Down-regulation of p27Kip1 by Two Mechanisms, Ubiquitin-mediated Degradation and Proteolytic Processing*

(Received for publication, December 10, 1998, and in revised form, February 15, 1999)

Michiko Shirane‡§, Yumiko Harumiya, Noriko Ishida‡§, Aizan Hirai**, Chikara Miyamoto‡‡, Shigetsugu Hatakeyama‡§, Kei-ichi Nakayama‡§ §§, and Masatoshi Kitagawa‡‡‡

From the ‡Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, §CREST, Japan Science and Technology Corporation, Kita-ku, Fukuoka 812-0012, the *Department of Molecular Biotherapy Research, Cancer Chemo therapy Center, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 170-8455, the †Second Department of Internal Medicine, Chiba University School of Medicine, Chiba 260-856, **Chiba Prefectural Togane Hospital, Togane 283-8588, and ‡‡‡Nippon Roche Research Center, Kamakura 247-0063, Japan

The intracellular level of p27Kip1, a cyclin-dependent kinase (CDK) inhibitory protein, is rapidly reduced at the G1/S transition phase when the cell cycle pause ceases. In this study, we demonstrated that two post-translational mechanisms were involved in p27Kip1 breakdown: degradation via the ubiquitin (Ub)-proteasome pathway and proteolytic processing that rapidly eliminates the cyclin-binding domain. We confirmed that p27Kip1 was ubiquitinated in vitro as well as in vivo. The p27Kip1 ubiquitination activity was higher at the G1/S boundary than during the G0/G1 phase, and p27Kip1 ubiquitination was reduced significantly when the lysine residues at positions 134, 153, and 165 were replaced by arginine, suggesting that these lysine residues are the targets for Ub conjugation. In parallel with its Ub-dependent degradation, p27Kip1 was processed rapidly at its N terminus, reducing its molecular mass from 27 to 22 kDa, by a ubiquitination-independent but adenine triphosphate (ATP)-dependent mechanism with higher activity during the S than the G0/G1 phase. This 22-kDa intermediate had no cyclin-binding domain at its N terminus and virtually no CKD2 kinase inhibitory activity. These results suggest that p27Kip1 is eliminated by two independent mechanisms, ubiquitin-mediated degradation and ubiquitin-independent processing, during progression from the G1 to S phase.

Cell cycle progression is controlled by a series of kinase complexes composed of cyclins and cyclin-dependent kinases (CDKs) (1). The enzymatic activities of cyclin/CDK complexes are regulated by many mechanisms that reflect both the diversity of the signals they integrate and the central importance of their roles in cell cycle control. These regulatory mechanisms include variations in cyclin levels, positive- and negative-acting phosphorylation of the kinase subunit, and the actions of CDK inhibitors (CKIs) (2). Of these, the CKIs appear to be the most diverse and flexible regulators. Mammalian CKIs are classified into two families: the Cip/Kip and Ink4 families. The former consists of p21Cip1/Waf1, p27Kip1, and p57Kip2, each of which has a conserved domain, called the CDK-binding/inhibitory domain, at its N terminus.

The CKI p27Kip1 plays a pivotal role in the control of cell proliferation (3–5). Transition from the G1 to S phase is promoted by G1 cyclin/CDK complexes, such as cyclin D/CDK4 and 6 and cyclin E/CDK2, and p27Kip1 inhibits the activities of these kinases directly by binding to them (6–9). The elimination of p27Kip1 during the late G1 phase is required for G1 cyclin/CDK complex activation and cell cycle progression from the G1 to S phase in various cell lines (10–13). Consistent with this idea is that forced expression of p27Kip1 blocks cell cycle progression during the G1 phase, whereas targeted p27Kip1 mRNA antisense vectors increase the fraction of cells in the S phase. Moreover, p27Kip1 down-regulation due to enhanced degradation in various malignant neoplasms, such as colorectal, breast, stomach, and non-small-cell lung cancers, was observed (14–18). Finally, we and others demonstrated that targeted disruption of the mouse p27Kip1 gene resulted in enhanced growth of mice, multiple organ hyperplasia, and a predisposition to tumors (19–21). These lines of evidence support the idea that p27Kip1 is a key molecule that negatively regulates cell cycle progression.

