Protein-tyrosine kinases (PTKs) play important roles in a variety of signal transduction pathways that are involved in cell growth, differentiation, cell death, and carcinogenesis (1–3). Since the products of many oncogenes are PTKs and since defects in normal PTKs are closely associated with carcinogenesis, increasing numbers of inhibitors of PTKs have been developed as potential anticancer drugs (2–8). Examples of the most useful anticancer drugs available to date are STI571 (Gleevec), which is used in the treatment of patients with chronic myeloid leukemia (4, 9), and ZD1839 (Iressa), which is used to treat non-small cell lung cancer (10). Oxindole inhibitors of PTKs, such as SU5416 and PD173074, suppress angiogenesis and are useful for the destruction of the vasculature that is needed for the growth and proliferation of tumor cells in vivo (11, 12). Most of the PTK inhibitors reported to date, including STI571, ZD1839, SU5416, and PD173074, have chemical structures that resemble the structure of ATP and compete with ATP for binding to the ATP binding site in the catalytic domain of the PTKs, with resultant inhibition of enzymatic activities. By contrast to these ATP-competitive inhibitors of PTK, the shikonin derivative β-hydroxyisovalerylshikonin (β-HIVS), isolated from the plant Lithospermum radix, inhibits the activity of v-Src in an ATP-non-competitive manner (13). This feature of β-HIVS is very useful for the inhibition of PTK activity in vivo because β-HIVS does not need to compete with ATP in the intracellular environment. In a previous study, we demonstrated that apoptosis is induced via suppression of the expression of apo-like kinase 1 (PLK1) after inhibition of PTK activity by β-HIVS (14). Polo-like kinase 1 and its homologs are involved in several aspects of mitosis, including activation of the anaphase-promoting complex (15), maturation of the centrosome (16) and formation of bipolar spindles (17, 18). In the present study, we continued our analysis of genes involved in β-HIVS-induced apoptosis using a DNA array and found that expression of a gene for tumor necrosis factor receptor-associated protein 1 (TRAP1) was significantly suppressed upon treatment of human leukemia HL60 cells with β-HIVS. TRAP1 was initially identified as a type I tumor necrosis factor receptor-binding protein by yeast two-hybrid screening, which is an efficient method for studying interactions among proteins (19, 20). An analysis of the cDNA sequence revealed that human TRAP1 is identical to heat shock protein 75 (HSP75), which is a member of the HSP family of molecular chaperones that interact with the retinoblastoma protein during mitosis and after heat shock (21). TRAP1 is substantially homologous to members of the 90-kDa family of heat-shock proteins (HSP90) and is expressed both in transformed cells and in a wide variety of normal tissues (19). HSP90 is an important molecular chaperone for proteins that are involved in numerous cellular processes (22–26). A number of cell signaling molecules, such as steroid hormone receptors and protein kinases, require HSP90 for maintenance in an active state within the cell (27, 28). The importance of HSP90 is also supported by its abundance in all species, with evolutionary conservation of its amino acid sequence from prokaryotes to mammals (29, 30). The HSP90 family of molecular chaperones gained a member upon the discovery of TRAP1/HSP75. However, TRAP1 does not form a stable complex with...
the classic co-chaperones of HSP90, such as p23 and Hop (31), even though HSP90 is able to form multicomponent complexes with these co-chaperones (32–35). Moreover, immunofluorescence experiments showed that human TRAP1 is localized in mitochondria and, indeed, mitochondrial localization sequences have been found at the amino terminus of this protein (31). Thus, it appears that TRAP1 has specific functions that differ from those of other members of the HSP90 family.

As noted above, apoptosis can be induced by a variety of extracellular stresses and signals. Heat shock is an extracellular stress that is involved in the induction of apoptosis and it results in the synthesis of a set of heat-shock proteins (HSPs). These proteins form a large family, which includes HSP90, HSP70, HSP60, and other small HSPs, and they play important roles in various aspects of cell homeostasis by functioning as molecular chaperones (36, 37). HSP70 protects cells from a number of apoptotic stimuli, such as heat shock, radiation, oxidative stress, withdrawal of growth factors, chemotherapeutic agents, ceramide, and tumor necrosis factor (38–40). These observations suggest that HSPs might play important roles in the regulation of apoptosis. In the present study, we investigated whether TRAP1, a member of the HSP family, has the ability to modulate a signaling pathway that induces apoptosis. Our results suggest that suppression of the expression of TRAP1 might be involved in the induction of apoptosis via changes in the functions of mitochondria.

