A retinoblastoma susceptibility gene product, RB, targeting protease is regulated through the cell cycle

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Abstract Degradation of cyclin B and cyclin-dependent kinase inhibitor, p27, at a specific time has been shown to play a critical role in regulating the cell cycle. SPase, a nuclear and cytosol protease with cathepsin B- and L-like proteolytic activity, has been identified in several cell lines. This proteolytic enzyme selectively degraded nuclear proteins such as retinoblastoma susceptibility gene product, RB, and transcription factor, SP-1. High levels of SPase activity were detected at the G_1/S, moderate levels at the G_1 and S phases, and undetectable activity at the M phase of synchronized CV-1 cells, suggesting that SPase activity is regulated through the cell cycle. Degradation of RB correlated with SPase activity throughout the cell cycle, suggesting that SPase regulates RB, which has a functional role in regulating cell cycle. These results demonstrated that SPase plays an integral role in regulating the nuclear regulator, RB, in controlling cell cycle progression.

Key words: Retinoblastoma susceptibility gene product RB; Transcription factor SP-1; Cysteinyl protease SPase; Cell cycle; Synchronization of cells; Proteolysis

1. Introduction

The balance between protein synthesis and degradation controls levels of cellular proteins. Cellular proteolysis is not only important for scavenging excess proteins, but also regulates gene expression, the cell cycle, apoptosis and cellular stress responses [1–3]. Protein degradation occurs by either lysosomal or non-lysosomal mechanisms [4]. One major family of lysosomal proteases is the cysteinyl proteases, which include cathepsin B, H, and L [5].

Effects of thiol or serine protease inhibitors on cell cycle and cell proliferation have been reported [6,7]. Although a role for intracellular proteases in the regulation of cell growth has been proposed [8], little evidence is currently available. Three trypsin-like proteases have been suggested to function at the G_1, S and G_2 phases in HeLa cells [9]. Proteolysis of important proteins involved in cell cycle functions, such as cyclin B and cyclin-dependent kinase inhibitor p27, is crucial in regulating cell cycle progression in a wide variety of cell types [10–12]. It has also been demonstrated that inactivation of calpain results in enhanced stability of the p53 protein that mediates downstream effects such as cell cycle arrest and apoptosis [13]. Recently, we purified and characterized a unique cathepsin-like protease, SPase, which selectively degrades important nuclear regulators such as transcription factor SP-1 and the retinoblastoma susceptibility gene product, RB [14]. Thus, a possible role for SPase is to affect the stability of important regulators involved in controlling cellular proliferation.

A specific protease, SPase, was isolated from nuclear extracts of the green monkey kidney cell line, CV-1 [14]. Studies of biochemical characteristics and substrate specificity indicate that SPase is a cathepsin B-like cysteinyl protease [14]. However, the two tryptic peptide sequences derived from the purified SPase are either identical or highly homologous to those of human cathepsin L, and share immunoreactivity with both anti-human cathepsin L and mouse cathepsin L antibodies [14]. SPase is localized to both cytoplasmic and nuclear fractions. Transcription factor SP-1 and the retinoblastoma susceptibility gene product, RB, are substrates of SPase, while other nuclear factors such as c-Jun and c-Fos are not. These results imply that SPase plays an integral role in regulating a set of proteins in the nuclei.

RB is a nuclear phosphoprotein that exhibits cell cycle-dependent alterations in its phosphorylation state [15–17]. Microinjection of the full-length or carboxyl-terminal half of the protein into cells inhibits G_1 phase progression, suggesting that RB may function during the early G_1 phase [18]. Involvement of RB at the G_2 and M phases of the cell cycle has also been recently suggested [19–21]. The extent of RB phosphorylation varies with the progression of cells through the cell cycle, suggesting that phosphorylated RB results in inactivation of RB function, thereby allowing cell proliferation [18]. The physiological interaction of D-type cyclins with RB may therefore help to direct cyclin-dependent kinases (cdks) to this substrate, resulting in RB phosphorylation at multiple sites and subsequent destabilization of the multimeric complexes [22–24]. Specific phosphatase acting on RB in the late stages of mitosis is also important for dephosphorylation [25].

In addition to being phosphorylated in a cell cycle-dependent manner, RB expression may be regulated at the post-transcriptional level [26]. This could be either at the level of translational control or protein turnover. It has been reported that ubiquitin-dependent proteolysis is involved in E7-E6- or E7-mediated degradation of RB [27,28]. These results raise the possibility that degradation of RB under certain conditions can be an alternative pathway to inactivate the normal function of RB in constraining cell growth.