A major question is: how are the intracellular levels of p27Kip1 regulated in a precisely timed fashion? Previous studies showed that p27Kip1 mRNA does not fluctuate during the cell cycle, implying the existence of posttranslational machinery that controls the p27Kip1 expression levels (3, 22). Genetic studies on yeast revealed that SIC1, a CKI controlling the G1/S transition, like mammalian p27Kip1, is degraded specifically by the ubiquitin (Ub)-proteasome system (23–25). Furthermore, the Ub-proteasome pathway was suggested to be involved in p27Kip1 degradation in mammals (26). The Ub-proteasome pathway is emerging as a major and universal mechanism that regulates selective and time-controlled elimination of short-lived key regulatory proteins, e.g. cell cycle proteins (cyclins (27, 28) and CKIs (29)) and transcriptional activators (IXB (30, 31), c-Jun (32), p53 (33), β-catenin (34), and others). This pathway requires adenosine triphosphate (ATP) and the covalent conjugation of target proteins with multiple Ub molecules (35–37). This multistep process involves Ub activation by a Ub-activating enzyme (E1), followed by transfer of Ub to a Ub-
conjugating enzyme (E2), and the third step is the transfer ofUb to a Ub ligase (E3), which catalyzes the formation of isopeptide bonds between the C-terminal glycine of Ub and the ε-amino groups of lysine residues on the target proteins. During subsequent cycles, additional Ub molecules are added to the substrate. Then, multi-ubiquitinated proteins are recognized by the 26 S (1500 kDa) proteasome complex and rapidly degraded into short peptides. 26 S proteasomes are multicatalytic protease complexes containing chymotrypsin-like, trypsin-like, and postglutamyl activities together with ATP.

Therefore, it is important to elucidate the mechanisms responsible for p27Kip1 breakdown, not only to improve our understanding of cell-growth control, but also for the discovery of new anti-cancer drugs. In this study, we found that p27Kip1 is down-regulated at the G1/S transition point by two pathways: Ub-mediated degradation and a novel ubiquitination-independent processing pathway that abrogates p27Kip1 function by eliminating its cyclin-binding domain.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ub and ubiquitin aldehyde (Ub-CHO) were purchased from Sigma and Pure Chemical Industries, Ltd. (Osaka, Japan). The proteasome inhibitors lactacystin and clasto lactacystin β-lactone were purchased from Kyowa Medics (Tokyo, Japan) and Calbiochem, respectively, and the calpain inhibitor ALLN (N-acetyl-Leu-Leu-nor-leucinal) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). The proteasome inhibitor ZLLLAL (Z-Leu-Leu-Leu-H aldehyde) and caspase inhibitors Ac-DEVD-CHO and Ac-YVAD-CHO were purchased from Peptide Institute Inc. (Osaka, Japan) and the protease inhibitors antipain, pepstatin, leupeptin, E64, chymostatin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

**Cell Culture and Synchronization of Cells**—NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum, and FM3A cells were cultured in RPMI medium containing 10% fetal calf serum (Life Technologies, Inc.). NIH3T3 cells were synchronized by subjecting them to contact inhibition during culture to confluence to arrest them at the G0/G1 phase, then releasing them from contact inhibition, allowing them to progress to the S phase, by replating at a density of approximately 30%. For cell cycle analysis, bromodeoxyuridine-pulsed NIH3T3 cells were fixed with 70% ethanol, treated with 2N HCl containing 0.5% Triton X-100, neutralized with borax buffer (pH 8.5), subjected to dual color staining with an anti-bromodeoxyuridine antibody (Amerham Pharmacia Biotech, UK Ltd., Bucks, United Kingdom) and 5 μg/ml propidium iodide, and then analyzed using a FACS-BAS-2000 imaging analyzer (Fuji Film, Kanagawa, Japan).

