely required for the execution of cytokinesis and that other pathways, such as post-translational modifications, may be able to independently inactivate CKAP2 during mitotic exit. It is also possible that the levels of ectopically expressed CKAP2 in our experiments may be below a threshold level required for it to exert a dominant effect to interfere cytokinesis.

What is the physiological significance of degradation of CKAP2? CKAP2 is a spindle-associated protein with a strong microtubule bundling activity (Figs. 6 and 7). Thus, down-regulation of the CKAP2 protein levels through its degradation during mitotic exit provides a mechanism to ensure that its activity on spindle microtubules is strictly regulated in the cell cycle. Indeed, stable accumulation of CKAP2 interferes with cell proliferation, as we were not able to isolate any stable CKAP2-GFP clones with an expression of the transgene at a level greater than 5% of the endogenous CKAP2 protein (data not shown). Strikingly, in the transient transfection experiments, ectopic expression of CKAP2, either the wild-type protein or its nondegradable variant, led to a stable prometaphase arrest with monopolar spindles, providing the direct evidence that the proper regulation of the CKAP2 protein level is essential for normal mitotic progression. This arrest by the monopolar spindle occurred at a cell cycle stage prior to the degradation of CKAP2, thereby precluding our effort to directly analyze the physiological importance of CKAP2 degradation.

We speculate that uncontrolled accumulation of the CKAP2 protein in the cell cycle could alter the structure and dynamics of the mitotic spindle and the central spindle during anaphase and cytokinesis (24), at which stages the cells undergo dramatic reorganization of the microtubule assembly for chromosome separation and cell division. Alternatively, degradation of CKAP2 may be required, in some cell types, for the execution of the certain G1 events, such as the passage through the restriction point, which is consistent with the proposed function of CKAP2 in controlling the phosphorylation of pRb and the accumulation of the p27 protein (25).

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