EXPERIMENTAL PROCEDURES

Reagents, Cell Lines, and Cell Culture—Atractyloside, camptothecin, genistein, N-acetyl-cysteine, VP16, and HSP70-specific monoclonal antibody (BRM-22) were purchased from Sigma. β-HIVS was isolated from Lathyrus odoratus (44) and dissolved in ethanol at a concentration of 10−4 M, as used for a stock solution. ST1571, kindly provided by Novartis (Basel, Switzerland), was prepared as a 10−4 M stock solution in sterile phosphate-buffered saline. A monoclonal antibody against human TRAP1 (TRAP1–6) was kindly provided by Drs. D. O. Toft and S. J. Felts (Mayo Graduate School, Rochester, MN) or obtained from Affinity BioReagents, Inc. (Golden, CO). The monoclonal antibody against human Bel-2 and cislipatin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Monoclonal antibodies against human HSP90 (F-8), human lamin A/C (636), and human AIF (E-1); and polyclonal antibodies against human Bcl-2 and cisplatin were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Monoclonal antibodies against human Bax (N-20), human Bak (N-20), and human Raf-1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody against GAPDH (MAB374) was purchased from Chemicon International, Inc. (Tremecula, CA). A monoclonal antibody against cytochrome oxidase subunit IV (A-21347) was purchased from Molecular Probes, Inc. (Eugene, OR). A monoclonal antibody against cytochrome c (THS 2C12) was obtained from BD PharMingen (San Diego, CA). A monoclonal antibody against phosphotyrosine (PY20) was obtained from ICN Biomedicals, Inc. (Aurora, OH). The cdNA array kit (Atlas Cancer 1.2 array) and the Total RNA Labeling System were purchased from Clontech (Palo Alto, CA). Effectene transfection reagent and RNAiFect transfection reagent were purchased from Qiagen, Inc. (Chatsworth, CA). Small cell lung cancer cells (H446, DMS520, and N303) were purchased from the American Type Culture Collection. Human leukemia HL60 and K562 cells, human embryonic kidney (HEK) 293 cells and HeLa cells were provided by the Japanese Cancer Resource Bank. All lines of cancer cells were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal calf serum in the presence of the Poly(A)’ mRNA was isolated from β-HIVS-treated and untreated HL60 cells, and radiolabeled cdNA was prepared with [γ−32P]ATP using the total RNA Labeling System. Atlas Cancer 1.2 array nylon membranes were incubated initially for 30 min at 68 °C in ExpressHyb hybridization solution that contained 100 μg/ml salmon testis cdNA. Individual radiolabeled probes were added to the hybridization solution and incubation was continued overnight at 68 °C. The array membranes were then washed three times at 68 °C with 2× saline-sodium citrate buffer (SSC) that included 1% SDS, twice at 68 °C with 0.1× SSC that included 1% SDS, and finally with 2× SSC at room temperature. The membranes were scanned with a Storm Phosphorimager (Molecular Dynamics, CA) after 48-h exposure to a Phosphorimager screen. Images were analyzed with the AIS system (Imaging Research Inc.).

Preparation of Cell Lysates—Cells were washed twice with phosphate-buffered saline and lysed in lysis buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml antipain, 50 mM NaF, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.15 M NaCl, 1% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 15,000 × g for 15 min, and the supernatant was subjected to Western blotting analysis.

Western Blotting Analysis—A cell lysate containing 30 μg of protein was fractionated by SDS-PAGE and then proteins were transferred to a nitrocellulose membrane. The membrane was first rinsed with TBST (20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 0.05% Tween 20) and then blocked with 5% (w/v) skim milk in TBST for 1 h at room temperature. The blocked membrane was subsequently probed for 1 h at room temperature with a 1:200 to 1:1,000 dilution of first antibodies in blocking solution. The membrane was then washed three times with TBST, incubated with 1 μg/ml of horseradish peroxidase-conjugated antibodies against rabbit IgG or horseradish peroxidase-conjugated antibodies against mouse IgG. After the membrane had been washed with TBST, bands of protein on the membrane were visualized with an ECL Western blotting detection kit (PerkinElmer Life Sciences, Inc., Boston, MA).

The siRNAs and Transfection of Cells—TRAP1-SI1 and TRAP1-SI2 siRNAs were produced by Qiagen, Inc. (Chatsworth, CA). The sequences encoding the siRNAs were as follows (numbers in parentheses indicate nucleotide positions within the open reading frame of the target mRNAs): TRAP1-SI1 (5′-AAACATGAGTTCGATTCAAGG-3′ (75–95); and TRAP1-SI2 siRNA, 5′-AACATGAGTTCCAGGGCGGAG-3′ (238–258). Cells were treated with siRNA according to the instructions provided with the RNAiFect transfection reagent (Qiagen, Inc.) with slight modifications. DMS514 cells and HEK 293 cells (2 × 106) were treated with 5 μg of siRNA in RPMI 1600 medium (Invitrogen) designated with 10% fetal calf serum. Three days after transfection, cells were lysed with lysis buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5% Triton X-100) and incubated for 20 min on ice. The suspension was centrifuged at 27,000 × g for 20 min, and the fragmented DNA was recovered from the supernatant. The pellet remaining in the centrifugation tube was sonicated for 60 s at 36.5 kHz. The amount of total DNA was determined by a fluorometric method with DAPI. The intensity of fluorescence was measured at 454 nm with excitation at 362 nm. The extent of DNA fragmentation was defined as the ratio of the amount of fragmented DNA to the total amount of DNA.

Analysis of Cell Death—Apoptotic cells were assessed by an examination under a fluorescence microscope. The extent of DNA fragmentation after staining with Hoechst 33342. Quantitation of nuclear morphology was performed by determining the resulting ratio of DNA fragmentation in the absence of detergent and with DABCO, a phosphate-buffered saline (without Ca2++ and Mg2+). The washed cells were suspended in lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS) that contained 0.1% RNase A and incubated for 60 min at 50 °C. The lysate was incubated for an additional 60 min at 50 °C in the presence of 1 mg/ml proteinase K. The extent of DNA fragmentation in the absence of detergent and with DABCO was determined by a fluorometric method with DAPI. The intensity of fluorescence was measured at 454 nm with excitation at 362 nm. The extent of DNA fragmentation was defined as the ratio of the amount of fragmented DNA to the total amount of DNA. Analysis of DNA fragmentation by agarose gel electrophoresis was performed as follows: Drug-treated or untreated cells were collected by centrifugation and washed with Dubelcco’s phosphate-buffered saline (without Ca2++ and Mg2+). The washed cells were suspended in lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% SDS) that contained 0.1% RNase A and incubated for 60 min at 50 °C. The lysate was incubated for an additional 60 min at 50 °C in the presence of 1 mg/ml proteinase K. The extent of DNA fragmentation in the absence of detergent and with DABCO was determined by a fluorometric method with DAPI. The intensity of fluorescence was measured at 454 nm with excitation at 362 nm. The extent of DNA fragmentation was defined as the ratio of the amount of fragmented DNA to the total amount of DNA.
Construction of Plasmid Vectors and Transfection—The 0.9-kb EcoRI fragment containing the open reading frame of human Bcl-2 cDNA was inserted into the HinII site of the pUC19 cloning vector. The insert was digested with XbaI and HindIII and subcloned into the XbaI-HindIII restriction sites of the pRcCMV expression vector. DMS114 cells were transfected with expression plasmids using the EffecteneTM transfection reagent, according to the manufacturer’s instructions.