SP-1 is a protein that activates transcription of viral and cellular genes that contain at least one SP-1 binding site con-

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sisting of a GC-rich decanucleotide sequence, 5′-GGGGCG-GGGC-3′ (GC-box), within their transcriptional control regions [29–31]. It has been shown that SP-1 is in both glycosylated and phosphorylated form [32,33], but it is still unclear whether these post-translational modifications play an important role in regulating SP-1-mediated transcription. Nevertheless, SV40-infected CV-1 cells show an increased amount of phosphorylated SP-1 by a dsDNA-dependent kinase [33]. Under certain physiological and biochemical conditions, phosphorylated SP-1 may influence the transcriptional initiation event and its intracellular stability by facilitating turnover by a specific protease [33].

RB is one of the substrates for SPase both in vitro and in vivo [14]. It is of interest to determine whether SPase is involved in cell cycle regulation by degradation of RB. In this report, we demonstrate that SPase activities are regulated through the cell cycle. Degradation of RB parallels SPase activity throughout the cell cycle. These results demonstrated that proteolysis of RB is a novel mechanism for controlling cell cycle progression.

2. Materials and methods

2.1. Materials

Aphidicolin, nocodazole, (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester (E-64d) and *N*-acetylleucylleucylnorleucinal (ALLN) were obtained from Sigma Inc. (St. Louis, MO). Isoleucine-free MEM select-amine kit, 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium chloride (NTB) were purchased from Gibco/Bethesda Research Laboratories (Gaithersburg, MD). Anti-RB (IF8) and anti-SP-1 (PEP2) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Prestained sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) standards were obtained from Bio-Rad (Hercules, CA), and ECL developing reagents were obtained from Amersham Life Science (Arlington Heights, IL). All other chemicals were of analytical grade.

2.2. Cell culture

CV-1 cells (green monkey kidney cell line), COS-1 cells (simian virus 40-transformed CV-1 cells) and BSC-1 cells (green monkey kidney cell line) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells were grown at 37°C in a humidified incubator with 6% CO_2.

2.3. Synchronization of cells

Cells were synchronized at the G_1 phase by isoleucine starvation for 48–60 h, then incubated with a complete medium supplemented with 5 μg/ml of aphidicolin for an additional 16 h to synchronize cells at the G_1/S boundary [34]. Cells were released from the G_1/S boundary by washing three times with phosphate buffered saline (PBS) and refeeding with the complete medium. Six h after the removal of aphidiocolin, cells were arrested at the S phase. M phase-arrested cells were collected after an additional 12–16 h of treatment with 0.04 μg/ml of nocodazole [35].

2.4. Oligonucleotides and electrophoretic mobility shift assays (EMSA)

Oligonucleotides of the wild-type human *c-jun* Sp-1 sequence, 5′-TGACGGGCGGGCCCGCCCCCCT-3′, were synthesized, deprotected, then purified through a Sephadex G-25 spin column. Complementary oligonucleotides were annealed and labeled at their 5′ ends using [γ-P^{32}]ATP (4500 Ci/mmol; ICN Biochemicals, Inc., CA) and T4 polynucleotide kinase (Promega, WI). Radiolabeled double-stranded oligonucleotides were purified through a Sephadex G-25 spin column. The specific activity of the oligonucleotide probes was typically 10^5 cpm/ng of DNA. Probes were stored at −20°C. For assay of SPase activity, EMSAs were performed as previously described [36].

2.5. Western blot analysis

Aliquots of nuclear extracts or recombinant proteins were electrophoresed on an SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with the anti-RB antibody, followed by a secondary antibody of either horseradish peroxidase- or alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G. Immunoreactive bands were detected with either 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium chloride (NBT) or ECL developing reagents.

