The orphan nuclear receptor SHP inhibits apoptosis during the monocytic differentiation by inducing p21^{WAF1}

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Abbreviation: SHP, small heterodimer partner

Abstract

Small heterodimer partner (SHP) is an atypical member of nuclear receptor superfamily that lacks a DNA-binding domain. In previous study, we showed that SHP, c-jun, p65 of NF- κ B subunits, and p21WAF1 expression was increased during monocytic differentiation with the exposure of human leukemia cells to a differentiation agent, PMA. In this study, c-Jun and p65 were shown to mediate the transcriptional activation of the SHP promoter. In addition, SHP induced the cell cycle regulatory protein levels and cooperatively increased an induction of p21WAF1 expression with p65. Furthermore, SHP protected differentiated cells from etoposide-induced cellular apoptosis through the induction and cytoplasmic sequestration of p21WAF1. Complex formation between SHP and p21WAF1 was demonstrated by means of coimmunoprecipitation. These results suggest that SHP prolongs a cellular survival of differentiating monocytes through the transcriptional regulation of target genes of cell survival and differentiation.

Keywords: apoptosis; cell differentiation; cyclin-de-

pendent kinase inhibitor p21; monocytes; nuclear receptor subfamily 0, group B, member 2

Introduction

The molecular mechanisms regulating cell differentiation involve a fine and complex balance of proteins and signal transduction pathways that modulate the progression through control of cell cycle and cell survival (Scott *et al.*, 1994; Cory, 1995). Human macrophages are differentiated into noncycling cells arrested at the G₁ checkpoint that derive from peripheral blood cycling human monocytes. Although little is known regarding the molecular mechanisms governing the monocyte differentiation to macrophage, promonocytic human cell lines, can be triggered to differentiate to human macrophages by defined stimuli (Oberg *et al.*, 1993; Henkel *et al.*, 1999). Agents such as phorbol esters (PMA) induce their exit from the cell cycle at G₁, leading to their differentiation, which is characterized by the appearance of macrophage-like features such as cell surface integrins, adherence to plastic, and the production of reactive oxygen intermediates (Freytag, 1988; Baeuerle and Henkel, 1994; Casini and Pelicci, 1999). U937, human leukemic lymphoma cell line with monoblastic characteristics, can be induced to differentiate towards morphologically mature macrophage-like cells after treatment with PMA (Larsson *et al.*, 1988; Lubbert *et al.*, 1991).

Treatment of myeloid leukemia cells with PMA is associated with changes in the expression of certain early- and late-response genes and level of modification of several transcription factors by signal pathway. PMA down-regulates c-myc transcripts in HL-60 cells and induces expression of the *c-jun* gene (Sherman *et al.*, 1990; Szabo *et al.*, 1991). Activation of Jun/AP-1 contributes to induction of *c-jun* transcription and monocytic differentiation (Angel *et al.*, 1988; Szabo *et al.*, 1991). The differentiated macrophages, in contrast to cycling monocyte, contain significant levels of NF- κ B in the nuclei. The differentiation to macrophages triggered by plastic adherence or phorbol esters leads to a progressive degree of NF- κ B nuclear translocation, a hallmark of differentiated macrophages. NF- κ B plays a key role in cell differentiation by conferring cell survival that in the case of macrophages is

mediated through p21^{WAF1/Cip1} (Pennington *et al.*, 2001).