Subcellular Fractionation—DMS114 cells were disrupted by nitrogen cavitation as described elsewhere with slightly modifications (48). All subsequent manipulations were performed at 4 °C. The cells were washed with ice-cold Dulbecco’s phosphate-buffered saline and resuspended in buffer A (20 mM HEPES-KOH (pH 7.5), 250 mM sucrose, 1.5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenethylsulfonil fluoride), and then they were placed in the cavitation vessel. The vessel was pressurized with nitrogen gas to 350 lb/in² and allowed to equilibrate for 5 min. The valve for cell outflow was opened gradually so that the suspension was released dropwise and collected in a tube. Each lysate prepared by nitrogen cavitation was centrifuged at 700 × g for 10 min to remove nuclei and unbroken cells and then at 13,000 × g for 20 min for isolation of mitochondria. The nuclear fraction, including unbroken cells, was resuspended in sucrose buffer (2.2 mM sucrose, 1 mM MgCl₂) and the suspension was centrifuged at 40,000 × g for 60 min for isolation of nuclei. The post-mitochondrial supernatant was centrifuged at 100,000 × g for 20 min and the resultant supernatant was used as the cytosolic fraction.

Analysis of the Release of Cytochrome c from Mitochondria—Isolated mitochondria were suspended in buffer B (65 mM KCl, 10 mM HEPES-KOH (pH 7.5), 2 mM KH₂PO₄, 1 mM MgCl₂, 50 μM EGTA, 125 mM sucrose, 5 mM succinic acid), and the protein concentration was adjusted to 1.25 μg/μL. 80 μL of the suspension of mitochondria were incubated with 40 μL of the indicated cytosolic fraction at 37 °C for 45 min. The mixture was then centrifuged at 100,000 × g for 20 min, and aliquots of the supernatant were analyzed by Western blotting with the cytochrome c-specific antibody. Analysis of the release of cytochrome c using digitonin lysis buffer, was performed as follows: Cells (1 × 10⁶) were washed with ice-cold Dulbecco’s phosphate-buffered saline and resuspended in 100 μL of ice-cold digitonin lysis buffer (0.02% digitonin in phosphate-buffered saline). After 5 min on ice, the cells were centrifuged at 10,000 × g for 5 min and the supernatant was subjected to Western blotting analysis with the cytochrome c-specific antibody (49).

RESULTS

Suppression of Expression of the Gene for TRAP1 in Human Leukemia HL60 Cells upon Treatment with β-HIVS—Our previous study showed that β-HIVS induces apoptosis in human leukemia cells by inhibiting the activity of a PLK1. We also showed that the effect of β-HIVS on PLK1 activity occurs upstream of mitochondria (14). Mitochondria play an important role in the regulation of apoptosis by releasing apoptogenic molecules that include cytochrome c (50–52). In the present study, we tried to clarify the involvement of mitochondria in β-HIVS-induced apoptosis. To identify genes that might be involved in mitochondrial functions during β-HIVS-induced apoptosis, we performed cDNA array analysis using HL60 cells, as described previously (14). We found that the expression of several genes was affected by treatment of cells with β-HIVS (data not shown). Among the genes affected by β-HIVS, the gene for TRAP1 was of particular interest because TRAP1 is a member of the HSP family of molecular chaperones and is reported to be a member of the HSP family and to be localized upstream of mitochondria (14). Mitochondria play an important role in the regulation of apoptosis by releasing apoptogenic molecules that include cytochrome c (50–52).

Analysis of the Release of Cytochrome c from Mitochondria—Isolated mitochondria were suspended in buffer B (65 mM KCl, 10 mM HEPES-KOH (pH 7.5), 2 mM KH₂PO₄, 1 mM MgCl₂, 50 μM EGTA, 125 mM sucrose, 5 mM succinic acid), and the protein concentration was adjusted to 1.25 μg/μL. 80 μL of the suspension of mitochondria were incubated with 40 μL of the indicated cytosolic fraction at 37 °C for 45 min. The mixture was then centrifuged at 100,000 × g for 20 min, and aliquots of the supernatant were analyzed by Western blotting with the cytochrome c-specific antibody (49).

The 0.9-kb EcoRI fragment containing the open reading frame of human Bcl-2 cDNA was inserted into the HinII site of the pUC19 cloning vector. The insert was digested with XbaI and HindIII and subcloned into the XbaI-HindIII restriction sites of the pRcCMV expression vector. DMS114 cells were transfected with expression plasmids using the Effectene™ transfection reagent, according to the manufacturer’s instructions.

