# Facilitation of proteasomal degradation of p27$^{Kip1}$ by N-terminal cleavage and their sequence requirements

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**Abstract The sequence requirement for N-terminal cleavage and the proteasomal degradation of p27$^{Kip1}$ and their relationship was investigated. Residues 5–8 were required for the cleavage and the mutation of S10 to E inhibited the cleavage. The C-terminal PEST sequence was necessary for the degradation and residue R165 was found to play an important role in the degradation. The inhibition of the cleavage by deleting residues 5–8 inhibited the degradation, while the fragment mimicking the cleavage product accelerated the degradation. Both the cleavage and degradation demonstrated a similar sensitivity toward proteasome inhibitors and ATP depletion. These two processes are thus suggested to be tightly linked and sequential.**


*Keywords:* p27$^{Kip1}$; Proteasome; Proteolysis

## 1. Introduction

p27$^{Kip1}$ is a cyclin-dependent kinase (Cdk) inhibitor which causes growth arrest in response to anti-mitogenic signals such as serum starvation and cell–cell contact [1,2]. The mitogenic stimulation causes a rapid downregulation of p27$^{Kip1}$ before the quiescent cells return to the cell cycle and progress into the S phase [3,4]. Among the transcriptional and post-transcriptional mechanisms regulating the level of p27$^{Kip1}$, the proteolytic degradation plays an important role in the rapid downregulation and inactivation of p27$^{Kip1}$ [5,6]. The ubiquitination-proteasome pathway plays an important role in the degradation of p27$^{Kip1}$, while the calpain-mediated ubiquitin-independent degradation mechanism has also been reported to degrade p27$^{Kip1}$ [5,7]. Skp2 was shown to mediate the ubiquitination of p27$^{Kip1}$ [7,8]. However, p27$^{Kip1}$ was found to be degraded in Skp2$^{-/-}$ mice during $G_1$/S transition [9]. On the other hand, the phosphorylation of p27$^{Kip1}$ has been reported to regulate degradation [10–13]. The phosphorylation of T187 was shown to induce ubiquitination and subsequent degradation [11]. However, the knock-in mice of p27$^{Kip1}$ with a mutation of T187 to A revealed a degradation mechanism independent of T187 phosphorylation, which works during the $G_1$/S transition [14]. On the other hand, the phosphorylation of S10 was shown to stabilize p27$^{Kip1}$ against degradation during the $G_1$/S transition [12,13]. In addition to the proteasome-mediated degradation, the N-terminal cleavage has also been reported to contribute to the functional downregulation of p27$^{Kip1}$ [6]. However, the sequence requirement for the proteasomal degradation and the N-terminal cleavage of p27$^{Kip1}$, and the relationship between these two proteolytic mechanisms remain to be elucidated.

We have previously cloned a second isoform of p27$^{Kip1}$ (p27$^{Kip1R}$), which was found to be resistant to both N-terminal cleavage and proteasomal degradation [15]. In a preliminary experiment, a fragment consisting of the region 1–162 common to both isoforms (Fig. 1(a)) was found to undergo only N-terminal cleavage, but it was resistant to proteasomal degradation. In the present study, taking our initial observation into account, we separately clarified some of the sequences required for the N-terminal cleavage and the proteasomal degradation. Furthermore, the initial observation allowed us, for the first time, to uncover the relationship between N-terminal cleavage and the proteasomal degradation. Our observations suggest that the two proteolytic processes are tightly linked and they may also be sequential.

## 2. Materials and methods

### 2.1. Construction of plasmids

All recombinant proteins of p27$^{Kip1}$, p27$^{Kip1R}$ and their mutants were expressed in bacteria as hexahistidine-tagged proteins using pQE32 (Qiagen, Hilden, Germany) as an expression vector. The full-length p27$^{Kip1}$ consisting of 198 residues (refer to as Kip1$^{1–198}$) was the same as previously described [1,15]. Because the original clone of p27$^{Kip1R}$ (pAD-K4) lacked the N-terminal 8 amino acids [15], the full length p27$^{Kip1R}$ (Kip1R$^{1–180}$) was constructed by connecting a 5′ part of p27$^{Kip1}$ and a 3′ part of pAD-K4 using an overlap extension PCR [16]. The truncation mutants were obtained by PCR amplification using specific primer sets. The site-directed mutants were obtained with an overlap extension PCR using complementary primers containing the intended mutations, as previously described [15,17]. The mutants used in the present study are Kip1$^{1–162}$ (a fragment containing residues