3. Results

3.1. Evidence for the proteolytic activity of SPase in CV-1 cells

We have demonstrated that transcription factor SP-1 and the retinoblastoma susceptibility gene product, RB, are substrates of SPase purified from CV-1 cell nuclear extracts [14]. To examine the effect of SPase on SP-1 binding activity, we performed electro-mobility shift assay (EMSA) with incubated CV-1 cell nuclear extracts prepared in the presence of membrane-permeable cysteinyl protease inhibitor, E-64d or ALLN, and COS-1 cell nuclear extracts containing SP-1. We observed intact SP-1 binding activity in the nuclear extracts prepared from the E-64d- or ALLN-pretreated CV-1 cells (Fig. 1A, lanes 3 and 4), suggesting that the protease inhibitor diminishes proteolytic activity of SPase in vivo and prevents proteolytic degradation of cellular proteins such as SP-1. The specificity of protease inhibitors to inhibit SPase activity was confirmed by the addition of anti-cathepsin L antibody to depletion of SPase since anti-cathepsin L antibody cross-hybridized with SPase [14]. The SPase-depleted CV-1 nuclear extracts failed to inhibition of SP-1 binding (data not shown), suggesting that the inhibition of SP-1 binding observed initially was due to the proteolytic activity of the SPase. To explore the significance of SP-1 degradation by SPase activity in vivo, we examined the integrity of RB in the nuclear extracts prepared from permeable cysteinyl protease inhibitor-pretreated CV-1 cells by Western blotting using the anti-RB (IF-8) antibody. IF-8 antibody was directed against RB amino acid residues 300–380. As shown in Fig. 1B (lanes 2 and 3), either E-64d- or ALLN-treated CV-1 nuclear extracts, prepared in the absence of leupeptin, remained

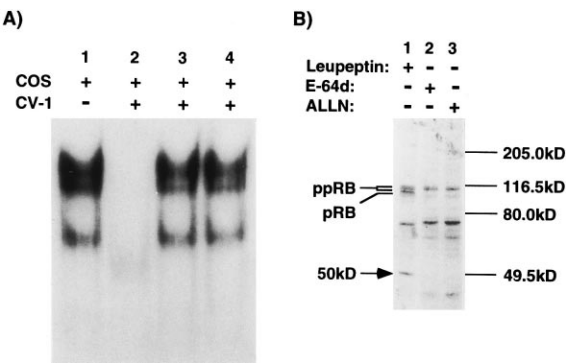

Fig. 1. Detection of SPase activity in the CV-1 cells. A: Four micrograms of COS-1 nuclear extracts were used in an EMSA with the h*cj*-Sp-1 probe (lane 1). A mixture of COS-1 and CV-1 nuclear extracts (4 μg each) was used in EMSA as a control (lane 2). CV-1 nuclear extracts prepared from cells treated with E-64d (50 μg/ml) or with ALLN (50 μM) for 5 h were used in lanes 3 and 4, respectively. B: Western blot analysis of RB degradation. CV-1 cells were grown in the absence or presence of E-64d (50 μg/ml) or ALLN (50 μM) for 24 h. The nuclear extracts were then prepared in the presence or absence of a protease inhibitor, leupeptin, as indicated. Ten micrograms of each nuclear extract were analyzed by the Western blot analysis probed with the anti-RB antibody (lanes 1–3).

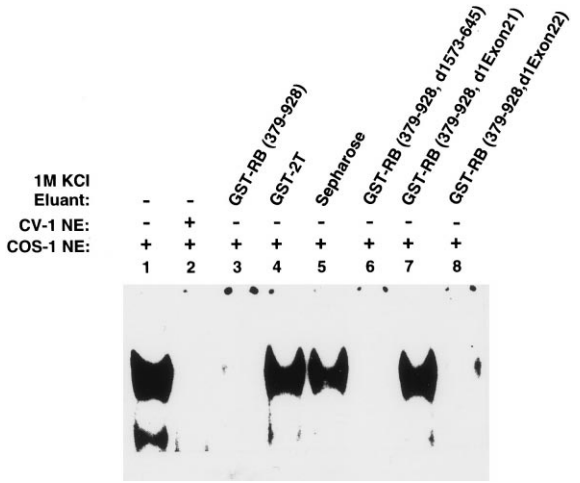

Fig. 2. SPase is a RB binding protein. Thirty micrograms of CV-1 nuclear extracts were incubated at 4°C for 1/2 h with 300 μl of glutathion S-transferase (GST)-sepharose beads immobolized with GST-RB (379–928) and its various mutants, GST-RB (379–928, dl573–645), GST-RB (379–928, dlExon21) or GST-RB (379–928, dlExon22). Proteins binding specifically to the beads were eluted with 1.0 M of KCl after extensive washing with a buffer containing 20 mM HEPES (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 20% glycerol, and 0.1 M KCl. Eluted fractions were dialyzed against EMSA binding buffer. One-third of the dialyzed samples were subjected to EMSA with h*cj*-Sp-1 probe to test the effect of SP-1 binding (lanes 3, and 6–8). The SP-1 DNA binding complex from 4 μg of COS-1 nuclear extracts and eluent from GST-2T sepharose or sepharose alone were used as controls for lack of SPase activity (lanes 1, 4 and 5). CV-1 nuclear extracts inhibiting the SP-1 DNA binding complex recovered from COS-1 nuclear extracts were used as a positive control for SPase activity (lane 2).