**Suppression of expression of the gene for TRAP1 by β-HIVS.** Total RNA was isolated from HL60 cells that had been treated with 10⁻⁶ M β-HIVS for the indicated times. Expression of TRAP1 and GAPDH mRNAs was examined by Northern blotting analysis. The level of expression of the gene for TRAP1 was normalized by reference to that of the gene for GAPDH (TRAP1/GAPDH) and is given as a percentage of the control value. The results are typical of results obtained in three independent experiments that gave similar results.

Involvement of TRAP1 in Apoptosis

In a previous study, we examined the effects of β-HIVS on the induction of cell death in 37 lines of cancer cells derived from human solid tumors, and we showed that β-HIVS increased the extent of apoptotic cell death (Fig. 3). We also investigated apoptotic cell death in DMS114 cells using a cell death detection ELISAPLUS kit (Roche Applied Science). This kit includes mouse monoclonal antibodies directed against DNA and histones and allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Assays using the ELISA™ kit confirmed that treatment of DMS114 cells with β-HIVS increased the extent of apoptotic cell death (Fig. 3). We also investigated apoptotic cell death in DMS114 cells using a cell death detection ELISA™ kit (Roche Applied Science). This kit includes mouse monoclonal antibodies directed against DNA and histones and allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Assays using the ELISA™ kit confirmed that treatment of DMS114 cells with β-HIVS increased the extent of apoptotic cell death (Fig. 3). We also investigated apoptotic cell death in DMS114 cells using a cell death detection ELISA™ kit (Roche Applied Science). This kit includes mouse monoclonal antibodies directed against DNA and histones and allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Assays using the ELISA™ kit confirmed that treatment of DMS114 cells with β-HIVS increased the extent of apoptotic cell death (Fig. 3).
Involvement of TRAP1 in Apoptosis

Effects of TRAP1-specific siRNAs on β-HIVS-induced Apoptosis—Since expression of TRAP1 was suppressed during the induction of apoptosis, we investigated the effects of PLK1-specific siRNAs on apoptosis. To examine the effects of siRNAs on the expression of TRAP1, we designed two siRNA sequences (TRAP1-SI1 and TRAP1-SI2) directed against the human gene for TRAP1, and introduced them into HEK 293 cells. As shown in Fig. 4A, treatment of HEK 293 cells with TRAP1-SI2 significantly suppressed the expression of TRAP1, as compared with that in cells treated with non-silencing siRNA or with vehicle alone. The extent of the suppression of the expression of TRAP1 by TRAP1-SI2 was greater than that by TRAP1-SI1. TRAP1-SI2 had no effect on the expression of HSP90 but treatment of HEK 293 cells with TRAP1-SI1 slightly suppressed the expression of HSP90. Levels of HSP70 and GAPDH in HEK 293 cells were unaffected by treatment with TRAP1 siRNAs under the same experimental conditions. Thus, TRAP1-SI2 had a strong and specific silencing effect on the expression of TRAP1. Fig. 4B shows the effects of β-HIVS on DMS114 cells after treatment of cells with vehicle, non-silencing siRNA and TRAP1-SI2. When DMS114 cells that had been treated with TRAP1-SI2 were exposed to 5 and 10 μM β-HIVS for 24 h, a significant increase in the induction of apoptosis was observed, as compared with that in cells exposed to β-HIVS after treatment with vehicle or with non-silencing siRNA. Fig. 4C shows the silencing effects of TRAP1-SI2 on the expression of TRAP1 in DMS114 cells that had been transfected with vehicle, non-silencing siRNA and TRAP1-SI2. The suppression of expression of TRAP1 was also observed in DMS114 cells after treatment with TRAP1-SI2. These results indicate that suppression of the expression of TRAP1 is intimately associated with the induction of apoptosis by β-HIVS.

Effects of TRAP1-specific siRNAs on apoptosis in HL60 cells. After treatment of HL60 cells with 10^{-6} M β-HIVS for the indicated times, DNA fragmentation (A and B) and the expression of TRAP1, HSP90, HSP70, and GAPDH (C) were examined, as described under “Experimental Procedures.” A, analysis of DNA fragmentation by agarose gel electrophoresis. B, analysis of DNA fragmentation, as determined by a fluorometric method with DAPI. The data are presented as means ± S.D. of the results of three independent experiments. C, Western blotting analysis with antibodies against TRAP1, HSP90, HSP70, and GAPDH. The results are typical of the results of three experiments that gave similar results.

Moreover, levels of TRAP1 were clearly depressed by treatment with VP16 for 6–15 h (Fig. 3E). These observations suggest that suppression of the expression of TRAP1 might be closely correlated with the induction of apoptosis by a variety of stimuli.