Small heterodimer partner (SHP) is an atypical orphan nuclear receptor that lacks a conventional DNA binding domain and consists only of putative ligand binding domain (Seol *et al.*, 1996). SHP is expressed in a wide variety of tissues, with highest expression in heart, brain, liver, lung, and adrenal gland (Johansson *et al.*, 2000). It has been reported that SHP represses the transcriptional activity of a number of nuclear receptors such as constitutive androstane receptor (CAR), estrogen receptor (ER), thyroid hormone receptor (TR), Retinoid X receptor (RXR), hepatocyte nuclear factor 4 α (HNF 4 α), androgen receptor (AR), liver receptor homolog 1 (LRH-1), ER-related receptor- γ (ERR γ), glucocorticoid receptor (GR), and liver X receptor (LXR) (Seol *et al.*, 1996; Lee *et al.*, 2000; Johansson *et al.*, 2000; Gobinet *et al.*, 2001; Borgius *et al.*, 2002; Brendel *et al.*, 2002; Lee and Moore, 2002; Sanyal *et al.*, 2002). Although SHP is well known as the repressor of various NRs, recently it is identified that SHP roles as a novel transcription coactivator of nuclear factor, NF- κ B and appears to functions as a distinct regulatory component of the transcriptional activities of NF- κ B in oxidized LDL-treated, resting macrophage cells (Kim *et al.*, 2001b). It was reported that during the procedure of promonocyte cell differentiation of HL-60 leukemia cells toward the monocyte lineage by phorbol ester, mRNA and protein levels of SHP were increased in a time-dependent manner. It seems that SHP plays a fundamental role in the processes of monocytic differentiation (Choi *et al.*, 2004). However, precise functions of SHP in monocytic differentiation have not been clearly elucidated. In the previous report, the role for SHP in the protection of cells against Nur77-mediated apoptosis has been proposed (Yeo *et al.*, 2005). Anti-apoptosis function of SHP would be related with the resistance of phorbol ester-differentiated monocytes against apoptosis.

A number of transcription factors have been implicated in monocyte differentiation together with the transcriptional regulation of the downstream target gene expression, including p21^{WAF1} (hereafter referred to as p21) (Freytag, 1988; Nguyen *et al.*, 1993; Scott *et al.*, 1994; Nerlov and Graf, 1998; Wang *et al.*, 1998; Kelly *et al.*, 2000). p21 inhibits cell cycle progression by binding to G₁ cyclin-CDK complexes through its N-terminal domain. In addition, recent studies have shown that p21 promotes the association of Cdk4 with D-type cyclins, and targets Cdk4 and cyclin D1 to the nucleus (LaBaer *et al.*, 1997). Thus, the cell cycle inhibitory activity of p21 is intimately correlated with its nuclear localization and this property appears to be responsible

for the early stages of the differentiation program (Jiang *et al.*, 1994; Steinman *et al.*, 1994; Halevy *et al.*, 1995; Andres and Walsh, 1996).

In this study, SHP-overexpressed cell represents resistance to apoptosis; moreover, p21 protein is significantly accumulated in the cytoplasmic compartment as a basal level and induced rapidly by the apoptosis signal in the SHP-overexpressed cell line. Additionally, it was confirmed that SHP was induced from an early phase of monocytic differentiation prior to a dramatic increase of p21. Together, this study shows that SHP increased by signal of differentiation or apoptosis plays a role to protect monocytic cells from cell death through rapid and significant accumulation of p21 in the cytoplasmic compartment.

Results

c-Jun and NF- κ B p65 increase the transcriptional activation of the SHP promoter

To investigate the influence on the basal level of cellular proteins related to monocytic differentiation, U937 cells were stimulated with PMA and assayed with immunoblotting. As shown as Figure 1A, c-Jun was induced and phosphorylated during the progression of differentiation. Furthermore, the level of p65 of NF- κ B subunits, SHP, and p21^{WAF1} were increased by the treatment of PMA. To gain insight that the differentiation signaling effects on the transcriptional activation of the SHP promoter, transient transfection assay was analyzed using the SHP promoter as a reporter construct with PMA. The result of Figure 1B shows that promoter activity of SHP is positively regulated by the treatment of PMA as a dose-dependent manner.

Cycling primary human monocytes have no NF- κ B in their nuclei. However, their differentiation into macrophages triggered by PMA significantly leads to nuclear translocation of NF- κ B. Its translocation into the nucleus by the PMA stimuli regulates various genes including monocytic differentiation and cell survival programs (Pennington *et al.*, 2001). To investigate whether NF- κ B regulates transcriptional level of SHP, p65 expression vector and SHP luciferase-based reporter were cotransfected. As a result, p65 increased the transactivation of the SHP promoter and p65 and c-Jun increased the expression level of SHP synergistically (Figure 1C). It is supported by the report that SHP plays a role as a coactivator of p65 in macrophage cells (Kim *et al.*, 2001b). Taken together, these results may indicate that the expression level of SHP is associated with transcriptional induction.

p21 induced by SHP in the late monocytic differentiation is sequestered in the part of the cytoplasm

It was reported that NF- κ B p65 directly interacts with SHP *in vitro* and *in vivo* and SHP plays a role as a coactivator of NF- κ B (Kim *et al.*, 2001b). To examine whether increased SHP by p65 contributes to induction of p21 transcriptional levels, SHP and p65 expression vector was cotransfected with p21 promoter-reporter vector. Single transcriptional activity of p65 on p21-promoter was not strong, in spite of the suggestion of the possibility that p65 directly regulates p21 gene expression (Pennington *et al.*, 2001). However, promoter activity of p21^{WAF1} was upregulated by p65 and

SHP synergistically (Figure 2A).