an intact RB with truncated 78-kDa proteins, but not the SPase-degraded 50-kDa fragment. In contrast, non-E-64d- or ALLN-treated extracts prepared in the presence of leupeptin, to prevent further degradation in vitro, generated the SPase-degraded 50-kDa fragment (Fig. 1B, lane 1). Therefore, SPase activity in CV-1 cells appears to be effectively inhibited by the permeable cysteinyl protease inhibitors, E-64d or ALLN, suggesting that SPase is indeed involved in both RB and SP-1 turnover in vivo.

3.2. SPase is an RB binding protein

Previous and the above studies indicated that the RB protein is a substrate for the specific protease, SPase. To provide further evidence that SPase binds to RB to form an enzyme-substrate complex, the glutathion S-transferase (GST)-RB (379–928) fusion protein and its various mutants, GST-RB (379–928, dl573–645), GST-RB (379–928, dlExon21) and GST-RB (379–928, dlExon22), were linked to glutathion sepharose for SPase binding analysis. GST-2T sepharose or sepharose alone was used as a negative control (Fig. 2, lanes 4 and 5). CV-1 nuclear extracts containing SPase activity were incubated at 4°C for 1/2 h with sepharose beads carrying various GST-RB fusion proteins. Proteins specifically binding to the beads were eluted with 1.0 M of KCl after extensive washing. Eluted fractions were dialyzed against EMSA binding buffer. One-third of the dialyzed samples were used for the effect of SP-1 binding assay with EMSA containing the h*cj*-Sp-1 probe [35]. These results demonstrated that 1.0 M of KCl eluent from GST-RB (379–928), GST-RB (379–928, dl573–645) or GST-RB (379–928, dlExon22) sepharose contains SPase activity sufficient to abolish SP-1 binding (Fig. 2, lanes 3, 6 and 8). In contrast, 1.0 M of KCl eluent from GST-RB (379–928, dlExon21) failed to inhibit SP-1 binding (Fig. 2, lane 7), suggesting that the RB exon 21 region is required for interaction with SPase to form an enzyme-substrate complex.

3.3. The activity of SPase is regulated through the cell cycle

The RB protein is an important cell cycle regulator, and is one of the substrates of SPase. To determine whether SPase activity is also regulated through the cell cycle, CV-1 cells were synchronized, and the activities of SPase were measured at different stages of the cell cycle. As shown in Fig. 3, high levels of SPase activity in synchronized CV-1 cells were detected at G_1/S, moderate levels at the G_1 and S phases, and no detectable levels at the M phase, suggesting that SPase activity is regulated through different phases of the cell cycle. The extent of synchronization of the cells was indicated by flow cytometry data, as shown in Fig. 3. The CDK2 protein has been expressed constitutively throughout the cell cycle in several cell lines [37]. We therefore examined the CDK2 protein levels as internal controls by Western blot analysis (Fig. 3, lower panel). Another green monkey kidney cell line, BSC-1, gave results similar to those of CV-1 cells (data not shown). Thus, SPase activity is regulated at different stages of the cell cycle.

3.4. Proteolysis of the RB or SP-1 proteins in SPase-containing cells is cell cycle stage dependent

To determine whether the level of RB or SP-1 correlates with the activity of SPase throughout the cell cycle, we examined the integrity of both RB and SP-1 at the different stages of synchronized CV-1 cells. Nuclear extracts from each stage

