Caspase-3 Specifically Cleaves p21$^{WAF1/CIP1}$ in the Earlier Stage of Apoptosis in SK-HEP-1 Human Hepatoma Cells

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SUMMARY

In the present study, we provide evidence that ginsenoside Rh_2 (G-Rh_2) as well as staurosporine induces apoptosis of human hepatoma SK-HEP-1 cells by caspase 3-mediated processing of p21$^{WAF1/CIP1}$ in the early stage of apoptosis. Immunoblottings showed that G-Rh_2 as well as staurosporine induced the processing of caspase-3 to an active form, p17. In stable Bcl-2 transfectants however, G-Rh_2 induced DNA fragmentation, while staurosporine did not. In the early stage of apoptosis, p21$^{WAF1/CIP1}$ was detected to undergo proteolytic processing specifically conducted by caspase-3. p21$^{WAF1/CIP1}$ translated *in vitro* was cleaved into a p14 fragment, when incubated with cell extracts obtained from either G-Rh_2- or staurosporine-treated cells. Cleavage was equally inhibited in both cases by adding Ac-DEVD-cho, a specific caspase-3 inhibitor, but not by Ac-YVAD-cho, a specific caspase-1 inhibitor. Similarly, p21$^{WAF1/CIP1}$ was efficiently cleaved by recombinant caspase-3 overexpressed in *E. coli*. Moreover, the endogenous p21$^{WAF1/CIP1}$ of untreated-cell extracts was also cleaved by recombinant caspase-3. Mutation analysis allowed identification of two caspase-3 cleavage sites, DHVD^{112}/L and SMTD^{149}/F, which are located within, or near the interaction domains for cyclins, Cdks, and PCNA. Taken together, these results show that G-Rh_2 as well as staurosporine increases caspase-3 activity, which in turn directly cleaves p21$^{WAF1/CIP1}$ resulting in elevation of Cdk kinase activity in the early stages of apoptosis. We propose that proteolytic cleavage of p21$^{WAF1/CIP1}$ is a functionally relevant event that allows unleashing the cyclin/Cdk activity from the inhibitor seen in the earlier stage of apoptosis, the event of which may be associated with the triggering mechanism for the execution of apoptosis.

Key Words: Ginsenoside Rh_2 (G-Rh_2), staurosporine, Apoptosis, Caspase-3, p21$^{WAF1/CIP1}$

The abbreviation used here:

Ac-YVAD-cho, Ac-Tyr-Val-Ala-Asp-aldehyde; Ac-YVAD-cmk, Ac-Tyr-Val-Ala-Asp-chloromethyl ketone; Ac-DEVD-cho, Ac-Asp-Glu-Val-Asp-aldehyde.

Introduction

There is evidence to suggest that cyclins and Cdks are required for apoptosis, under certain circumstances [1, 2]. For example, specific pharmacological inhibitors of cyclin-dependent kinases, such as oloumoucine and flavopiridol, protect differentiated PC12 cells from apoptosis [3]. In addition, several apoptotic inducing factors, such as granzyme B, Fas, staurosporine, and topoisomerase I inhibitors have been reported to activate cyclin/Cdk complexes in the earlier stage of apoptosis [4-7]. In support of this view, Cdk protein kinase inhibitors, such as $p21^{WAF1/CIP1}$ and $p16^{INK4A}$ have been recently suggested to be functionally associated with apoptosis. Moreover, $p21^{WAF1/CIP1(-/-)}$ mutant cells or cells that down-regulate the expression of $p21^{WAF1/CIP1}$ are shown to be more susceptible to apoptosis than wild cell types in response to PGA_2 or ionizing irradiation, and this phenomenon can be rescued by ectopic expression of $p21^{WAF1/CIP1}$ [11, 12]. Thus, it appears that $p21^{WAF1/CIP1}$ is somehow functionally linked to apoptotic processes. Despite the accumulated data, it still remains largely unknown how these cell cycle-regulating components are functionally linked to the execution of apoptosis and the activation of aspartate specific cysteine proteases (caspases) that play a central role during the process of apoptosis.