**Purification of Proteasome**—Human p27Kip1 was affinity purified with a glutathione-Sepharose CL-4B (Amersham Pharmacia Biotech), and the GST tag was cleaved using PreScission Protease (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. Myc-tagged p27Kip1 and HA-tagged Ubs were transiently expressed. Each DNA was incubated with LipofectAMINE reagent (Life Technologies, Inc.) in serum-free medium (Opti-MEM, Life Technologies, Inc.) for 30 min at room temperature, after which the mixture was incubated with the required cells for 16 h, followed by incubation in complete medium for 32 h.

**Preparation of Cell Extracts**—For the in vitro ubiquitination assay, the required cells were washed with phosphate-buffered saline, suspended in double-distilled water, and frozen and thawed three times; the resulting lysate was subjected to centrifugation at 100,000 x g for 4 h at 4 °C, and the supernatant (S100P) was retrieved and frozen at −80 °C. For the in vivo ubiquitination assay and analysis of endogenous p27Kip1 levels, the required cells were incubated in lysis buffer containing 0.1% Nonidet P-40 on ice for 15 min, cleared by centrifugation at 15,000 rpm for 15 min at 4 °C, and the protein concentration of the supernatant was determined by the Bradford method ( Protein Assay; Bio-Rad). For the in vitro degradation assay, NIH3T3 cells were homogenized in phosphate-buffered saline followed by centrifugation at 100,000 x g for 1 h at 4 °C and the resulting supernatant (S100) was retrieved and frozen at −80 °C.

**Ubiquitination Assay**—Each reaction mixture or cell lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Immobilon-P membranes (Millipore, Bedford, MA), which were probed with the required anti-p27Kip1 (N-20, C-19; Santa Cruz Biotechnology Inc., Santa Cruz, CA, or clone 57, hereafter designated TDL; Transduction Laboratories, Lexington, KY), anti-ε-Myc (9E10; Santa Cruz) or anti-α-tubulin (TU-01; Funakoshi, Tokyo, Japan) antibody. The Renaissance blotting system (NEF Life Science Products) was used to visualize the proteins.

**In Vitro Ubiquitination Assay**—Mouse recombinant p27Kip1 was incubated with the FM3A or NIH3T3 cell extract in the presence of an ATP-regenerating system (50 mM Tris (pH 8.3), 5 mM MgCl2, 5 mM ATP, 10 mM creatine phosphate, 0.2 unit/ml creatine kinase) together with 1 mg/ml Ub, 100 μg/ml Ub-CHO, 2 mM dithiothreitol, a proteasome inhibitor (10 μg/ml each of antipain, pepstatin, leupeptin, and PMSF) and a proteasome inhibitor mixture (250 μM ALLN, 250 μM ZLLLAL, and 25 μM clasto-lactacystin β-lactone). The reactions were carried out at 37 °C for 1 h and terminated by adding SDS sample buffer, and each reaction mixture was subjected to SDS-PAGE on a 10% gel, followed by immunoblotting analysis with the anti-p27 mAb TDL.

**In Vivo Ubiquitination Assay**—NIH3T3 cells were incubated with or without 100 μM ALLN for 12 h in a CO2 incubator, lysed, and then subjected to Western blotting with the TDL mAb. COS7 cells were transiently transfected with myc-tagged p27 and HA-tagged Ub and 36 h later, 50 μM lactacystin was added to the culture, which was incubated for another 12 h in a CO2 incubator. Then, the cells were lysed in 1% Triton X-100, neutralized above, and subjected to immunoblotting with the anti-ε-myc antibody 9E10.

**In Vitro Degradation Assay**—Mouse recombinant p27Kip1 was incubated with the NIH3T3 cell extract (S100) at 37 °C for 30 or 60 min and subjected to immunoblotting with the required anti-p27Kip1 antibody (N-20, C-19, or TDL).

**In Vivo Degradation Assay**—NIH3T3 cells were metabolically labeled with 100 μCi/ml Tran35S-label (ICN Pharmaceuticals Inc., Costa Mesa, CA) in methionine-free medium for 3 h, and then chased in complete medium for 0 or 3 h in a CO2 incubator. Cell lysates were immunoprecipitated with the TDL mAb, followed by Protein G-Sepharose (Amersham Pharmacia Biotech) affinity purification and SDS-PAGE, as described above, autoradiography, and quantification using a BAS2000 image analyzer.