To obtain more information regarding increased expression, localization, and regulation of p65, SHP, and p21 in monocytic differentiation, U937 cells were stimulated with PMA for indicated times and nuclear and cytoplasmic fractions were obtained. As shown as Figure 2B, p65 was located in the cytoplasm in early differentiation, while in the late differentiation p65 translocated to the nucleus. Furthermore, the protein level of p21 was induced in the nucleus significantly at 24 h after treatment of PMA, and then decreased as time goes. In contrast, in the cytoplasm p21 was induced gradually after 24 h of PMA treatment. SHP was increased in the nucleus slightly from 12 to 24 h.

Figure 1. Increased expression of SHP, c-Jun, NF- κ B, and p21 during U937 promonocytic cell differentiation. (A) U937 cells grown in suspension become adherent following exposure to 5 nM PMA by 24 h with persistence of the phenotype for up to 3 days after the first appearance of the PMA treatments. The protein expressions of SHP, c-Jun, NF- κ B p65, and p21 were examined with their cognate antibodies and phosphorylation of c-Jun was detected with anti-phospho-c-Jun antibody. Western blotting of β -tubulin was used as a loading control. (B) Human SHP promoter reporter plasmid was transfected. After 24 h following transfection in absence or presence of several concentrations of PMA, as indicated, cells were harvested for luciferase assay. (C) Cells were transfected with indicated dose of p65 and c-Jun expression vector and human SHP promoter-luciferase plasmid together. Luciferase activity was measured and the values are expressed as the means $\pm$ S.D. for at least three independent experiments.

However, after 24 h of PMA treatment, SHP was significantly increased in the cytoplasm (Figure 2B). In order to analyze relationship between SHP and p21 in cytoplasmic localization, physical interaction between p21 and SHP was investigated by coimmunoprecipitation. As shown in Figure 2C, p21 was detected in the immune complexes of SHP from SHP-overexpressed cells. Also, we verified that p21 interacted with SHP during PMA-induced monocytic differentiation of U937 cells in

endogenous cellular levels (Figure 2D).

SHP-overexpressed cells are resistant to apoptosis induced by apoptogenic stimuli

In the previous report, p21, originally identified as a cell cycle inhibitor, acts as an inhibitor of apoptosis in the cytoplasm (Asada *et al.*, 1999). To identify the roles of the cytoplasmic translocation of p21 and SHP in cellular apoptosis, we established

Figure 2. p21 induced by SHP in the late monocytic differentiation is sequestered in the part of the cytoplasm. (A) The p65 and SHP upregulate p21 promoter activity synergistically. The p21 promoter (100 ng) was cotransfected with the indicated amount of expression plasmid encoding SHP and p65 in the presence or absence of etoposide treatment. After 48 h of incubation, cell lysates were obtained and analyzed. Luciferase activity was normalized for transfection efficiency by corresponding β -gal activity. Data shown are the mean of three independent determinations. (B) The protein levels and cellular localizations of SHP and p21 are related with monocytic differentiation. U937 cells were treated with 2.5 nM PMA and incubated for the indicated time and nuclear and cytoplasmic fractions were obtained. Each fraction was analyzed by 12% SDS-PAGE and immunoblotted. (C) The interaction between SHP and p21 was identified by coimmunoprecipitation method. Total cell lysates were obtained and immunoprecipitated with anti-p21 or anti-HA antibody and then analyzed with anti-HA or anti-p21 antibody by Western blot analysis. (D) U937 cells were treated with PMA for the indicated times and were assessed to coimmunoprecipitation using antibodies specific for the SHP. IgG served as a negative control. The precipitates were analyzed via immunoblotting with antibodies specific for p21.