Involvement of over ten families of caspases in the multistep apoptotic processes has been documented [13, 14]. A number of substrates for caspases have been identified, including enzymes involved in DNA repair system, nuclear and cytoplasmic structure proteins, and other proteins such as PARP, DNA-dependent protein kinase (DNA-PK), D4-GD1, U1-70kDa, PKC, and PAKs [15-20]. More recently, Rb protein, a mediator of cell cycle progression and regulation, has been identified as a substrate of caspase-3 (-like) protease during apoptosis induced by either staurosporine or tumor necrosis factor (TNF) [21, 22]. Thus, it is evident that caspase-dependent cleavages of the cell cycle regulating proteins are also functionally important molecular events in apoptosis.

Ginsenoside Rh_2 (G-Rh_2), a ginseng saponin with a dammarane skeleton, isolated from the root of *Panax ginseng* C.A. Meyer, has been reported to induce cell-growth inhibition as well as differentiation in various cancer cell types [23-27]. In earlier reports, we showed that G-Rh_2 at a concentration of 1 (μM inhibits the proliferation of SK-HEP-1 human hepatoma cells by down-regulating cyclin E/Cdk2 kinase activity at the G1/S transition stage, and that the down-regulation is partly a consequence of increased protein levels of $p27^{Kip1}$, an inhibitor of Cdk2 kinase [25]. We have also shown that G-Rh_2 at a concentration (12μM~7.5 μg/ml) 12-fold higher than that required to arrest the cell cycle can induce apoptotic cell-death, and that this cell-death is associated with proteolytic processing of caspase-3 to an active form, p17 [28]. However, processing of caspase-3 as well as apoptotic cell-death was not blocked by ectopic expression of Bcl-2 in SK-HEP-1 cells [28]. Thus, the underlying mechanism of G-Rh_2 is not fully understood, although there are several lines of evidence to suggest that the compound may act through glucocorticoid receptor binding [26]. It is interesting

however, to notice that G-Rh_2 can arrest the cell cycle by specifically inducing the Cdk kinase inhibitor at lower concentration, and induce apoptosis at higher concentration via proteolytic activation of caspase-3 under the same experimental conditions. Thus, the compound provides a means to study the link between the cell cycle regulatory machinery and the caspase-3-mediated mechanism of apoptosis.

Here, we describe evidence that $p21^{WAF1/CIP1}$ undergoes proteolytic cleavage conducted by caspase-3 in the earlier stage of apoptosis induced by either G-Rh_2 or staurosporine. Caspase-3 cleaves $p21^{WAF1/CIP1}$ at two sites, $DHVD^{112}$/L and $SMTD^{149}$/F, the first cleavage site of which is located between the N-terminus and the nuclear translocation signal and the second site is located within the interaction domains for PCNA and Cdk4/cyclin D. Taken together, we propose that proteolytic cleavage of $p21^{WAF1/CIP1}$ is a functionally relevant event that permits the release of the cyclin/Cdk complex from its inhibitor $p21^{WAF1/CIP1}$, resulting in elevated cyclin/Cdk kinase activity in the earlier stage of apoptosis.

Experimental Procedures

Materials

G-Rh_2 was generously provided by the Korean Ginseng and Tobacco Research Institute (Taejon, Korea), and dissolved in 80% ethanol and stored at -20℃. Dulbecco's Modified Eagle's Medium (DMEM), G418 and calf serum were from Gibco BRL (Grand Island). Staurosporine and general biochemicals were from Sigma (St. Louis). Mouse monoclonal anti-human $p21^{WAF1/CIP1}$ was from Upstate Biotechnology Inc. (Lake Placid). The tetrapeptide caspase inhibitors, Ac-DEVD-cho, Ac-YVAD-cho, and Ac-YVAD-cmk were purchased from Takara Shuzo Co. (Shiga). The fluorogenic substrates, DEVA-MCA and YVAD-MCA were purchased from Peptide Institute (Osaka).