**RESULTS**

**Ubiquitination of p27Kip1 in Vivo and in Vitro**—The results of the previous study suggested that p27Kip1 is down-regulated by the Ub/proteasome pathway (26). Originally, we developed an assay system for estimating p27Kip1 ubiquitination activity in vitro. Careful manipulation was required to detect the unstable intermediates; the key factors for detection of ubiquitinated p27Kip1 were inhibition of isopeptidase activity, a supply of excess Ub, and the exclusion of proteasomes. Isopeptidase, which associates with 26 S proteasomes and hydrolyzes...
immunoblotting with the anti-p27 antibody of the FM3A cell lysate from which the proteasomes had been removed by ultracentrifugation (S100Pr−). The reaction mixtures were analyzed by immunoblotting with the anti-p27′ mAb, blotted, and probed with an avidin-HRP conjugate. The amount of ubiquitinated p27 was increased when ubiquitin was a limiting factor in this reaction. When GST-Ub (34 kDa) was added to the reaction mixture instead of Ub (8 kDa), mono-ubiquitinated p27 was detected (Fig. 1A) and smeared bands of multi-Ub chains, made it difficult to detect ubiquitinated p27Kip1. In this study, the addition of Ub-CHO, an isopeptidase inhibitor, to the reaction mixture dramatically improved the amount of ubiquitinated p27Kip1 detected in vitro (compare lanes 4 and 8 in Fig. 1A). The addition of exogenous Ub to the reaction mixture also increased the formation of Ub-p27Kip1 conjugates (compare lanes 6 and 8 in Fig. 1A), suggesting that Ub was a limiting factor in this reaction. When GST-Ub (34 kDa) was added to the reaction mixture instead of Ub (8 kDa), a species with a higher molecular mass appeared (compare lanes 8 and 10 in Fig. 1A). Thus, this result confirms that the bands with lower electrophoretic mobilities were ubiquitinated p27Kip1, not aggregates of p27Kip1′ or non-specific products resulting from cross-reaction with the mAb. Proteasomes were removed from the cell cytoplasmic extract by differential centrifugation at 100,000 × g for 4 h, and the proteasome activity of the resulting proteasome-depleted supernatant (S100Pr−) was very low. The exclusion of proteasomes also dramatically increased Ub-p27Kip1 conjugate formation (data not shown).

In order to confirm that the protein with the higher molecular mass we detected was ubiquitinated p27Kip1, identical reaction mixtures containing biotinylated-Ub instead of Ub were subjected to immunoprecipitation-immunoblotting analysis. The resulting products were immunoprecipitated with the TDL mAb, blotted, and probed with an avidin-HRP conjugate. Then mono-ubiquitinated p27Kip1 and smeared bands of multi-ubiquitinated p27Kip1 were detected (Fig. 1B, lane 1).

Ubiquitinated p27Kip1 was also detected in vivo. ALLN, which inhibits proteasome and calpain activities, induced the accumulation of Ub-p27Kip1 conjugates in NIH3T3 cells (Fig. 1C). Ubiquitinated p27Kip1 was also observed in the transient p27Kip1′ cDNA transfection assay using COS7 cells (Fig. 1D), in which co-expression of Ub and p27Kip1′ led to moderately enhanced ubiquitination of p27Kip1 (Fig. 1D, lane 2) and the selective proteasome inhibitor lactacystin further enhanced the ubiquitinated p27Kip1 level (Fig. 1D, lane 3). These data indicate that p27Kip1′ is ubiquitinated in vivo as well as in vitro. Of note, mono- and di-ubiquitinated p27Kip1′ was also accumulated in the ALLN- and lactacystin-treated cells (Fig. 1, C and D, respectively), suggesting that the degradation of the mono-ubiquitinated p27Kip1′ might also be dependent on the proteasome. We also observed accumulation of a p27Kip1′ fragment with a lower molecular mass (approximately 22 kDa, referred to hereafter as p27Δ22) (Fig. 1C), whereas the specific proteasome inhibitor lactacystin resulted in the decrease in p27Δ22 (Fig. 1D), suggesting that this smaller p27 fragment was produced by proteasomes and subsequently degraded by a calpain-like protease.
20 h after release, roughly corresponding to the G1/G0, G1/S, and G2/M phases, respectively. The p27Kip1 ubiquitination activities of these lysates were determined by the in vitro ubiquitination assay, as described above under “Experimental Procedures” and in the legend to Fig. 1. The ubiquitination activity was higher in cells at the G1/S boundary (10 h) than during any other phase (Fig. 2C, lane 2). Therefore, we concluded that p27Kip1 ubiquitination activity is regulated in a cell cycle-dependent manner, increases at or near the G1/S boundary stage, and declines thereafter.