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*Abbreviations:* Cdk, cyclin-dependent kinase; ATPγS, a non-hydrolysable ATP analogue

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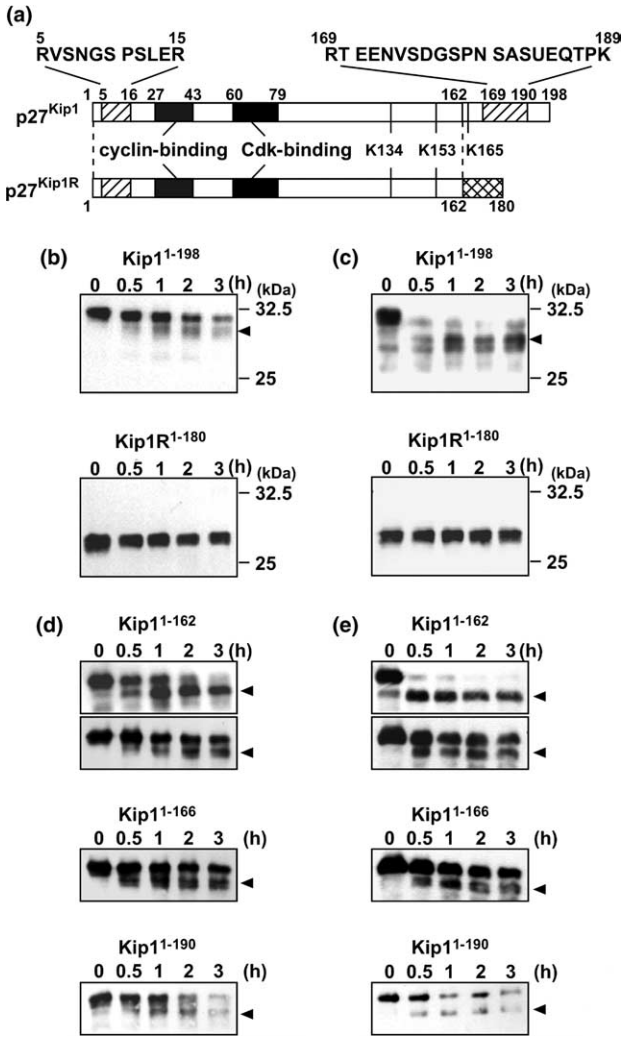


Fig. 1. Time course of the degradation of $p27^{Kip1}$, $p27^{Kip1R}$ and the C-terminally truncated $p27^{Kip1}$. (a) The schematic presentation of the structure of two isoforms of $p27^{Kip1}$. The region 1–162 is common to the two isoforms [15]. Closed boxes, the cyclin-binding and Cdk-binding regions [12,24]; the hatched boxes, potential (residues 169–190) and poor (residues 5–15) PEST sequences as determined by a PEST-FIND program [21]; cross-hatched box, a C-terminal region specific to $p27^{Kip1R}$. The amino acid sequences of the putative PEST sequences are shown. (b–e) Representative immunoblots showing the time course of degradation of $Kip1^{1–198}$ and $Kip1R^{1–180}$ (b, c) and the C-terminally truncated $p27^{Kip1}$ (d, e) catalyzed by the extracts of endothelial cells in the late $G_1$ phase (b, d) and HeLa cells in the S phase (c, e). The photos are representatives of three independent experiments. The triangles indicate a cleavage product. In (d) and (e), two representative degradation patterns of $Kip1^{1–162}$ are shown to indicate that the activity of cleavage varied somewhat from preparation to preparation of the cell extracts.

1–162 of $p27^{Kip1}$), $Kip1^{1–166}$, $Kip1^{1–190}$, $Kip1^{5–162}$, $Kip1^{9–162}$, $Kip1^{18–162}$, $Kip1^{9–198}$, $Kip1^{18–198}$, $Kip1^{1–162/R5A}$ ($Kip1^{1–162}$ containing a mutation of R5 to A), $Kip1^{1–162/S10A}$, $Kip1^{1–162/S10E}$, $Kip1^{18–198/K165R}$, $Kip1^{18–198/K153R/K165R}$ and $Kip1^{18–198/K134R/K153R/K165R}$ (Fig. 1(a)). The nucleotide sequences of all constructs were determined to contain no unintended mutation.