Cell Culture

SK-HEP-1 cells were maintained as a monolayer culture in DMEM supplemented with 5% (v/v) heat-inactivated calf serum and gentamicin 50 (μg/ml. The cells were cultured for 12 h in serum-free DMEM and then treated with 7.5 (μg/ml (12 μM) of G-Rh_2, or 500 nM of staurosporine, for the indicated time under the same culture conditions.

Assay for Caspase3 Activity

The assay of enzyme activity was performed as described everywhere [29]. Briefly, cells were harvested, washed three times with PBS, and suspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. After addition of 10 mM digitonin, cells were incubated at 37℃ for 10 min. Lysates were centrifuged at 15,000 rpm for 3 min, and cleared supernatants were collected. Protein concentration was determined by the BCA assay and adjusted to 60 μg/ml. The lysates were incubat-

ed at 37℃ for 30 min with fluorescent-conjugated specific substrates: 50 μM of DEVD-MCA for caspase-3.

Expression Vectors

To construct the in vitro translation system, the full-length $p21^{WAF1/CIP1}$ cDNA was inserted into the pCITE-4 vector (Novagen) carrying an S-TagTM N-terminal fusion sequence. For this, the insert encoding the N-terminal region of S-Tag^{TM}-$p21^{WAF1/CIP1}$, which lacks the start codon ATG of $p21^{WAF1/CIP1}$, was generated by PCR and subcloned in-frame into the *Bam*HI/*Hinc*II sites of pCITE-4a(+) (Novagen). The 5' PCR primer (AAGGATCCTCAGAACCGGCTGGGGAT) containing a *Bam*HI restriction site and the 3' PCR primer (AAAAGCTTCAGGCTTCCTGTGGGCGG) containing a *Hind*III restriction site were prepared. Substitutions of Asn for Asp^{112}, and/or of Ala for Asp^{149} in $p21^{WAF1/CIP1}$ were accomplished by converting the aspartic acid codon GAC to AAC, and/or GAT to GCT by site-directed mutagenesis (Clontech Laboratories).

Expression and Purification of [^{35}S]-S-Tag^{TM}-$p21^{WAF1/CIP1}$, and Preparation of Recombinant Caspase-3

The resulting fusion protein of S-Tag^{TM}-$p21^{WAF1/CIP1}$ was translated in the TNT T7-coupled reticulocyte lysates system (Promega) in the presence of [^{35}S]methionine (Amersham) or cold methionine according to the instructions of the manufacturer. The translated S-Tag^{TM}-$p21^{WAF1/CIP1}$ was purified with S-protein Agarose (Novagen). The activated caspase-3 was prepared as previously described [18]. The recombinant caspase-3 overexpressed in bacteria was active, as confirmed by an enzyme assay with fluorogenic substrates, DEVD-MCA and YVAD-MCA.

Cleavage Assay of $p21^{WAF1/CIP1}$

In vitro translated, purified [^{35}S]-S-Tag^{TM}-p21WAF1/CIP1 was incubated with 50 (μg of apoptotic cell extracts or with E. coli extracts expressing recombinant caspase-3 in 20 μl of reaction mixture containing buffer A. The reaction mixtures were incubated at 30℃ for 1 h in the absence or presence of increasing concentrations of tetrapeptide inhibitors, Ac-DEVD-cho, Ac-YVAD-cho, or Ac-YVAD-cmk. The reaction mixtures were resolved on 15% SDS-PAGE and visualized by autoradiography.

Immunoblot Assay

As previously described [28], cells were harvested by scraping them into ice-cold PBS containing 1 mM PMSF and 0.5 μg/ml of protease inhibitors: aprotinin, pepstatin A, and antipain. Cells were pelleted and resuspended in lysis buffer (0.5% Triton X-100, 20 mM Tris-Cl pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 50 mM (βglycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, and protease inhibitors: leupeptin, pepstatin A, PMSF, and antipain). $p21^{WAF1/CIP1}$ proteins were detected by the ECL

method (Amersham Life Science), using a mouse monoclonal anti-human $p21^{WAF1/CIP1}$ antibody.