SHP-overexpressing cells in HeLa cell lines. For this experiment, we used two kinds of stable cell clone isolated previously (S-1 and S-16). In SHP-overexpressing clones selected with G418, p21 protein level was significantly induced. To confirm the effect of increased p21 protein level on cell cycle and survival, the apoptosis susceptibility of mock and SHP overexpressing cells (S-1 cells) were tested by the method of DNA fragmentation assay and flow cytometry analysis. When mock transfectants were treated etoposide or H₂O₂, a large portion of cells underwent apoptosis; however, S-1 cells relatively showed resistance against apoptosis (Figure 3A and B). This result strongly suggests that SHP is an important regulator of apoptotic cell death. Furthermore, in the condition of etoposide-mediated apoptosis, the protein level of SHP, but not mRNA levels of SHP, was remarkably assessed (Figure 3C). As shown in Figure 3D, SHP is conducive to rapid induction of p21 with treatment of etoposide. These results suggest that cellular sensitivity to apoptosis in SHP-overexpressed cells is relevant to increased p21^{WAF1} expression.

p21 induced by apoptotic stimuli is sequestered in the cytoplasmic localization

In order to investigate the relationship with rapid induction of p21 and SHP in the presence of apoptotic stimuli, we tested the p21 promoter activity by SHP in the presence of etoposide. As shown in Figure 4A, the activity of reporter genes encoding p21 promoter was enhanced by SHP along with etoposide. Moreover, SHP was conducive to rapid induction of p21 proteins with treat-

Figure 3. SHP-overexpressed cells are resistant to apoptosis induced by apoptogenic stimuli. (A) SHP stably overexpressed clone was selected by the resistance against G418. Control and S-1 cells were incubated with or without etoposide or H₂O₂ and cells were harvested and subjected to extranuclear DNA extraction. Purified DNAs were electrophoresed in a 2% agarose gel and visualized by UV fluorescence after staining with ethidium bromide. (B) SHP inhibits apoptosis induced by treatments of etoposide. Cells were treated with etoposide for 16 h and fixed with 95% ethanol, washed twice with cold PBS, and treated RNase A. Cells were analyzed using flow cytometry for DNA content by PI staining. (C) U937 cells were treated with etoposide (100 μg/ml) for the indicated time intervals. After incubation, whole-cell lysates were assayed for SHP protein levels using western blotting. TBP expression is shown as a protein-loading control (upper panels). Total RNA from U937 cells were converted to ss-cDNAs by reverse transcriptase. These cDNAs were used as templates for PCR using SHP primers; β-actin primers were used as mRNA-loading control (lower panels). (D) Control and S-1 cells were treated with or without etoposide (100 μg/ml) in mild condition for 16 and 38 h. At the end of incubation, whole-cell lysates were obtained and assayed for p21 and Bax expression by Western blot analysis. TBP expression is shown as a protein-loading control (S-1, SHP stable clone No. 1).

ment of etoposide. Noteworthy, great part of increased p21 was detected in the cytoplasmic fraction; however, the protein level of p21 was increased in the nucleus either. In addition, basal protein level of p21 in the S-1 cells was much higher as compared with control cells (Figure 4B). To clarify the level and cellular localization of SHP and p21 related to monocytic differentiation and apoptosis, nuclear and cytoplasmic fractions were

obtained. The presence of cytoplasmic p21 is related to the acquisition of the resistance against etoposide stimuli, the same expressional pattern of SHP and p21 gave the insight to further studies. When etoposide was induced in U937 with or without PMA, SHP and p21 were induced in the cytosolic compartment (Figure 4C). In order to determine whether SHP prevents the cell death as sequestering the cytoplasmic accumulation of p21,