Determination of Ubiquitination Site(s) on p27Kip1—In an attempt to determine which lysine residue(s) in p27Kip1 is/are the target(s) for Ub conjugation, the 13 lysine residues in human p27Kip1 were substituted by arginine in clusters, as shown in Fig. 3A: KR1 (K25R, K47R, K59R), KR2 (K68R, K73R, K81R), KR3 (K96R, K100R), KR5 (K134R, K153R, K165R), and KR6 (K189R, K190R). These mutants were subjected to the in vitro ubiquitination reaction followed by immunoblotting analysis and probing with the TDL mAb, which recognizes the amino acid (aa) stretch around position 60. The ubiquitination level of KR5 was significantly lower than those of wild-type (wt) p27Kip1 and KR2, -3, and -6 (Fig. 3B, lanes 1–12). As KR1, which contained mutations at position 59, did not react with this mAb (Fig. 3B, lanes 3 and 4), another anti-p27Kip1 mAb, C-19, was used to probe this mutant (Fig. 3B, lanes 13–16). The ubiquitination level of KR1 was comparable to that of wt p27Kip1 (Fig. 3B, lanes 14 and 16). A time-course experiment confirmed that the ubiquitination of KR5 was impaired markedly in comparison with that of wt p27Kip1 (Fig. 3C). As cleavage of the GST tag was incomplete as a result of the purification of recombinant p27Kip1, GST-p27Kip1 remained. The possibility that Ub was conjugated to the GST portion of the GST-p27Kip1 fusion protein was excluded by the reduction in ubiquitination of KR5 mutant. These results indicate that Ub conjugation of p27Kip1 targets some or all the lysine residues at positions 134, 153, and 165.

Proteolytic Processing of p27Kip1—We also observed that bands with higher electrophoretic mobilities (p27Δ22k in Fig. 1, C and D) appeared in parallel with p27Kip1 ubiquitination in vivo and in vitro, suggesting the activity responsible for processing p27Kip1 in the cell lysates that contributes to the down-regulation of p27Kip1 in parallel with that responsible for Ub-dependent degradation. Therefore, we investigated this processing activity. The small fragment shown in Fig. 1D seemed to be a truncated product lacking the N-terminal portion, because the myc epitope attached to the C terminus of p27Kip1 was retained. We investigated the processing mechanism by incubating recombinant p27Kip1 protein with the NIH3T3 cell extract and analyzing the reaction product (p27Δ22k in Fig. 4), which was readily detected, suggesting this processing reaction was probably rapid and further degradation of the p27Δ22k fragment was rate-limiting. The molecular masses of the product of exogenous p27Kip1 and the derivative of endogenous p27Kip1 in NIH3T3 cells were both approximately 22 kDa, indicating that the reaction in vitro faithfully reproduced p27Kip1 processing in vivo (Fig. 4A, lanes 1 and 3). The amount of intact p27Kip1 decreased as the amount of the processed product increased. The sum of p27Kip1 expression level was also reduced, indicating that the further degradation process from the 22-kDa intermediate occurred. This processing reaction was ATP-dependent, because the addition of an ATP-regenerating system to the reaction mixture promoted it, resulting in a reduction in the amount of wt p27Kip1 and an increase in p27Δ22k protein production (Fig. 4B, lane 3). In contrast, ATP-yS, which suppresses ATP regeneration, inhibited the processing activity, as the amount of wt p27Kip1 protein...
FIG. 3. In vitro ubiquitination of p27(Kip1), KR mutants. A, lysine residues in p27(Kip1) were substituted with arginine in clusters, as indicated. B, in order to locate the ubiquitination site(s) in p27(Kip1), recombinant KR mutants (KR1, -2, -3, -5, and -6) and wt p27(Kip1) proteins were subjected to the in vitro ubiquitination assay, as described in the legend to Fig. 1A. The TDL mAb was used for analysis of KR2, -3, -5, and -6, the epitopes of which are located near aa 60; and the anti-p27(Kip1) antibody C-19, which recognizes the C terminus of p27(Kip1), was used to analyze KR1. C, time-course analysis of ubiquitination with wt p27(Kip1) and the KR5 mutant in vitro.