Results

$G\text{-}Rh_2$ as well as Staurosporine Induces Apoptosis via Proteolytic Activation of Caspase-3

In a previous report, we showed that in the same way as staurosporine' $G\text{-}Rh_2$ induces apoptosis via proteolytic cleavage of caspase-3 into the active p17 form in SK-HEP-1 human hepatoma cells. Here, we determined the protease activity in the cells in response to these agents and found that proteolytic cleavage of caspase-3 is associated with activation of the protease. Caspase-3 activity was significantly elevated at times as early as 0.5 h and reached a maximum, equivalent to 60-fold activation, after 1 h of treatment with 7.5 (μg/ml of $G\text{-}Rh_2$ (Fig. 1A). This time course corresponded to the time course of proteolytic cleavage of caspase-3 and coincided with the morphological changes in the cells induced by $G\text{-}Rh_2$ [28]. Similarly, elevation of protease activity was evident at 4 h and reached a maximum, which was 25- to 30-fold higher than the basal level, after 8 h in cells treated with 500 nM of staurosporine. (Fig. 1A, right). The results suggested that $G\text{-}Rh_2$ as well as staurosporine induce apoptosis via proteolytic activation of caspase-3.

Proteolytic Cleavage of $p21^{WAF1/CIP1}$ during Apoptosis

We examined whether $G\text{-}Rh_2$-induced apoptosis is associated with irreversible perturbation of the cell cycle through deregulation of components functioning at the G1/S transition. Interestingly, $p21^{WAF1/CIP1}$, an inhibitor of Cdk protein kinase underwent proteolytic cleavage and generated a smaller fragment, p14 in the cells in response to $G\text{-}Rh_2$ (Fig. 1B, upper panel). The time course of the appearance of the p14 fragment followed that of caspase-3 activation, suggesting that caspase-3 may be responsible for the cleavage of $p21^{WAF1/CIP1}$ in these cells. Similar results were observed when the cells were treated with staurosporine 500 nM (Fig. 1B, lower panel). The results strongly suggested that proteolytic cleavage conducted by caspase-3 may be a general molecular event during apoptosis.

We then examine whether caspase-3 is directly responsible for the cleavage of $p21^{WAF1/CIP1}$. [^{35}S]-S-Tag™-$p21^{WAF1/CIP1}$ translated *in vitro* (denoted as [^{35}S]$p21^{WAF1/CIP1}$) was incubated with apoptotic cell extracts obtained from cells induced with either $G\text{-}Rh_2$ (Fig. 2A) or staurosporine (Fig. 2B). In both cases, [^{35}S]$p21^{WAF1/CIP1}$ was equally processed into a smaller fragment (apparent molecular weight: 14 kDa) after incubation with the cell extracts (Fig. 2). The processing was prevented by adding increasing concentrations of Ac-DEVD-cho, a specific inhibitor of caspase-3, but was not by Ac-YVAD-cho or Ac-YVAD-cmk, inhibitors of caspase-1. These results strongly suggested that $p21^{WAF1/CIP1}$ is a biologically relevant substrate of caspase-3 (-like) protease.

The results were confirmed by that [^{35}S]p21WAF1/CIP1 translated *in vitro* underwent proteolytic cleavage by recombinant caspase-3 overexpressed by *E. Coli* (Fig. 3, left). The results clearly