Figure 4. p21 induced by apoptotic stimuli is sequestered in the cytoplasmic localization. (A) SHP upregulated the promoter activity of p21. The reporter plasmid for human p21 promoter was transfected into HeLa cells. In 24 h after transfection in the absence or presence of etoposide for 12 h, cells were harvested for luciferase assay. Luciferase activity was measured and the values are expressed as the means $\pm$ S.D. for at least three independent experiments. (B) p21 is sequestered in the cytoplasmic compartment in SHP overexpressing cells. To examine the localization of p21, control and S-1 cells were treated with 100 μg/ml etoposide for 16 h. After incubation, whole cell lysates were assayed by western blot analysis. The expression of β-tubulin is shown as a loading control for cytoplasmic protein. (C) U937 cells were treated with or without 2.5 nM PMA for 48 h and then induced apoptosis with 50 μg/ml etoposide. Nuclear and cytoplasmic fractions were prepared and applied with Western blot analysis using antibodies against SHP and p21. The protein level of β-tubulin was measured for a loading control and a marker of cytoplasmic fraction. (D) Control and S-1 cells of equal numbers were transfected with siRNA for p21 to knockdown endogenous p21 expression. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to determine the effect of SHP and p21 on the cell viability exposed to the indicated doses of etoposide for 16 hr. The values are expressed as the means $\pm$ S.D. * $P < 0.05$ compared with the indicated control. The decreased protein expression of p21 with specific siRNA for p21 with the treatments of 100 μg/ml etoposide in control and S-1 cells was confirmed with Western blot analysis using anti-p21 antibody (lower panels).

cells were transfected with or without SHP and siRNA for p21 in the absence or presence of etoposide. As shown in Figure 4D, treatment of etoposide significantly inhibited the viability of cells as measured by MTT assay. However, SHP-overexpressed cells did not prevent the apoptotic cell death in the treatment of etoposide when the increases of p21 were inhibited using siRNA for p21. Taken together, these results suggest that SHP may inhibit proapoptotic signals mediated by induction of p21.

Discussion

From the point of view of regulation, the process of cell differentiation could be conceptually composed of two steps: the commitment to differentiation and the subsequent expressions of genes that determine the phenotype of differentiated cells. The control of commitment is crucial for the timing of differentiation, whereas the control of subsequent gene expression is crucial for the determination of the particular differentiated phenotype. In this study, it was suggested that SHP takes a part in the process of monocytic differentiation and plays a critical role for viability specified to monocytic cells.

It was identified that SHP expression is abundant in murine macrophage cell line, RAW 264.7 (Kim *et al.*, 2001b) and is upregulated during PMA-induced monocytic differentiation of HL-60 cells (Choi *et al.*, 2004). By this report, it needed to identify the protein level of SHP in the stage of differentiation during promonocyte to monocyte. Concomitantly, the level of SHP protein was induced in the process of PMA-induced monocytic differentiation of U937 cells (Figure 1A). Other reports suggested that PMA-induced monocytic differentiation is deeply associated with the induction of c-Jun by activation of SAPK. Coexpression of c-Jun and SHP was also observed in heart, kidney, epididymus, prostate, and pancreas in addition to monocyte/macrophage. Based on this coexpression and its ability to transactivate the SHP promoter, it was confirmed that c-Jun is a potent regulator of SHP expression in this study (Figure 1C).

The present study provides a number of novel observations pertaining to the regulation and functional roles of NF- κ B in monocytic cell differentiation. In the monocyte differentiation, NF- κ B translocates to the part of nucleus and plays a critical role for the gene expression needed to differentiation. Also, it was reported that NF- κ B mediates PTEN expression in TNF- α -induced monocytic differentiation (Lee *et al.*, 2007). It was

identified that SHP acted as a novel transcription coactivator of NF- κ B (Kim *et al.*, 2001b). Interestingly, NF- κ B and c-Jun together increased activity of SHP promoter synergistically (Figure 1C). It suggested the possibility that a certain signal pathway including activation of NF- κ B and c-Jun also targets on the regulation of SHP genes. We identified that SHP contributed on the transactivation of p21 promoter with NF- κ B p65 via physical interaction between SHP and p65 (Data not shown). The protective effect of NF- κ B is, in part, mediated through p21, since this protein was found to be regulated in an NF- κ B-dependent manner and to confer survival features during macrophage differentiation (Pennington *et al.*, 2001). In this study, it was suggested that SHP and NF- κ B may directly drive the transcription of p21.