Collectively, our results suggest that p27(Kip1) was degraded by at least two pathways: Ub-proteasome-mediated degradation and proteolytic processing, by which p27(Kip1) was first processed by proteasomes in an ATP-dependent manner to produce an approximately 22-kDa fragment (p27Δ22k) and then degraded to small peptides by a calpain-type protease. These results indicate the existence of a novel proteolytic processing pathway, in addition to the Ub-proteasome pathway, that regulates intracellular p27(Kip1) expression levels.

We carried out further experiments to determine whether the rapid processing of p27(Kip1) to p27Δ22k affects the function of p27(Kip1). First, in an attempt to locate the processing site, three antibodies that recognize different positions of p27(Kip1) were used. The epitope recognized by the anti-p27(Kip1) mAb TDL may be located near the aa at position 60, as discussed above, and the anti-p27(Kip1) Abs N-20 and C-19 were raised against the N (aa 2–21) and C (aa 181–198) termini (Fig. 5B) of p27(Kip1). The p27Δ22k fragment was detected by C-19 and TDL, but not by N-20 (Fig. 5A), which suggests that the processing site was located on the N-terminal side some distance from position 60. In view of its deduced molecular mass, the processing site seemed to lie near positions 35–40. Therefore, this processing reaction eliminates the cyclin-binding domain.

In order to examine the function of the processed p27(Kip1), the CDK2-inhibitory activities of recombinant p27(Kip1) and N terminus-deletion mutant p27Δ22k (Fig. 5B) were tested. Intact p27(Kip1) inhibited the catalytic activity of CDK2 in a concentration-dependent manner (Fig. 5C, lanes 7–11), whereas the inhibitory activity of p27Δ22k was approximately 100 times lower than that of wt p27(Kip1) (Fig. 5C, lanes 2–6). Thus, the conversion of native p27(Kip1) to p27Δ22k by proteolytic processing may interfere with the CDK inhibitory activity of p27(Kip1).

Cell Cycle-Dependent, Ubiquitination-Independent Processing of p27(Kip1)—We also examined whether the activity that degraded endogenous p27(Kip1) in NIH3T3 cells in vivo was cell cycle-dependent. As expected, the p27(Kip1)-degradative activity was higher in the S than in the G1/G0 phase (Fig. 6, A and B). In the S phase, the amount of p27Δ22k produced increased markedly in parallel with the amount of p27(Kip1) degraded. This result suggests the processing activity probably contributes to the down-regulation of p27(Kip1) as the cell cycle progresses from the G1 to the S phase. In vitro experiments suggested that the degradative processing was executed by 26 S proteasomes, as this rapid reaction was ATP-dependent and inhibited by proteasome inhibitors.

In order to determine whether this degradative processing reaction was mediated through ubiquitination, wt p27 and its ubiquitination-deficient mutant KR5 were transiently expressed in NIH3T3 cells, resulting in comparable levels of p27Δ22k in both groups, suggesting that the reaction that produced p27Δ22k was ubiquitination-independent. Collectively, our data suggest the existence of a novel mechanism that negatively regulates intracellular p27(Kip1) levels, i.e. a Ub-independent proteolytic processing pathway, in addition to the Ub-mediated degradation pathway.