*2.2. Expression and purification of recombinant protein*

The recombinant proteins were expressed in *Escherichia coli* JM109 for 4 h as previously described [1,15]. The cells were homogenized in buffer A (6 M guanidine HCl, 0.1 M sodium phosphate and 10 mM Tris–HCl, pH 8.0) and then subjected to affinity purification using a $Ni^{2+}$-loaded HiTrap affinity column on an ÄKTA prime liquid chromatography device (Amersham Pharmacia Biotech, Tokyo, Japan). The column was washed in buffer A and in buffer B (6 M urea, 0.1 M sodium phosphate and 10 mM Tris–HCl, pH 6.3) and then eluted in buffer B containing 0.5 M imidazole-HCl. The pooled peak fractions were dialyzed against 30 mM Tris–HCl and 30 mM NaCl, pH 7.5. Because of the low solubility after dialysis, $Kip1R^{1–180}$ was re-natured on the column as previously described [18], and the column eluates were then directly used in an assay.

*2.3. Cell culture and cell cycle synchronization*

The endothelial cells of the porcine thoracic aortas (5–16 passages) and HeLa cells were cultured as previously described [1,15]. Endothelial cells were synchronized at the late $G_1$ phase of the cell cycle by harvesting cells at 15 h after seeding the growth arrested confluent endothelial cells at a 50% confluent density [1,15]. HeLa cells were synchronized at the S phase by harvesting the cells 3 h after the release from the 24-h block by 2 mM hydroxyurea [1,15,19].

*2.4. In vitro degradation assay*

The hypotonic cell extract was prepared in 20 mM HEPES, 5.0 mM KCl, 1.5 mM $MgCl_2$, and 1.0 mM dithiothreitol, pH 7.5 as previously described [15]. The in vitro degradation assay was started by mixing 10 μl of cell extract supplemented with 25 mM phosphocreatine and 10 μg/ml creatine kinase, 30 ng of recombinant protein and 1 mM ATP unless otherwise specified at 30 °C for each time point. The samples were withdrawn at 0, 0.5, 1, 2 and 3 h, and were then subjected to an immunoblot analysis using anti-$p27^{Kip1}$ antibody (BD Biosciences, Tokyo, Japan) and enhanced chemiluminescence technique, as previously described [1,15]. The densities of the bands were determined with the NIH image ver1.61 (National Institute of Health, Bethesda, MD, USA) after obtaining the chemiluminescence images on X-ray film with a CCD camera.

*2.5. Statistical analysis*

Student's *t* test was used to determine statistical significance between the two groups. *P* values of less than 0.05 were considered to be significant.

## 3. Results

*3.1. Differences in the degradation pattern between $p27^{Kip1}$ and $p27^{Kip1R}$*

Fig. 1 demonstrates representative patterns of degradation of $Kip1^{1–198}$ and $Kip1R^{1–180}$ catalyzed by the extract of endothelial cells (Fig. 1(b)) and HeLa cells (Fig. 1(c)). In both cell types, $Kip1^{1–198}$ degraded within 3 h. It is noteworthy that an intermediate product was observed in both cell types during the degradation of $Kip1^{1–198}$. The product (28.1 kDa) was approximately 2.5 kDa smaller than $Kip1^{1–198}$ (30.7 kDa), as estimated on SDS–PAGE. On the other hand, $Kip1R^{1–180}$ was resistant to degradation, and no such intermediate product as that seen with $Kip1^{1–198}$ was observed.

*3.2. The region 1–162 is sensitive to cleavage but resistant to degradation*

The difference in the susceptibility to degradation between two isoforms was considered to be due to the difference in the C-terminus. We thus first examined the degradation of $Kip1^{1–162}$, the region common to two isoforms to determine the structural basis for the difference in the degradation pattern (Fig. 1(a)). In both endothelial cells (Fig. 1(d)) and HeLa cells (Fig. 1(e)), $Kip1^{1–162}$ was cleaved to a fragment which was approximately 2.5 kDa smaller than $Kip1^{1–198}$ with no further degradation, although the activity of cleavage varied somewhat from preparation to preparation of the cell extracts. The

extension of 4 amino acids to the C-terminus of $p27^{Kip1}$ ($Kip1^{1–166}$) did not restore the susceptibility to degradation, while the addition of 163–190 ($Kip1^{1–190}$) restored the susceptibility toward degradation similar to that observed with $Kip1^{1–198}$ (Fig. 1(d) and (e)). The intermediate product was also observed during the degradation of $Kip1^{1–190}$. It is noteworthy that the reduction of molecular size by the cleavage was ~2.5 kDa and similar in all mutants despite the difference in the C-terminal length. This observation thus suggests that the cleavage removed a ~2.5 kDa fragment from the N-terminus.