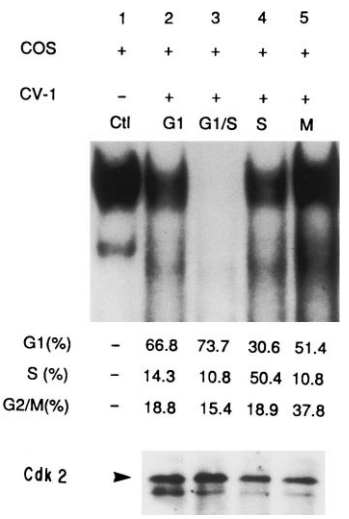

Fig. 3. Inhibition of SP-1 binding activity by CV-1 cell nuclear extracts from different stages of the cell cycle. The h*cj*-Sp-1 probe was used in the EMSA. Four micrograms each of nuclear extracts were subjected to EMSA, including COS-1 nuclear extract as control (lane 1), CV-1 nuclear extracts prepared from the G_1 phase (lane 2), the G_1/S block (lane 3), the S phase (lane 4), and the M phase (lane 5). CV-1 cells were synchronized as described in Section 2. The proportions of cells at different stages of the cell cycle in the synchronized CV-1 cells (data from FACS) are indicated below the EMSA. As shown in the bottom panel, equal amounts of CDK2 proteins were detected by Western blot analysis in 10 μg of CV-1 nuclear extracts prepared from the G_1, G_1/S, S and M phases of the cell cycle.

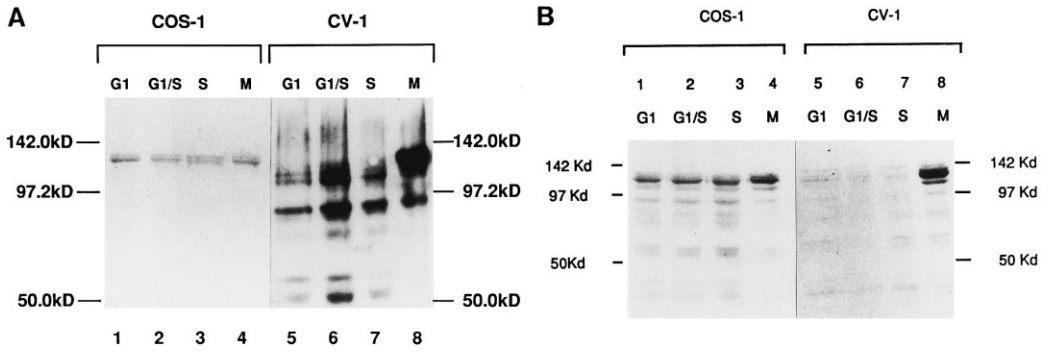

Fig. 4. Degradation of the RB and SP-1 proteins at different stages of the cell cycle in CV-1 cells. Equal amounts (15 μg) of nuclear extract proteins from COS-1 and CV-1 cells synchronized at the G_1, G_1/S, S or M phases of the cell cycle were loaded onto a 10% SDS-PAGE (lanes 1–4 for COS-1 cells, and lanes 5–8 for CV-1 cells). Western blot analysis was performed probing with the anti-RB antibody (panel A) or the anti-SP-1 antibody (panel B).

of the cell cycle were prepared in the presence of 5 μM leupeptin to prevent further degradation by SPase in vitro. The integrity of RB and SP-1 in vivo was analyzed using Western blot from the nuclear extracts prepared above, and probed with the anti-RB or the anti-SP-1 antibody (Fig. 4A and B). As shown in Fig. 4A, the RB protein was highly or partially degraded to a 50-kDa fragment in the nuclear extracts prepared at the G_1, G_1/S, or S phases (Fig. 4A, lanes 5, 6 and 7). In contrast, the RB protein retained intact without resulting in a degraded 50-kDa RB fragment at the M phase (Fig. 4A, lane 8). In COS-1 cells, which have undetectable SPase activity, the RB protein remained intact and at a constant level throughout the cell cycle (Fig. 4A, lanes 1–4). Similarly, almost undetectable levels of SP-1 proteins were observed in nuclear extracts prepared at the G_1, G_1/S and S phases (Fig. 4B, lanes 5–7), as compared with a high level of intact SP-1 at the M phase (Fig. 4B, lane 8). Expression of SP-1 throughout the cell cycle also remained unchanged in COS-1 cells (Fig. 4B, lanes 1–4). These results are consistent with those regarding the regulation of SPase activity during cell cycle progression, suggesting that degradation of RB or SP-1 by SPase is also cell cycle stage dependent in CV-1 cells.