Effects of TRAP1-specific siRNAs on β-HIVS-induced Apoptosis—Although TRAP1 was identified originally as a tumor necrosis factor receptor-binding protein (19), it has been reported that TRAP1 is also present in the cytoplasm, where it interacts with the retinoblastoma protein during mitosis or heat shock (21). To examine the distribution of TRAP1 in DMS114 cells, we investigated the subcellular localization of TRAP1 by subcellular fractionation and Western blotting analysis. As shown in Fig. 5A, we detected TRAP1 in the mitochondrial fraction but not in isolated nuclei or in the cytosolic fraction. Fig. 5B shows the effects of β-HIVS on the expression of TRAP1, Bax, and cytochrome c in the mitochondria and cytosol of DMS114 cells. Treatment of DMS114 cells with β-HIVS increased the amount of cytochrome c in the cytosolic fraction and suppressed the expression of TRAP1 in mitochondria. No accumulation of TRAP1 was observed in the cytosolic fraction after treatment of cells with β-HIVS. These results indicate that the amount of TRAP1 in mitochondria drops markedly, without its release into the cytosolic fraction, during apoptosis. Although Bax has been reported to translocate from the cytoplasm to the mitochondrial membrane during apoptosis that is induced by DNA-damaging agents (53, 54), no translocation of Bax to the mitochondrial fraction was detected during treatment with β-HIVS (Fig. 5B). We also observed a marked decrease in the level of TRAP1 in mitochondria after treatment of DMS114 cells with VP16 (Fig. 5C). To our surprise, we found most of the Bax protein in the cytosolic fraction of DMS114 cells and no translocation of Bax to mitochondria was observed even after treatment of cells with VP16 (Fig. 5C). We confirmed that treatment of HeLa cells with VP16 induced apoptotic cell death (Fig. 5E) and caused the translocation of Bax from the cytosol to the mitochondrial fraction, with the resultant release of cytochrome c (Fig. 5D).

TRAP1-specific siRNA Enhanced the Release of Cytochrome c from Mitochondria—Mitochondria play a crucial role in apoptosis via the release of apoptogenic molecules that include cytochrome c (50–52). As demonstrated above, suppression of the expression of TRAP1 in mitochondria sensitized DMS114 cells to β-HIVS-induced apoptosis (Figs. 4 and 5). Therefore, we next examined the effects of β-HIVS and VP16 on the release of cytochrome c from mitochondria in DMS114 cells that had been treated with TRAP1-specific siRNAs. When DMS114 cells that had been treated with TRAP1-SI2 siRNA were exposed to β-HIVS, we observed a significant increase in the release of cytochrome c, as compared with that in cells exposed to β-HIVS after treatment with non-silencing siRNA (Fig. 6, A and C). There was no significant enhancement of the release of cytochrome c upon treatment with β-HIVS of DMS114 cells that were also treated with non-silencing siRNA, as compared with control cells (Fig. 6A). VP16 also enhanced the release of cytochrome c from mitochondria in DMS114 cells that had been...
transfected with TRAP1-specific siRNA (Fig. 6, B and D). Thus, the phenomenon whereby reduction of the expression of TRAP1 increased the amount of cytochrome c released during apoptosis was not restricted to cells treated with β-HIVS.

The results described above demonstrated that the release of cytochrome c from mitochondria in DMS114 cells was enhanced when levels of TRAP1 were depressed by TRAP1-specific siRNA. To investigate the details of the mechanism of the release of cytochrome c from mitochondria in response to β-HIVS and VP16, we constructed an assay system in vitro using isolated mitochondria and the cytosolic fraction from DMS114 cells. When mitochondria from DMS114 cells were treated with atractyloside, which is a specific inhibitor of adenine nucleotide translocase (ANT) and directly induces the release of cytochrome c from mitochondria (55), cytochrome c was released in a dose-dependent manner (Fig. 7A). By contrast, exposure of isolated mitochondria to β-HIVS or to VP16 did not induce the release of cytochrome c (Fig. 7A), indicating that β-HIVS and VP16 could not directly induce the release of cytochrome c from mitochondria. Fig. 7B shows the effects of a cytosolic fraction, obtained from DMS114 cells that had been treated with β-HIVS, on the release of cytochrome c from isolated mitochondria. We found that the cytosolic fraction obtained from DMS114 cells that had been exposed to β-HIVS...
induced the release of cytochrome c from isolated mitochondria. We observed the similar release of cytochrome c using a cytosolic fraction from cells that had been treated with VP16. No significant release of cytochrome c was induced by the control cytosol (Fig. 7C). We next examined the involvement of TRAP1 in the release of cytochrome c, using mitochondria isolated from DMS114 cells that had been transfected with TRAP1-specific siRNA. Treatment of DMS114 cells with TRAP1-specific siRNA suppressed the expression of TRAP1 in isolated mitochondria, whereas the expression of TRAP1 was unaffected in mitochondria obtained from cells treated with non-silencing siRNA (Fig. 7E). As shown in Fig. 7, D and F, the release of cytochrome c from mitochondria that had been isolated from DMS114 cells treated with TRAP1-specific siRNA was enhanced by incubation with the cytosolic fraction obtained from cells that had been exposed to either β-HIVS or VP16, as compared with the release of cytochrome c from mitochondria from cells treated with non-silencing siRNA. There was a slight increase in the release of cytochrome c from mitochondria from cells that had been transfected with TRAP1-specific siRNA even without incubation with a cytosolic fraction. Taken together, our findings suggest that changes in levels of TRAP1 in mitochondria might be associated with the regulation of the release of cytochrome c from mitochondria.

**Expression of Bcl-2 Inhibits Both the Release of Cytochrome c and the Suppression of Expression of TRAP1 during Apoptosis**—Since the anti-apoptotic protein Bcl-2 is known to prevent the release of cytochrome c from mitochondria and to inhibit subsequent apoptosis (56, 57), we investigated whether overexpression of Bcl-2 might affect suppression of the expression of TRAP1 during β-HIVS-induced apoptosis. We constructed two expression plasmids (pBcl-2-1 and pBcl-2-2) and introduced them into DMS114 cells. As shown in Fig. 8A, transfection of cells with the expression plasmids resulted in overexpression of Bcl-2, whereas barely any Bcl-2 was detected in control cells and in cells transfected with the vector or treated with vehicle alone. Both the induction of apoptosis and the release of cytochrome c in response to β-HIVS were inhibited in cells that had been transfected with Bcl-2-expression plasmids, as compared with those events in control cells and in cells transfected with the vector alone (Fig. 8, B and C). Fig. 8D shows the effects of overexpression of Bcl-2 on the expression of TRAP1 during β-HIVS-induced apoptosis. Suppression of the expression of TRAP1 by β-HIVS was inhibited in DMS114 cells that had been transfected with the Bcl-2-expression vector, as compared with that in cells transfected with the vector alone, suggesting that Bcl-2 might be involved in the regulation of the expression of TRAP1 in DMS114 cells.