DISCUSSION

The p27(Kip1) expression level alters dramatically during the cell cycle, particularly from the G1 to the S phase, suggesting
that rapid elimination of p27Kip1 is probably a prerequisite for the activation of cyclin/CDK kinase complexes and cell cycle progression. The mechanism responsible for the regulation of p27Kip1 expression remains elusive. By analogy with CKIs in Saccharomyces cerevisiae (Sic1) and Schizosaccharomyces pombe (Rum1), protein degradation via the Ub-proteasome pathway has been speculated to be critical for controlling the expression levels of mammalian CKIs (23–25). Pagano et al. showed that p27Kip1 was ubiquitinated and that its ubiquitination was augmented by a Ub-conjugating enzyme (E2), Ubc3, because most of the chymotrypsin-like activity present in the cytosol fractions appears to be attributable to proteasomes (37).

In this study, we demonstrated the presence of an alternative p27Kip1 degradation pathway operating at the G1/S transition point of the cell cycle. The Ub-independent p27Kip1 processing activity produced the 22-kDa fragment of C-terminal p27Kip1 rapidly, and was ATP-dependent and sensitive to proteasome-specific and chymotrypsin-specific inhibitors, suggesting that 26 S proteasomes conduct this processing reaction, because most of the chymotrypsin-like activity present in the cytosol fractions appears to be attributable to proteasomes (37). This is highly similar to the case of cyclin B1 (49 kDa) that is processed at the N terminus, producing a 42-kDa truncated...
Down-regulation of p27^Kip1 by Two Mechanisms

In our A. NIH3T3 cells were synchronized at the G1/S or S phase then metabolically labeled with Tran35S-label and chased, as described under "Experimental Procedures." B, the levels of p27^Kip1 (solid bar) and p27Δ22k (shaded bar), as percentages of the intact p27^Kip1 level before degradation (0 h), in each phase are shown. C, NIH3T3 cells were transiently transfected with myc-tagged wt p27 and p27Δ22k, metabolically labeled with Tran35S-label, separated by SDS-PAGE, and analyzed using a BAS2000 imaging analyzer. The molecular masses of myc-p27 and myc-p27Δ22k were 28 and 23 kDa, respectively.

Acknowledgments—We thank Dr. B. Boheman for the plasmid used in this study. Dr. T. Nakata for fruitful discussion, N. Nishimura and other laboratory members for technical assistance, and M. Kimura and A. Takimoto for secretarial assistance.