4. Discussion

The cell cycle is a highly regulated event, and all steps of progression through the cell cycle must be rigorously controlled [10]. Emerging evidence suggests that in all cells, serine/threonine kinases and proteasomes involved in phosphorylation and degradation of cyclins, respectively, are central to control of the cell cycle [12,38,39]. SPase, a cathepsin-like enzyme, has been localizing to the cytoplasm and the nucleus in several cell lines [14]. The involvement of the cathepsins in degradation of highly active regulatory proteins [40–42] led to the hypothesis that SPase rapidly degrades short-lived nuclear regulatory proteins, and thus plays an important role in regulation of the cell cycle.

In this report, we demonstrate that proteolysis of the RB protein by a newly identified cathepsin-like nuclear protease, SPase, is regulated throughout the cell cycle in SPase-containing cell lines such as CV-1, BSC-1, MDCK and MDOK (Fig. 3 and Fu et al., unpublished data). The activities of SPase parallel the degradation of the RB protein at the G_1, G_1/S and S phases of synchronized CV-1 cells (Fig. 4A). In contrast, no detectable SPase activity (Fig. 3, lane 5) without the degraded 50-kDa RB was observed at the M phase of the cell cycle (Fig. 4A, lane 8). A similar result was also observed when SP-1 was analyzed by Western blotting of nuclear extracts prepared from synchronized cells (Fig. 4B, lane 8). These results suggested that SPase regulates cell cycle mediating proteolysis of RB.

The E2F-1 protein is a transcription factor that regulates several genes involved in DNA synthesis during the cell cycle [43]. It is known that E2F-1 interacts with an underphosphorylated form of RB at the G_1 phase of the cell cycle [44]. Upon phosphorylation of RB at the G_1/S block, the E2F-1-RB complex becomes dissociated, and the free E2F-1 molecule turns on the progression of the cell cycle to the S phase [45]. Our previous data [14] demonstrated that SPase preferentially digests hyperphosphorylated RB. Therefore, high levels of SPase activity at the G_1/S phase could be important in removing hyperphosphorylated RB at this specific stage. Furthermore, with a limited proteolysis of RB, the cleaved or truncated RB species retain their functions as underphosphorylated RB and still interact with E2F-1. However, after severe proteolysis of RB, the degraded RB may release the cell from the G_1/S block, which is analogous to the function of the phosphorylated RB (Fu et al., unpublished data). Therefore, the degree of RB proteolysis may generate a different pathway for the progression of the cell cycle in certain cell types.

Degraded RB with a fragment of approximately 60 kDa has been observed in TPA-treated U937 cells [46] or in a retinoic acid-treated non-small cell lung carcinoma cell line [47]. Moreover, instability of exogenously expressed RB in the prolonged culture of reconstituted retinoblastoma cells has been implicated in the loss of tumorigenic suppression function [48]. Recently, it has been reported that hypophosphorylated RB in the anti-cancer drug-treated HL-60 and U937 cells is cleaved into 68- and 48-kDa fragments by an interleukin 1B-converting enzyme-like protease [49]. The RB cleavage is also tightly associated with the initiation of apoptosis, suggesting that a protease may be involved in mediating apoptosis [50,51]. In addition, Hinds et al. [52] reported that ectopic expression of cyclin D1 can overcome RB-induced growth arrest but not cause RB phosphorylation, suggesting that RB can be regulated by mechanisms other than phosphorylation for growth control. Therefore, the findings in this report suggest that the specific proteolysis of RB by SPase is one of the mechanisms involved in controlling cell cycle progression.

There are several potential explanations for how SPase regulates cell cycle progression through proteolysis of RB. Since phosphorylated RB has been suggested to be a preferential substrate for SPase [13], SPase could be one controlling factor for the levels of phosphorylated RB at different stages of the cell cycle, and might therefore regulate cell cycle function. In this case, the interplay of RB with other regulatory proteins involved in cell cycle control, such as D cyclins or E2F-1, could be altered when RB is in a truncated form. The truncated and degraded forms of RB may act as dominant-negative mutants to compete for the normal function of RB in regulating the cell cycle. In addition, over-expression of RB has been reported to possibly cause G_2/M arrest [53]. Thus, SPase could regulate cell cycle progression at the G_2/M checkpoint by removing the excess RB prior to the G_2/M phase. Finally, the absence of SPase activity and RB degradation at the M phase of the cell cycle may be important for mitosis and the continuation of the next cell cycle. To further test these possibilities and define the biological functions of degraded RB by SPase in the cell cycle control, the effects of the exogenously expressed SPase gene in COS-1 cells on cell cycle progression are currently being investigated.

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