**Effect of Inhibitors of Tyrosine Kinase on the Expression of TRAP1**—Our previous study showed that tyrosine kinases might be one of direct targets of β-HIVS in the induction of apoptosis (13). To study the mechanism of down-regulation of the expression of TRAP1 by β-HIVS, we investigated the effects of inhibition of tyrosine kinases on the expression of TRAP1. As shown in Fig. 9A (left panel), the extent of the tyrosine phosphorylation of proteins in DMS114 cells was clearly reduced after treatment of the cells with β-HIVS and the expression of both TRAP1 and PLK1 was suppressed in a dose-dependent manner. These results were consistent with our previous finding that the expression of PLK1 is suppressed, via inhibition of tyrosine kinase activity, by β-HIVS (14). The results in Fig. 9A (right panel) show the effects on the expression of PLK1 and TRAP1 of an inhibitor of tyrosine kinase, namely, genistein, which is a natural isoflavone that specifically inhibits the activities of protein tyrosine kinases (58). Treatment of DMS114 cells with genistein suppressed the expression of PLK1 and such suppression was closely correlated with inhibition of the tyrosine phosphorylation of proteins, as also observed upon treatment of cells with β-HIVS. However, even though the tyrosine phosphorylation of proteins was inhibited, the level of expression of TRAP1 was unaffected by genistein. Fig. 9B shows the effects of β-HIVS and of genistein on the induction of apoptosis under the same conditions. The number of apoptotic cells increased upon treatment with β-HIVS and this effect was dose-dependent. By contrast, no significant enhancement of the induction of apoptosis was observed upon treatment with genistein. Thus, neither suppression of the expression of PLK1 nor inhibition of tyrosine kinase activity was directly correlated with the induction of apoptosis, and our results suggest that suppression by β-HIVS of the expression of TRAP1 in DMS114 cells might be correlated with the induction of apoptosis. We also examined the effect of another inhibitor of tyrosine kinase,
STI571, which selectively inhibits the tyrosine kinase activity of p210<sub>bcr/abl</sub> (9). In chronic myeloid leukemia (CML) K562 cells, the activity of the tyrosine kinase that is encoded by the Bcr-Abl fusion gene is deregulated (60, 61). As shown in Fig. 9C, both the expression of PLK1 and levels of tyrosine phosphorylation of proteins in K562 cells were reduced by STI571 and these effects were dose-dependent, as described previously (14). By contrast, the expression of TRAP1 in K562 cells was unaffected by STI571 (Fig. 9C). These results suggest that suppression of the expression of TRAP1 in DMS114 cells by β-HIVS might not simply be caused by the inhibition of tyrosine phosphorylation.

Inhibition of β-HIVS-induced Suppression of the Expression of TRAP1 by an Antioxidant—It has been suggested that reactive oxygen species (ROS) might play an important role in the regulation of apoptosis (62–65). Therefore, we examined the
effects of the antioxidant N-acetyl cysteine (NAC) on apoptosis and suppression of the expression of TRAP1 in response to β-HIVS. As shown in Fig. 10A, the extent of apoptosis induced by treatment with β-HIVS and with VP16 was markedly reduced in the presence of NAC. Moreover, even though camptothecin (CPT), which is an inhibitor of DNA topoisomerase I, and the alkylating agent cisplatin also induced apoptosis in DMS114 cells, NAC had no significant inhibitory effect on the induction of apoptosis. Fig. 10, B and C show the effects of β-HIVS, CPT, and cisplatin on the expression of TRAP1 in DMS114 cells. The extent of the suppression of expression of TRAP1 by β-HIVS was much greater than that by CPT and by cisplatin. As shown in Fig. 10D, the suppression of the expression of TRAP1 by β-HIVS was inhibited in the presence of NAC. Taken together, these findings indicate that ROS, formed as a result of treatment with β-HIVS, might be important regulators of the expression of TRAP1.

**DISCUSSION**

In the present study, while attempting to identify genes that are involved in the mitochondrial functions that are associated with β-HIVS-induced apoptosis, we found that β-HIVS suppressed the expression of the gene for TRAP1. The level of expression of TRAP1 fell after treatment of HL60 and DMS114 cells with β-HIVS and the effects of β-HIVS were both dose- and time-dependent. VP16 also suppressed the expression of TRAP1 in DMS114 cells. By contrast, the results in Figs. 2 and 3 indicate that DMS114 cells required higher doses of β-HIVS for induction of apoptosis than those required to induce apoptosis in HL60 cells. The level of expression of TRAP1 was also higher in untreated DMS114 cells than that in untreated HL60 cells (data not shown). These findings suggest the possibility that high-level expression of TRAP1 might be involved in anti-apoptotic effects, as is the case for Bcl-2. This possibility is supported by the results in Fig. 4, which indicate that suppression of the expression of TRAP1 by siRNA-sensitized DMS114 cells to β-HIVS-induced apoptosis.