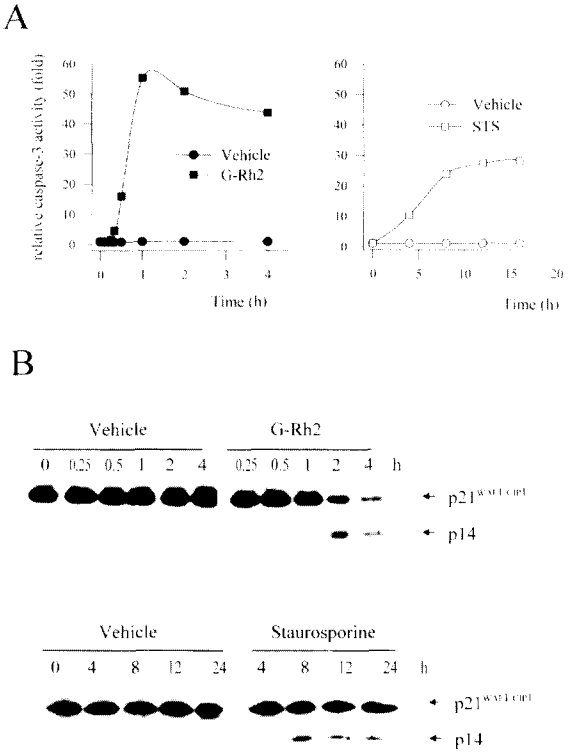

Fig. 1. Activation of caspase-3 and proteolytic cleavage of $p21^{WAF1/CIP1}$ in the apoptosis of SK-HEP-1 cells induced by either G-Rh_2 or staurosporine. A, activation of caspase-3 in the cells induced by G-Rh_2 or staurosporine (STS). The cells were treated with G-Rh_2 7.5 (μg/ml (left) or staurosporine 500 nM (right) for the indicated time, respectively. Using the cell lysates, the proteolytic activity of caspase-3 was measured as described under "Experimental Procedures." The results shown here were obtained from at least three independent experiments, and relative caspase-3 activity was calculated against the activity in vehicle-treated cell lysates at 0 time. B, proteolytic cleavage of $p21^{WAF1/CIP1}$ induced by G-Rh_2 or staurosporine. After treatment of the cells as described above, cell lysates were resolved by 15% SDS-PAGE, and then $p21^{WAF1/CIP1}$ and the cleaved fragments were visualized by immunoblotting using mouse monoclonal anti-human $p21^{WAF1/CIP1}$ antibody.

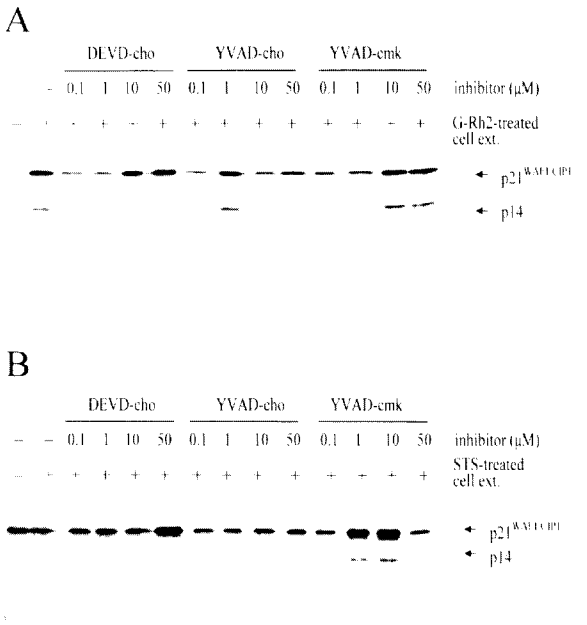

Fig. 2. Effects of caspase inhibitors on cleavage of $p21^{WAF1/CIP1}$ in an *in vitro* system. The apoptotic cell extracts, which were treated with either G-Rh_2 for 2 h (A) or staurosporine for 8 h (B), were prepared. The cell extracts were incubated for 1 h at 30℃, with purified [^{35}S]-S-Tag^{TM}-$p21^{WAF1/CIP1}$ in the absence or the presence of caspase inhibitors: Ac-DEVD-cho, Ac-YVAD-cho, or Ac-YVAD-cmk at concentrations of 0.1-50 (μM. The reaction mixtures were resolved on 15% SDS-PAGE gels, and subjected to autoradiography.

demonstrated that caspase-3 protease is directly responsible for the proteolytic processing of $p21^{WAF1/CIP1}$ that occurs in SK-HEP-1 cells during apoptosis induced by either G-Rh_2 or staurosporine.