REFERENCES

1. Sherr, C. J., and Roberts, J. M. (1995) Genes Dev. 9, 1149–1163
2. Sherr, C. J. (1994) Cell 79, 551–555
3. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994) Cell 78, 59–66
4. Tsuchiya, H., and Hunter, T. (1994) Cell 78, 67–74
5. Kato, J. Y., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. J. (1994) Cell 79, 487–498
6. Koff, A., Soderberg, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franz, B. R., and Roberts, J. M. (1992) Science 257, 1689–1694
7. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kato, J. Y. (1994) Mol. Cell. Biol. 14, 2086–2076
8. Yamamoto, K., Hirai, A., Ban, T., Saito, J., Tahara, K., Terano, T., Tamura, Y., Saito, Y., and Kitagawa, M. (1986) Endocrinology 117, 2303–2304
9. Tanaka, T., Tateuno, I., Noguchi, Y., Uchida, D., Oeda, T., Narumiya, M., Yasuda, T., Higashi, H., Kitagawa, M., Nakayama, K.-I., Saito, Y., and Hirai, A. (1998) J. Biol. Chem. 273, 26772–26778
10. Nourse, J., Firpo, E., Plascak, W. M., Coats, S., Polyak, K., Lee, M. H., Massague, J., Crabtree, G. R., and Roberts, J. M. (1994) Nature 372, 570–573
11. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995) Genes Dev. 9, 1831–1841
12. Coats, S., Flanagan, W. M., Nourse, J., and Roberts, J. M. (1996) Science 272, 877–880
13. Hirai, A., Nakamura, S., Noguchi, Y., Yasuda, T., Kitagawa, M., Tateuno, I., Oeda, T., Tahara, K., Terano, T., Narumiya, S., Koh, L. D., and Saito, Y. (1997) J. Biol. Chem. 272, 13–16
14. Cattavaro, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncarli, C., Sandhu, C., Shaw, P., Yeger, H., Moravá-Prozet, I., Kapusta, L., Fransen, E., Pritchard, K. J., and Slagerman, J. (1997) Nat. Med. 3, 227–230
15. Porter, P. L., Malone, K. E., Heagerty, P. J., Alexander, G. M., Gati, A., Labbe, E., Fingar, D. J., Lipton, S. A., and Roberts, J. M. (1997) Nat. Med. 3, 222–227
16. Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Druetta, G. F., Jessup, J. M., and Pagano, M. (1997) Nat. Med. 3, 231–234
17. Steiger, P. S., and Abrams, J. S. (1997) Nat. Med. 3, 152–154
18. Kawasaki, H., Tamaru, J., Tanaka, T., Hirai, A., Saito, Y., Kitagawa, M., Mikata, A., Harigaya, K., and Kuriyama, T. (1998) Am. J. Pathol. 153, 505–513
19. Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Nishimoto, T., Morgan, D. O., Franz, B. R., and Roberts, J. M. (1992) Science 257, 1689–1694
20. King, R. W., Jackson, P. K., and Kirschner, M. W. (1994) Cell 79, 59–66
21. Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak, K., Saito, Y., and Nakayama, K. (1996) Cell 85, 505–513
22. Feldman, R. M., Correll, C. C., Kaplan, K. B., and Deshaies, R. J. (1997) Cell 89, 221–230
23. Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997) Science 276, 209–219
24. Komiminami, K., and Toda, T. (1997) Genes Dev. 11, 1548–1560
25. Polyak, M., Tam, S. W., Bearor-Mero, P., Del-Sal, G., Chau, V., Yew, P. P., Draetta, G. F., and Rolfe, M. (1995) Science 269, 682–685
26. King, R. W., Jackson, P. K., and Kirschner, M. W. (1994) Cell 78, 505–511
27. Won, K. A., and Reed, S. I. (1996) EMBO J. 15, 4182–4193
28. Blagosklonny, M. V., Wu, G. S., Omura, S., and el-Deiry, W. S. (1996) Biochem. Biophys. Res. Commun. 227, 564–569
29. Chenn, V. D., Hagner, J., Palomina, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995) Genes Dev. 9, 1586–1597
30. Thanos, D., and Maniatis, T. (1995) Cell 80, 529–532
31. Treier, M., Stazewski, L. M., and Bohmann, D. (1994) Cell 78, 787–798
32. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) Cell 75, 495–505
33. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) EMBO J. 16, 3797–3804
34. Herschko, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
35. Weissman, A. M. (1997) Immuno Today 18, 189–198
36. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65,
Down-regulation of p27Kip1 by Two Mechanisms

38. Kitagawa, M., Higashi, H., Takahashi, I. S., Okabe, T., Ogino, H., Taya, Y., Hishimura, S., and Okuyama, A. (1994) *Oncogene* 9, 2549–2557
39. Kitagawa, M., Higashi, H., Jung, H. K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yushida, E., Nishimura, S., and Taya, Y. (1996) *EMBO J.* 15, 7060–7069
40. Donato, N. J., and Perez, M. (1998) *J. Biol. Chem.* 273, 5067–5072
41. Levkau, B., Koyama, H., Raines, E. W., Clurman, B. E., Herren, B., Orth, K., Roberts, J. M., and Ross, R. (1998) *Mol. Cell* 1, 553–563
42. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M., and Clurman, B. E. (1997) *Genes Dev.* 11, 1464–1478
43. Vlach, J., Hennecke, S., and Amati, B. (1997) *EMBO J.* 16, 5334–5344
44. Tokumoto, T., Yamashita, M., Tokumo, M., Katsu, Y., Horiguchi, R., Kajiwara, H., and Nagahama, Y. (1997) *J. Cell Biol.* 138, 1313–1322
45. Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992) *Nature* 360, 597–599
46. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) *Cell* 78, 773–785
47. Uren, A., Jakus, J., de Mora, J. F., Yeudall, A., Santos, E., Gutkind, S., and Heidaran, M. A. (1997) *J. Biol. Chem.* 272, 21669–21672