During monocyte differentiation, the cell cycle inhibitory protein p21 translocates from the nucleus to the cytoplasm (Asada *et al.*, 1999). Interestingly, whereas in the nucleus p21 functions as a cell cycle brake by binding to multi-cyclin/Cdk complexes and PCNA-DNA polymerase δ subunit (Sherr and Roberts, 1995), in the cytoplasm p21 can promote the assembly of cyclin D/Cdk4 complexes and their nuclear translocation (LaBaer *et al.*, 1997). It was previously reported that in monocytes, p21 is expressed in the cytoplasm, where it acts as an inhibitor of apoptosis (Asada *et al.*, 1999). Another novel function of cytoplasmic p21 associated with increased neurite outgrowth in developing neurons has been recently reported (Tanaka *et al.*, 2002). Thus, cytoplasmic p21 exerts biological effects distinct from those of nuclear p21, and it is important to determine the regulatory mechanisms responsible for its cytoplasmic or nuclear expression. In this result, p21 and SHP was interacted *in vivo* (Figure 2C and D), it is suggested that colocalization and interaction of p21 and SHP have the possibility to enhance cytoplasmic function of p21.

Recently, another important role for p21 in the protection of cells against apoptosis has been proposed. Accordingly, increased susceptibility to p53-mediated apoptosis in p21 deficient cells was observed in colorectal carcinomas and melanomas (Polyak *et al.*, 1996; Gorospe *et al.*, 1997). Inhibition of stress-mediated apoptosis in MCF-7 cells treated with prostaglandin A2 is also associated with p21 expression. In addition, p21 antisense therapy radiosensitizes human colon cancer cells exposed to the oxidative stress from apoptosis (Migliaccio *et al.*, 1999; Kim *et al.*, 2001a). Interestingly in differentiated human macrophages, p21 is predominantly located in the cytosol where it exerts an anti-apoptotic function by means of inhibiting the proapoptotic kinase Ask1 (Asada *et al.*,

1999). Interestingly, when SHP-overexpressed cells were resistant to apoptosis, the protein level of p21 was significantly induced (Figure 3A and B). Particularly, several proteins related to the cell cycle were increased; however, there was no significant change of proliferation in the SHP-overexpressed cells (data not shown). Moreover, in the condition of evoked apoptosis, SHP was induced significantly in the level of native SHP protein as well as ectopic SHP protein (Figure 4B). It corresponds with the peculiar resistance of monocytic differentiated cells, therefore it is suggested that the increase of SHP in the monocytic differentiation contributes to the cell survival. As a reference of these reports, the cytoplasmic localization of p21 in the SHP-overexpressed cells seems to have an important relationship with resistance to apoptosis (Figure 4C and D). In summary, this study demonstrates that SHP participates in the monocytic differentiation program and contributes to the acquisition of resistance to apoptosis by induction of p21.

Methods

U937 cell culture and induction of differentiation

U937 promyelocytic leukemia cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin 100 U/ml and streptomycin 100 µg/ml (Gibco BRL). To induce monocyte differentiation, U937 cells were seeded at 2×10^5 /ml and grown in the presence of 2.5 nM PMA for up to 3 days. At the end of differentiation experiment, U937 cells were harvested and resuspended in an appropriate buffer for each experiment.

Plasmid, reagent, and antibodies

pSUPER/p21 siRNA400 was kindly donated by Dr. Michèle Sabbah (Fritah *et al.*, 2005). pGL2B/hSHP-Luc (-2.2 kb) was kindly provided by Dr. Heung Sik Choi (Chonman National University, Korea). pCMX1/c-Jun, pcDNA3/p65, pGL3B/p21-Luc (-2300), or pcDNA3/HA/SHP was previously described, each other (Jang *et al.*, 2001; Choi *et al.*, 2004; Kim *et al.*, 2007; Park *et al.*, 2007).

The transfection reagent, PolyFect, was purchased from QIAGEN. All other reagents were purchased from Sigma. Antibodies against c-Jun (sc-1694), phosphor-c-Jun (sc-822), p65 (sc-372), SHP (sc-22040), β -tubulin (sc-5274), TBP (sc-273), p21 (sc-6246), or Bax (sc-493) were purchased from Santa Cruz Biotechnology, INC. and β -actin (A2066) or HA (1 867 423) antibodies were obtained from Sigma and Roche, each other.

Transient and stable transfection

Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% antibiotics. The cells were seeded in 24-well plates with growth medium and cotrans-

fected with β -gal vector, mammalian expression vectors for p65 of NF- κ B subunits, c-jun, and reporter vectors. Total amount of expression vectors were kept constant by adding pcDNA3.1/HisC. Relative luciferase and β -galactosidase activities were determined. All the transfection results represent the mean of three independent experiments.