Identification of caspase-3 cleavage sites on $p21^{WAF1/CIP1}$

We then searched for putative cleavage motifs of $p21^{WAF1/CIP1}$ by analyzing its amino acid sequence and identified the sequence DHVD^{112}L as a possible candidate. As expected, the *in vitro* translated-mutant protein, where D^{112} was substituted by N (denoted as D112N: Fig. 4B) failed to generate the p14 fragment after incubation with recombinant caspase-3, but unexpectedly generated a larger fragment, p19. Similar cleavage patterns were also observed when the mutant D^{112}N or wild type

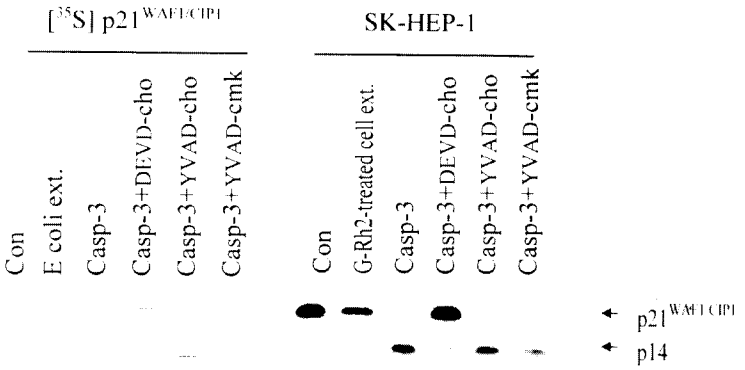

Fig. 3. Cleavage of $p21^{WAF1/CIP1}$ by recombinant caspase-3 in an *in vitro* system. Left, cleavage of [^{35}S]-S-Tag™-$p21^{WAF1/CIP1}$ by caspase-3. [^{35}S]-S-Tag™-$p21^{WAF1/CIP1}$ (denoted as [^{35}S]$p21^{WAF1/CIP1}$) was incubated in vitro with either *E. coli* extracts or *E. coli* extract containing recombinant caspase-3 (Casp-3) in the absence or presence of 50 (μM of tetrapeptide inhibitors, Ac-DEVD-cho, Ac-YVAD-cho, or Ac-YVAD-cmk. The reaction mixtures were resolved by 15% SDS-PAGE and visualized by autoradiography. Right, cleavage of endogenous $p21^{WAF1/CIP1}$ in the control cells by recombinant caspase-3. The cell extracts from untreated cells were incubated with the cell extracts from G-Rh_2-treated cells or *E. coli* extracts containing recombinant caspase-3 in the absence or presence of caspase inhibitors. After resolving the reaction mixtures on 15% SDS-PAGE, the endogenous $p21^{WAF1/CIP1}$ and its fragments were visualized by immunoblotting.

$p21^{WAF1/CIP1}$ was incubated with apoptotic cell extracts induced by either G-Rh_2 or staurosporine (Fig. 4B, left). The results suggested that there exist additional cleavage site(s) on $p21^{WAF1/CIP1}$ for caspase-3. As a possible additional putative cleavage site, we selected $SMTD^{149}$/F because D^{149} is followed by the hydrophobic amino acid F and a fragment with a size 19kDa would be expected if cleavage occurred at D^{149}/F site. For this, we prepared two additional mutants: D^{149}A, in which A replaces D149, and $D^{112}ND1^{49}$A, a double mutant in which both D^{112} and D^{149} are replaced by N and A, respectively. Caspase-3 generated p19 fragment from the D112N mutant, but failed to generate the p19 fragment from the double mutant D112ND149A (Fig. 4B, right), demonstrating that $SMTD^{149}$/F is an authentic cleavage site for caspase-3.