### 3.3. Sequences requirement for the N-terminal cleavage

The cleavage of the mutants of $Kip1^{1–162}$ was examined to determine the sequences required for the N-terminal cleavage without interference by the proteasomal degradation (Fig. 2). The deletion of 4 amino acids from the N-terminus ($Kip1^{5–162}$) did not inhibit the N-terminal cleavage, while the deletion of 8 residues ($Kip1^{9–162}$) completely inhibited the N-terminal cleavage in both cells types (Fig. 2). The residues 5–8 were thus necessary for the cleavage. The further deletion of 10 residues ($Kip1^{18–162}$) was also resistant to cleavage. However, $Kip1^{18–162}$ demonstrated a similar mobility on the SDS–PAGE to that of the cleavage product derived from $Kip1^{1–162}$ when applied side-by-side (data not shown). $Kip1^{18–162}$ was thus considered to represent a cleavage product. The mutation of R5 to A of $Kip1^{1–162}$ ($Kip1^{1–162/R5A}$) completely inhibited the N-terminal cleavage. The mutation of S10 to E ($Kip1^{1–162/S10E}$) inhibited the N-terminal cleavage, while the mutation to A ($Kip1^{1–162/S10A}$) had no apparent effect.

### 3.4. Effect of N-terminal cleavage on the proteasomal degradation of $p27^{Kip1}$ and the requirement of K165 for degradation

In order to elucidate the relationship between the N-terminal cleavage and proteasomal degradation, we compared the degradation of $Kip1^{1–198}$, $Kip1^{9–198}$ and $Kip1^{18–198}$ (Fig. 3). According to the observations shown in Fig. 2, $Kip1^{9–198}$ was considered to be resistant to the N-terminal cleavage, while $Kip1^{18–198}$ was considered to mimic the N-terminally cleaved $p27^{Kip1}$. The degradation of $Kip1^{9–198}$ was significantly slower than that observed with $Kip1^{1–198}$. The degradation of $Kip1^{18–198}$ was significantly enhanced compared to that observed with $Kip1^{1–198}$.

K134, K153 and K165 have been previously reported to play an important role in the ubiquitination and subsequent degradation of $p27^{Kip1}$ [6]. The mutation of K165 to A markedly inhibited the degradation of $Kip1^{18–198}$. However, the degradations of $Kip1^{18–198/K153R/K165R}$ and $Kip1^{18–198/K134R/K153R/K165R}$ were similar to that observed for $Kip1^{18–198/K165R}$ (data not shown).

### 3.5. Effect of protease inhibitors and ATP depletion on the N-terminal cleavage and proteasomal degradation

To separately determine the sensitivity of the N-terminal cleavage and proteasomal degradation to the protease inhibitors

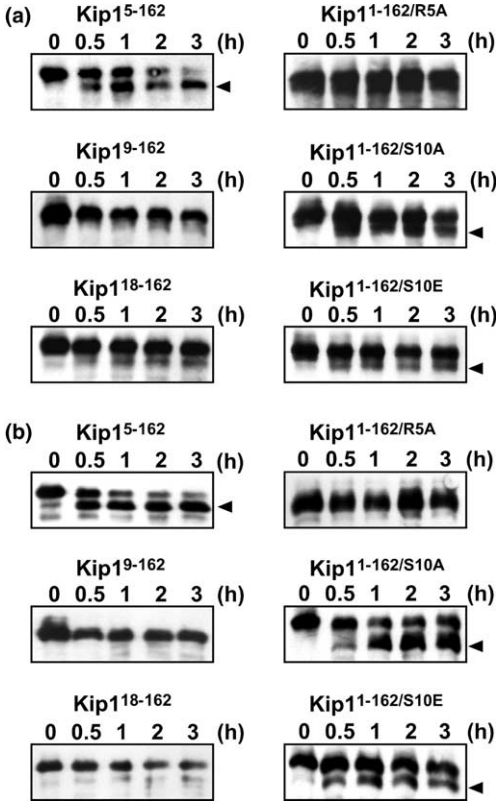


Fig. 2. Sequence requirement for the N-terminal cleavage of $Kip1^{1–162}$. Representative immunoblots showing the time course of degradation for the mutants of $Kip1^{1–162}$ catalyzed by the extract of endothelial cells in the late $G_1$ phase (a) and HeLa cells in the S phase (b).