Heat shock proteins are highly conserved molecular chaperones that play important roles in the proper folding and assembly of proteins under stress conditions. Recent reports have suggested the involvement of HSPs in the regulation of apoptotic processes (38–43). HSP70 blocks the activation of caspase-3 and subsequent apoptotic cell death, even though...
HSP70 does not inhibit the release of cytochrome c from mitochondria (66). Thus, HSP70 appears to prevent the induction of apoptosis by acting downstream of the release of cytochrome c and upstream of the activation of caspase-3. A similar mechanism for suppression of apoptosis that involves HSP27, another member of the HSP family, has also been reported. HSP27 blocks etoposide-induced apoptosis by preventing the cytochrome c and dATP-triggered activity of caspase-9, downstream of the release of cytochrome c (67). By contrast to the functions of HSP70 and HSP27, which act as general inhibitors of apoptosis, HSP90 appears to operate via a more specific inhibitory mechanism. The serine/threonine kinase Akt is a downstream effector of phosphoinositide 3-kinase and is thought to mediate many biological effects that are associated with anti-apoptotic processes. Inhibition of binding of Akt to HSP90 leads to the dephosphorylation and inactivation of Akt, resulting in the increased sensitivity of cells to the induction of apoptosis (68). A recent report describes the negative regulation of the cytochrome c-mediated oligomerization of Apaf-1 and the activation of procaspase-9 by HSP90 (43). It is conceivable, therefore, that each individual HSP, namely, HSP90, HSP70, and HSP27, acts at a different step to inhibit apoptosis.

**Fig. 7. Effects of the expression of TRAP1 on the release of cytochrome c from isolated mitochondria.** A, mitochondria isolated from DMS114 cells were exposed to atractyloside, β-HIVS, and VP16, separately and at the indicated concentrations, and incubated at 37 °C for 45 min. Supernatants obtained after centrifugation were analyzed by Western blotting with cytochrome c-specific antibody. B, mitochondria isolated from DMS114 cells were incubated at 37 °C for 45 min with a cytosolic fraction, obtained from DMS114 cells that had been exposed to 10 μM β-HIVS for 24 h. Supernatants obtained by centrifugation were analyzed by Western blotting with cytochrome c-specific antibody. C, mitochondria isolated from DMS114 cells were incubated at 37 °C for 45 min with a cytosolic fraction (protein concentration, 2.5 μg/μl), obtained from DMS114 cells that had been exposed to 10 μM β-HIVS or to 100 μM VP16 for 24 h. Supernatants obtained by centrifugation were analyzed by Western blotting with cytochrome c-specific antibody. D, mitochondria (Mt) isolated from DMS114 cells that had been treated with non-silencing siRNA and TRAP1-specific siRNA (TRAP1-SI2) were incubated at 37 °C for 45 min with a cytosolic fraction (protein concentration, 1.25 μg/μl), obtained from DMS114 cells that had been exposed to 10 μM β-HIVS or 100 μM VP16 for 24 h. Supernatants obtained by centrifugation were analyzed by Western blotting with cytochrome c-specific antibody. E, proteins in mitochondria, isolated from DMS114 cells that had been treated with non-silencing siRNA and TRAP1-specific siRNA (TRAP1-SI2), were analyzed by Western blotting with TRAP1-specific antibody and antibody specific for subunit IV of cytochrome oxidase (OX). F, intensities of bands of cytochrome c in D were quantified with the NIH Image program. All results are typical of the results of three experiments, which gave similar results.
FIG. 8. Effects of the expression of Bcl-2 on the β-HIVS-induced suppression of expression of TRAP1. A, after transfection of DMS114 cells with vehicle, vector, and Bcl-2-expression plasmids (Bcl-2-1 and Bcl-2-2) separately, the levels of expression of Bcl-2, TRAP1, and GAPDH were analyzed by Western blotting. B, after transfection of DMS114 cells with vehicle, vector, and Bcl-2-expression plasmids (Bcl-2-1 and Bcl-2-2) separately, cells were treated with 15 μM β-HIVS for 24 h, and the extent of apoptosis was determined by staining with Hoechst 33342. All results shown are means ± S.D. of results from three independent experiments. C, after transfection of DMS114 cells with vector and a Bcl-2-expression plasmid (Bcl-2-1) separately, cells were treated with 10 μM β-HIVS for 8 h and the release of cytochrome c was analyzed using digitonin lysis buffer as described under “Experimental Procedures.” The results are typical of the results of three experiments that gave similar results.

FIG. 9. Effects of inhibitors of tyrosine kinase on the expression of TRAP1. A, after treatment of DMS114 cells with β-HIVS and with genistein, separately and at various concentrations for 24 h, tyrosine-phosphorylated proteins and the levels of expression of TRAP1, PLK1, and GAPDH were analyzed by Western blotting with antibodies against phosphotyrosine (PY20), TRAP1, PLK1, and GAPDH, respectively. The asterisks indicate bands of tyrosine-phosphorylated proteins. B, after treatment of DMS114 cells with β-HIVS and with genistein, separately and at various concentrations for 24 h, the extent of apoptosis was determined by staining with Hoechst 33342. The results shown are means ± S.D. of results from three independent experiments. C, after treatment of K562 cells with STI571 at various concentrations for 24 h, tyrosine-phosphorylated proteins and the levels of expression of TRAP1, PLK1, and GAPDH were analyzed by Western blotting with antibodies against phosphotyrosine (PY20), TRAP1, PLK1, and GAPDH, respectively. The asterisks indicate bands of tyrosine-phosphorylated proteins. The results shown in A and C are representative of the results of three independent experiments, which gave similar results.
Moreover, exposure of DMS114 cells to either \( \beta \)-HIVS or VP16 caused an increase in the amount of cytochrome \( c \) in the cytosolic fraction and suppressed the expression of TRAP1 in mitochondria, without any accumulation of TRAP1 in the cytosol. While cytochrome \( c \) is released from mitochondria into the cytosol in response to apoptotic stimuli, the expression of TRAP1 in mitochondria was suppressed without the apparent release of TRAP1 into the cytosolic fraction during apoptosis that was induced by either \( \beta \)-HIVS or VP16. These observations suggest that changes in the level of expression of TRAP1 in mitochondria might be responsible for the release of cytochrome \( c \). This hypothesis is supported by our experiments in a cell-free system with isolated mitochondria and various cytosolic fractions. Release of cytochrome \( c \) from mitochondria isolated from DMS114 cells, in which the expression of TRAP1 had been suppressed by siRNA, was enhanced by incubation with a cytosolic fraction obtained from cells that had been exposed to \( \beta \)-HIVS or VP16, as compared with the release of cytochrome \( c \) from mitochondria isolated from cells treated with non-silencing siRNA.