Collectively, we have shown here that caspase-3 directly cleaves $p21^{WAF1/CIP1}$ at two sites; $DHVD^{112}$/L and $SMTD^{149}$/F, to give an N-terminal 14 kDa fragment during apoptosis induced by either staurosporine or G-Rh_2.

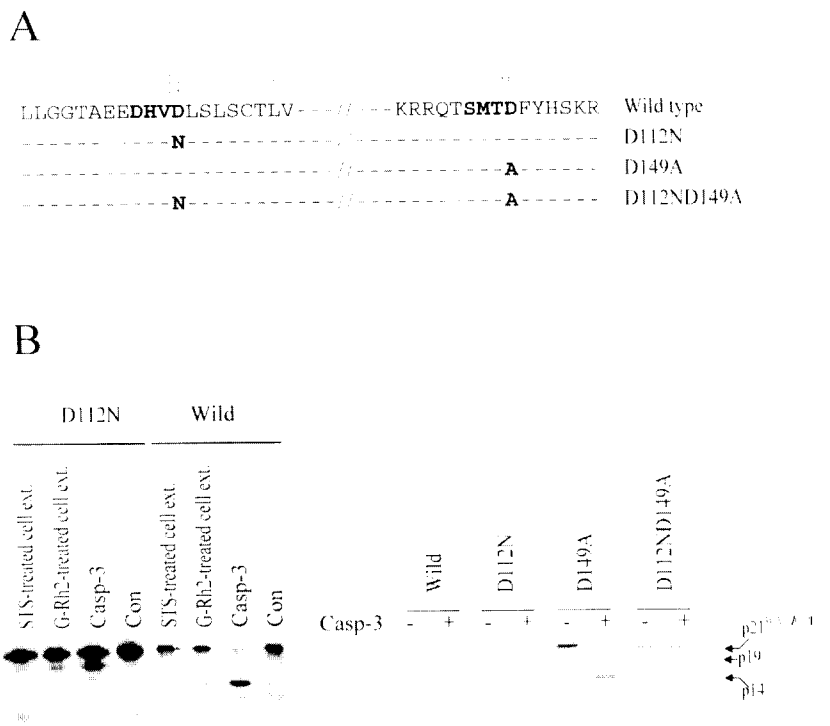

Fig. 4. Cleavage assays of p21$^{WAF1/CIP1}$ mutants by endogenous or recombinant caspase-3. A, schematics of wild type and mutants of p21$^{WAF1/CIP1}$. Putative caspase-3 cleavage sites were mutated as described under "Experimental Procedures." B, left, wild type and mutant p21$^{WAF1/CIP1}$ were translated in vitro in the presence of [^{35}S]methionine and purified by S-protein agarose beads. Wild type and mutant [^{35}S]p21$^{WAF1/CIP1}$ were incubated for 1 h at 30℃ with recombinant caspase-3 (Casp-3), or G-Rh$_2$- or staurosporine (STS)-treated cell extracts. The results were visualized by autoradiography. Right, wild type and mutants of [^{35}S]p21$^{WAF1/CIP1}$ were incubated for 1 h at 30℃ with recombinant caspase-3 (Casp-3). The reaction mixtures were then resolved by 15% SDS-PAGE, and subjected to autoradiography.

Discussion

In earlier reports, we showed that the staurosporine-induced proteolytic activation of caspase-3 is blocked by ectopic expression of Bcl-2 protein, whereas in the case of G-Rh$_2$, proteolytic activation is not blocked by overexpression of Bcl-2 protein. Consistent with this notion, we observed here that staurosporine-induced activation of caspase-3 is associated with activation of caspase-1, whereas this is not the case for G-Rh$_2$ [28]. These findings clearly suggest that these two agents act through different signaling pathways, but end up with a common caspase-3-mediated cleavage of p21$^{WAF1/CIP1}$.