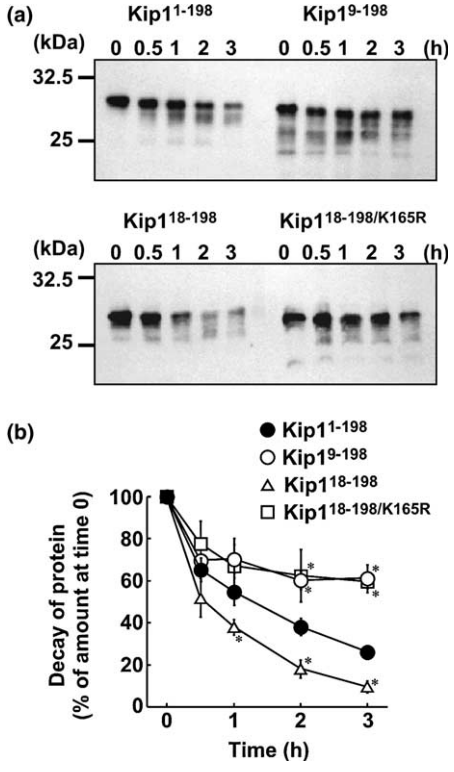


Fig. 3. Effects of the N-terminal cleavage on the degradation of $p27^{Kip1}$. Representative immunoblots (a) and summary (b) of the time course of degradation of $Kip1^{1–198}$, $Kip1^{9–198}$, $Kip1^{18–198}$ and $Kip1^{18–198/K165R}$ catalyzed by the extract of endothelial cells in the late $G_1$ phase. The data are means ± S.E. ($n = 5$). *$P < 0.05$ vs. $Kip1^{1–198}$.

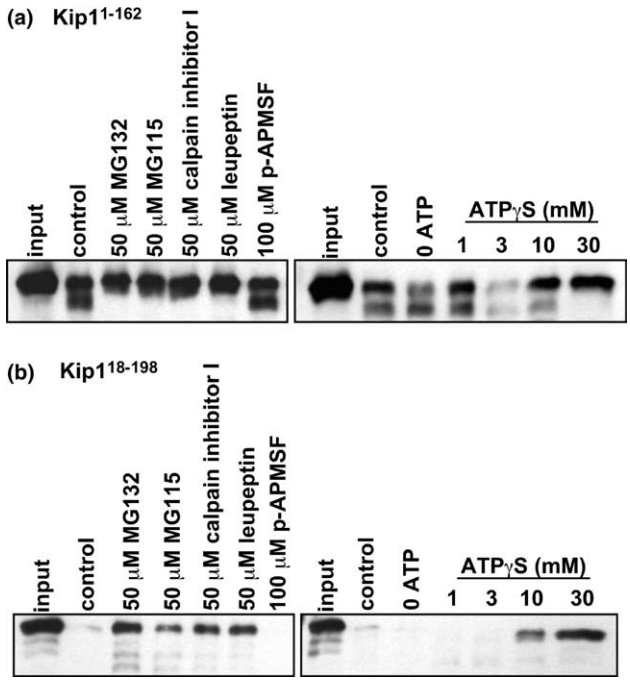


Fig. 4. Effects of proteasome inhibitor and ATP depletion on the N-terminal cleavage and the degradation of $p27^{Kip1}$. Representative immunoblots showing the N-terminal cleavage of $Kip1^{1–162}$ (a) and the degradation of $Kip1^{18–198}$ (b) catalyzed by the extract of endothelial cells in the late $G_1$ phase. The recombinant proteins were incubated with the cell extract for 3 h under the indicated conditions. Input, the sample obtained at time 0; control, the sample obtained after 3 h incubation with the cell extract in the absence of inhibitors and the presence of 1 mM ATP; 0 ATP, the samples obtained with no supplementation of ATP. ATPγS was added at the indicated concentrations in place of ATP.

(MG132, MG115, calpain inhibitor I, leupeptin or p-APMSF) and ATP depletion, the cleavage of $Kip1^{1–162}$ and the degradation of $Kip1^{18–198}$ were examined (Fig. 4). Interestingly, both N-terminal cleavage and proteasomal degradation showed a similar sensitivity toward the same set of protease inhibitors (MG132, MG115, calpain inhibitor I, and leupeptin) and the addition of ATPγS, a non-hydrolyzable ATP analog.