The anti-apoptotic protein Bcl-2 is known to regulate apoptotic signaling by blocking the release of cytochrome \( c \) from mitochondria, ensuring activation of caspase-9 and subsequent apoptosis (56, 57). The results obtained in the present study show that the release of cytochrome \( c \) and the subsequent induction of apoptosis by \( \beta \)-HIVS were prevented in cells that had been transfected with Bcl-2-expression plasmids, as compared with those events in cells transfected with the vector

![Figure 10](https://example.com/fig10.png)
alone. Furthermore, overexpression of Bcl-2 in DMS114 cells prevented the suppression of the expression of TRAP1 in response to β-HIVs, implying that Bcl-2 might be one of the regulators of expression of TRAP1. It has been reported that anti-apoptotic members of the Bcl-2 family inhibit the release of cytochrome c from mitochondria by preventing the translocation of Bax-like proteins to the mitochondria or their activation (59, 69). Bax is involved in the DNA damage-induced release of cytochrome c and subsequent induction of apoptosis as a result of its translocation from the cytoplasm to the mitochondrial membrane (53, 54). No translocation of Bax to the mitochondrial fraction was observed in DMS114 cells even after treatment of the cells with VP16, which causes DNA damage. Therefore, the mechanism responsible for the release of cytochrome c from mitochondria in DMS114 cells might be different from that in other cell lines.

It has been reported that β-HIVs inhibits the activity of several tyrosine kinases, such as v-Src and a receptor for EGF in vitro (13), and that it exerts its apoptosis-inducing activity via the inhibition of tyrosine kinases (14). Thus, it is possible that tyrosine kinases might be direct targets of β-HIVs in the induction of apoptosis. Indeed, when DMS114 cells were exposed to β-HIVs, the extent of tyrosine phosphorylation of proteins was reduced while the number of apoptotic cells increased. However, genistein and STI571, two specific inhibitors of protein tyrosine kinases, did not suppress the expression of TRAP1, even though they significantly inhibited the activity of tyrosine kinases. Moreover, VP16, an inhibitor of topoisomerase II that does not inhibit the activity of tyrosine kinases, also suppressed the expression of TRAP1. These results suggest that inhibition of the activity of tyrosine kinases might not be necessary for suppression of the expression of TRAP1. By contrast, treatment of DMS114 and K562 cells with an inhibitor of tyrosine kinase, such as genistein or STI571, suppressed the expression of PLK1, even though the level of expression of TRAP1 in mitochondria was unaffected by treatment with these inhibitors, as described above. In a previous study, we showed that the effect of β-HIVs on the activity of PLK1 occurs upstream of mitochondria via inhibition of tyrosine kinase activity (14). Therefore, our present results indicate that suppression of the expression of PLK1 upstream of mitochondria is not linked to suppression of the expression of TRAP1.

N-Acetyl-cysteine, a scavenger of ROS, efficiently prevented apoptosis induced by β-HIVs and VP16 but not apoptosis induced by CPT and cisplatin, suggesting that ROS might be involved in apoptosis that is induced by β-HIVs and VP16. It is noteworthy that treatment of DMS114 cells with CPT and with cisplatin caused only slight suppression of the expression of TRAP1 whereas treatment with β-HIVs significantly suppressed the expression of TRAP1. These observations suggest that suppression of the expression of TRAP1 might be closely correlated with production of ROS by β-HIVs and VP16. Furthermore, the β-HIVs-induced suppression of the expression of TRAP1 was blocked by NAC, indicating the involvement of ROS in the regulation of the expression of TRAP1. The molecular mechanism responsible for the production of ROS in response to β-HIVs remains to be elucidated. We are currently investigating this mechanism and the relationship between the production of ROS and the suppression of the expression of TRAP1.

In conclusion, our results indicate that β-HIVs and VP16 suppress the expression of TRAP1 during apoptosis in human leukemia HL60 cells and human lung cancer DMS114 cells. The β-HIVs-induced suppression of the expression of TRAP1 is probably mediated by ROS rather than by inhibition of tyrosine kinases. Such suppression is responsible for the induction of apoptosis since TRAP1-specific siRNA increased the apoptosis-inducing activity of β-HIVs. Reduction of the level of expression of TRAP1 by siRNA enhanced the release of cytochrome c from mitochondria during the apoptotic process, suggesting that TRAP1 might be involved in apoptosis via regulation of the release of cytochrome c from mitochondria. Further studies are required to clarify the details of the molecular mechanisms of action of TRAP1.