Earlier reports have also shown that proteolytic degradation of endogenous and transfected $p21^{WAF1/CIP1}$ is associated with ubiquitination in cells being released from G1 arrest, and that degradation is inhibited by treatment with a proteasome inhibitor, suggesting that $p21^{WAF1/CIP1}$ is a substrate of ubiquitin-mediated proteolysis [30]. However, in our experimental system, $p21^{WAF1/CIP1}$ is directly cleaved by caspase-3. Thus, it is conceivable that the proteolytic degradation of $p21^{WAF1/CIP1}$ is conducted by a ubiquitin-mediated mechanism in the process of cell cycle progression, and by caspase-3 in apoptosis.

It is surprising however, that $p21^{WAF1/CIP1}$ is cleaved at $SMTD^{149}/F$ by caspase-3 as the amino acid sequence $SMTD^{149}/F$ does not agree with the consensus cleavage motif DXYD/Z. This may be compensated by the fact that D^{149} is followed by F, a hydrophobic amino acid that favors caspase-3 cleavage. It is interesting to note here that D at the first cleavage site, $DHVD^{112}/L$ of human $p21^{WAF1/CIP1}$ is not conserved among species. In mouse and rat $p21^{WAF1/CIP1}$ [31, 32], $DHVD^{112}/L$ is substituted with $DHVA^{110}/L$ and $DHVA^{112}/L$, respectively, suggesting that this site in mouse and rat is not cleaved by caspase-3 because D, an essential amino acid for proteolytic cleavage, is lacking at the cleavage site. In contrast, the amino acid sequence and the presence of D at the cleavage site of $SMTD^{149}/F$ of human $p21^{WAF1/CIP1}$ are highly conserved in mouse and rat as $SLTD^{144}/F$ and $SLTD^{149}/F$, respectively. Thus, it is very likely that these sites in mouse and rat $p21^{WAF1/CIP1}$ can be also cleaved by caspase-3. For these reasons, we assume that the second cleavage site, $SMTD^{149}/F$, but not the first cleavage site, $DHVD^{112}/L$ may be the universal cleavage site of $p21^{WAF1/CIP1}$ among mammalian species

Recently, several lines of evidence have suggested that cell cycle regulating components are functionally implicated in apoptosis. First, some of apoptosis-inducing factors such as granzyme B, Fas, staurosporine, and topoisomerase I inhibitors activate cyclin/Cdk complexes in the earlier stage of apoptosis [4-7]. Second, Cdk protein kinase inhibitors, such as $p21^{WAF1/CIP1}$ and $p^{16INK4A}$ are functionally associated with apoptosis [10]. These enigmatic phenomena may be explained by the fact that $p21^{WAF1/CIP1}$ undergoes caspase-3-mediated proteolytic cleavage, because the cleavage of $p21^{WAF1/CIP1}$ can unleash cyclin/Cdk protein kinases from the inhibitor. In support of this view, the two cleavage sites of $p21^{WAF1/CIP1}$, $DHVD^{112}/L$ and $SMTD^{149}/F$, found in this study are located within functional domains. The first cleavage site, $DHVD^{112}/L$ is located between the N-terminal domain (p14), which is known to play an important role in the interactions of Cdk2, cyclins D and E, and SAPK/JNK, and the amino acid sequence which contains the nuclear translocation signal [33-36]. The second cleavage site, SMTD149/F, is located within the functional domains responsible for interaction with PCNA and cyclin D/Cdk4 [33, 35, 37].

Taken together, we propose that the proteolytic cleavage of $p21^{WAF1/CIP1}$, which is directly conducted by caspase-3 is a common mechanism through which G-Rh_2 and staurosporine induce apoptosis in SK-HEP-1 cells. And the proteolytic cleavage of $p21^{WAF1/CIP1}$ in the earlier stage of apoptosis may be a part of the mechanism by which the protective effect of the $p21^{WAF1/CIP1}$ inhibitor on apoptosis can be

unleashed that triggers apoptosis.

Acknowledgments

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