## 4. Discussion

The key finding in the present study is that the region 1–162 common to $p27^{Kip1}$ and $p27^{Kip1R}$ was resistant to degradation but susceptible to limited proteolysis. The limited proteolysis was suggested to remove an approximately 2.5 kDa fragment from the N-terminus. This fact enabled us to separately determine the structural basis for the N-terminal cleavage and the proteasomal degradation and to examine their relationship. The most important and novel finding of the present study is that the inhibition of the N-terminal cleavage inhibited proteasomal degradation, while the fragment mimicking the product of N-terminal cleavage degraded faster than the wild type. Our findings thus suggest that the N-terminal cleavage helps $p27^{Kip1}$ to undergo further degradation by proteasome. However, the fragment resistant to N-terminal cleavage still degraded. The N-terminal cleavage may thus not be an absolute requirement for proteasomal degradation. Since both N-terminal cleavage and proteasomal degradation exhibited the same sensitivity to proteasome inhibitors and ATP depletion, these two processes are thus suggested to be tightly linked and a sequential process probably catalyzed by the same machinery, namely proteasome.

The similar N-terminal cleavage of $p27^{Kip1}$ has been previously reported [6]. This report suggested that the cleavage removed an approximately 5 kDa fragment containing a cyclin-binding domain from the N-terminus, thus inactivating $p27^{Kip1}$ as a Cdk inhibitor. On the other hand, the N-terminal cleavage seen in the present study is suggested to remove approximately 20 residues but not the cyclin-binding domain, thus leaving $p27^{Kip1}$ active as a Cdk inhibitor [20]. Therefore, a further degradation is needed to inactivate $p27^{Kip1}$. Although the N-terminal cleavage per se does not inactivate $p27^{Kip1}$, it facilitates the inactivation of $p27^{Kip1}$ by accelerating proteasomal degradation.

The extension of residues 163–190 to $Kip1^{1–162}$ was found to restore susceptibility toward degradation. This extension contains a region 169–189 that is found to be a possible PEST sequence with a PEST-FIND score of +11.06 (Fig. 1(a)) [21,22]. A previous report suggested the possibility that this PEST sequence is involved in the calpain-mediated cleavage of $p27^{Kip1}$ [23]. Our findings suggest that the PEST sequence at the C-terminal region of $p27^{Kip1}$ plays an important role in the proteasomal degradation but not in the N-terminal cleavage of $p27^{Kip1}$. On the other hand, the proteasomal degradation of $p27^{Kip1}$ is dependent on ubiquitination [5–7]. K134, K153 and K165 were previously reported to serve as major ubiquitination sites [6]. Our findings suggest that K165 plays the most critical role among them. The critical role of K165 is consistent with the fact that K134 and K153 reside in the region common to $p27^{Kip1}$ and $p27^{Kip1R}$, while K165 resides in the C-terminal region specific to $p27^{Kip1}$ and at the proximity of the PEST sequence (Fig. 1(a)).

The present study clarified that residues 5–8 are necessary for the N-terminal cleavage and that the mutation mimicking the phosphorylation at S10 stabilizes $p27^{Kip1}$ against the proteolytic cleavage. The residues 5–8 are located in the region 5–15 that is found to be a poor PEST sequence (PEST-FIND score of −9.92) [21,22]. The PEST sequence is defined as hydrophilic stretches containing at least one P, one E or D and one S or T, and flanked by K, R or H residues [21]. The inhibition of the N-terminal cleavage in $Kip1^{1–162/R5A}$ may support the involvement of the PEST sequence. However, the significance of this region as a PEST sequence remains to be determined. The mutation mimicking the phosphorylation at S10 has been previously reported to stabilize $p27^{Kip1}$ [12,13]. Our observations suggest that this stabilization of $p27^{Kip1}$ is primarily due to the inhibition of N-terminal cleavage, which in turn stabilizes $p27^{Kip1}$ against proteasomal degradation.

$p27^{Kip1R}$ was resistant to both N-terminal cleavage and proteasomal degradation. This resistance to proteasomal degradation is probably due to the lack of a PEST sequence seen in the C-terminal region specific to $p27^{Kip1}$. The C-terminal region specific to $p27^{Kip1R}$ is thus suggested to inhibit the N-terminal cleavage. However, its mechanism remains to be elucidated.

In conclusion, N-terminal cleavage and proteasomal degradation of $p27^{Kip1}$ are suggested to be tightly linked and are also considered to occur as a sequential process mediated by the

same proteolytic mechanism. This cleavage facilitates further degradation of $p27^{Kip1}$ by proteasome.

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