For establishment of the pcDNA3/HA/SHP expressing stable cell line, cells were transfected with 11 µg of SHP expression plasmid (pcDNA3/HA/SHP) using calcium phosphate precipitation method. After 48 h from transfection, cells were cultured in the presence of 400 µg/ml G418.

Coimmunoprecipitation

Nuclear and cytosolic extract were prepared by a modification of the method of Dignam *et al.* (1983). For nuclear extract preparation, cells were washed twice and collected. Cells were lysed in buffer A containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 10% Nonidet P-40 (NP-40). The nuclei were collected by centrifuge and lysed in 100 µl of buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM PMSF, 25% Glycerol, 420 mM NaCl, 0.2 mM EDTA). Immune complexes were immunoprecipitated from clarified cell lysates with protein G-sepharose bead (Invitrogen) preincubated with antibody to p21^{WAF1} (Santa Cruz Biotechnology, INC) and HA (Roche). The beads were washed extensively with buffer A, boiled in the loading buffer, separated by SDS-PAGE, and subjected to Western blot analysis.

DNA fragmentation assay

Cells (5×10^5) were collected after treatment with etoposide for 16 h, resuspended in 450 µl lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-Cl, pH 7.5) on ice for 30 min. After centrifugation at 15,000 rpm for 20 min, the supernatant was collected and treated proteinase K (final concentration 0.5 µg/ml) and 1% SDS for 2 h at 37°C. DNA was obtained by phenol extraction following precipitation. The DNA was precipitated with 1/2 volume of 10 M ammonium acetate, 2.5 volume of isopropyl alcohol for 30 min at -20°C and then centrifuged at 15,000 rpm for 20 min. The pellet was dissolved in 15 µl of TE buffer (10 mM Tris-Cl, pH 8.0, 10 mM EDTA) containing RNase A (final concentration 0.5 µg/ml) and incubated for 2 h at 37°C. After this digestion, the extract was fractionated by electrophoresis on 2% agarose at 40 V for 3 h. DNA was visualized under UV light after staining with ethidium bromide (EtBr).

RNA isolation and RT-PCR

Total RNA from U937 cells was prepared using TRIzol (Invitrogen) according to the manufacturer's recommendation. The cDNA was synthesized from 2 µg of total RNA with Moloney murine leukemia virus (MMLV) Reverse Transcriptase (Promega, Madison, WI) using a random hexamer at 37°C for 1 h. The PCR primers for SHP gene amplification were: 5'-AGCTATGTGCACCTCATCGACC-

TGC-3' (sense), 5'-CAAGCAGGCTGGTCGGAATGGACT-TG-3' (antisense); for β -actin gene amplification: 5'-GAC-TACCTCATGAAGATC-3' (sense), 5'-GATCCACATCTGCT-GGAA-3' (antisense). The SHP cDNAs were amplified by PCR under the following conditions: 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s in a thermal cycler. The PCR products were examined by electrophoresis on 1.2% agarose gels.

Western blot analysis

U937 and HeLa cells were suspended in the lysis buffer (150 mM NaCl, 1% NP-40, 1 mM EDTA, 50 mM Tris, pH 7.5) containing protease inhibitors, and quickly sonicated on ice. Protein concentrations were measured using a commercial protein assay dye (Bio-Rad). The 30 μ g of cytoplasmic or nuclear protein was electrophoresed on 10% SDS-PAGE, or 30 μ g of soluble total cellular proteins was electrophoresed, and was electroblotted thereafter on PVDF membranes (Millipore). Binding of the primary antibody was detected using the ECL advance Western Blotting Detection kit (Amersham Biosciences).

MTT assay

For cell viability assay, cells were seeded in a 24-well tissue culture plate and incubated for 24 h. Cells were transfected with the indicated expression vectors and then etoposide was treated in the indicated concentration. After 24 h, MTT solution (0.5 mg/ml) was added to each well. After incubation for 2 h at 37°C, formazan crystals in viable cells were soluble in 200 μ l of DMSO. The soluble formazan product was spectrophotometrically quantified using an ELISA reader at 570 nm.

Statistical analysis

The data is expressed as a mean $\pm$ SD. The significant differences between the groups were determined using an unpaired Student's *t*-test. A *P* value < 0.05 was considered significant.

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