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Ha-*ras*^{Val12} Oncogene Increases Susceptibility of NIH/3T3 Cells to Lovastatin

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This study demonstrates that Ha-*ras*^{Val12} oncogene overexpression sensitizes NIH/3T3 fibroblasts to lovastatin (LOV) cytotoxicity. This sensitization is through apoptosis, which was characterized by increasing CPP32 (caspase-3) activity and DNA fragmentation. Bcl-2 overexpression increased the resistance of the Ha-*ras* transformants to LOV and rescued the cells from apoptosis, further confirming that the LOV-sensitive cells died of apoptosis. Further analysis showed that Ha-*ras* activity inversely correlated with WAF1 activity. LOV treatment suppressed Ha-*ras* activity but induced WAF1 activity and disrupted the cell population in G_0/G_1 and S phases. The Ha-*ras* transformants expressing either dominant negative Ras^{Asn17} or Raf-1^{CB4} showed reverted susceptibility to LOV. These data confirm the involvement of Ras and demonstrate that Raf-1 signalling is required for LOV-induced cell death. Taken together, the possible action of LOV-induced apoptosis is through suppressing Ha-*ras* activity and increasing WAF1 activity, which alters cell cycle progression and finally activates suppressed apoptotic pathway in a Fas/Fas-L- and p53-independent fashion. © 1998 Academic Press

As an essential requirement, either cell survival or apoptosis needs Ras activity (1). Downstream of Ras, multiple signalling pathways are classified into cell survival and death pathways. Briefly, phosphatidylinositol 3-kinase (PI3 kinase)/Akt and Rac1/NF-κB pathways are anti-apoptotic pathways (2, 3), whereas c-Jun amino-terminal kinase (JNK)/c-*Jun* pathway is a pro-apoptotic pathway (4). Raf-1/MAPK pathway is required for both anti-apoptotic (5) and pro-apoptotic events (6, 7). Therefore, Ras can either positively or negatively regulate the phenotypic responses of cells upon the exogenous stimuli (5).

Lovastatin (LOV), a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, has been widely used as an anti-atherosclerotic drug due to its cholesterol-lowering property (8). LOV exhibits multiple effects on major signalling pathways within the cells by blocking the post-translational isoprenylation of several Ras family proteins involved in signal transduction and making them unable to execute their mitogenic or oncogenic activity. For example, LOV has been used to inhibit growth and trigger apoptosis of human malignant glioma and prostate cancer cells (9, 10). LOV has also been used to block Ras signalling to evaluate the effect of Ras on cell responses (11).

Accumulated data show that rapid dividing tumor cells containing oncogenes (such as Ha-*ras*) are more sensitive to anticancer agents (12), and these chemotherapeutic agents kill cells predominantly through apoptosis (13). However, the real effects of the oncogene and its downstream effectors on cell responses triggered by diverse stimuli are not clarified because the oncogenes they observed were constitutively expressed, therefore, only the end-point responses were observed. This limitation was overcome in this study by manipulating the Ha-*ras* transgene in either a positive or a negative manner using an inducible Ha-*ras*^{Val12} oncogene (7, 14), accompanied with a mutant Ha-*ras*^{Val12} specific ribozyme (15), a dominant negative Ras^{Asn17} (16), or a dominant negative Raf-1^{CB4} (17) in NIH/3T3 fibroblasts to study the relationship between Ha-*ras* activity and the action of LOV. We demonstrate that Ha-*ras* overexpression indeed sensitizes cells to LOV cytotoxicity in a Raf-1-dependent pathway. Most of the LOV-treated cells accumulated in G_0/G_1 phase undergo apoptosis due to WAF1 activation. Bcl-2 overexpression may rescue these cells from LOV-induced apoptosis.

MATERIALS AND METHODS

Cells. Ha-*ras*^{Val12} oncogene cloned from the human T24 bladder carcinoma cell line (driven by an SV40 promoter with the *E. coli lac*

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repressor binding operator) was introduced into NIH/3T3 cells. Two cell lines designated 2-12 and 7-4 have been established (Fig. 1A). These transfectants, after isopropyl β-D-thiogalactoside (IPTG; Gold biotechnology, St. Louis, MO, USA) induction, showed similar expression levels of Ha-*ras* oncogene and characteristics of transformation (7, 14). The ribZ4 cell line (a derivative of 2-12 cells) containing a ribozyme plasmid, which specifically cleaves the mutant Ha-*ras*^{Val12} at the mutant codon 12, showed minimal expression level of Ha-*ras* transgene and characteristics of non-transformed cells (15). Moreover, a dominant negative Ha-*ras*^{Asn17} gene containing cell line (Ras 1) (16) and a dominant negative *raf-1*^{CB4} gene (17) containing cell line (Raf M) were established from 7-4 cells to block Ras signalling at different levels. These two cell lines showed reduced Ha-*ras* activity and with NIH/3T3-like phenotype (7). Furthermore, a constitutively expressed *bcl-2* gene was introduced into 7-4 cells and a cell line named 7-4-2 was selected, which expresses high level of Bcl-2 (7). All the cells were maintained in α-minimal essential medium (α-MEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 10% calf serum (GIBCO BRL) at 37°C in a 5% CO_2 incubator.

Plasmids. The plasmid Py2-luc consisting of multiple polyoma virus elements (Ets+ AP-1) and a luciferase reporter gene driven by a minimal mouse c-*fos* promoter was used to evaluate Ha-*ras* activity (kindly provided by Dr. C. A. Hauser) (18). Empty Δ56Fos-dE-luc reporter served as a control for evaluating Ras signalling. The WWP-Luc plasmid containing the luciferase reporter driven by the wild type WAF1 promoter was used to evaluate WAF1 activity (kindly provided by Dr. S. L. Chen). The plasmid pNFκB-Luc containing the luciferase reporter driven by a basic promoter element (TATA box) joined to five tandem repeats of NF-κB binding elements upstream of TATA box was used to evaluate NF-κB activity (STRATAGENE, CA, USA).

DNA transfection. Cells (2×10^5) in a 60-mm plate were cotransfected with desired reporter plasmids (3 μg/plate) and pSG5lacZ β-galactosidase reporter DNA (0.5 μg/plate, as the internal control) by Lipofectin method (GIBCO BRL) for 5 h. The cells were then cultured overnight in α-MEM containing 10% calf serum in the presence or absence of IPTG (2.5 mM), then treated with LOV (50 μM, kindly provided by Dr. S. J. Lin) in the presence or absence of IPTG and incubated for 48 h. The concentration used for IPTG was 2.5 mM, for LOV was 50 μM throughout this study, unless it was described otherwise. After harvesting the cells, the cell lysates were assayed to determine the activity of gene expression. β-galactosidase activity was determined to calibrate the transfection efficiencies.

MTT assay. The LOV cytotoxicity was determined by colorimetric MTT assay as described with modifications (19). Briefly, cells (5×10^3/well) were plated in the 96-well plates and incubated in α-MEM with or without IPTG overnight. Fifty microliter of serial tetraplicate dilutions of LOV was added. The cells were incubated for 48 h at 37°C and then pulsed with 10 μl of MTT (5 mg/ml; Sigma, St. Louis, MO, USA) and incubated for an additional 4 h at 37°C. MTT was measured spectrophotometrically with a Dynatech Mr 5000 microplate reader (Dynatech laboratories, VA, USA) at 590 nm after lysis of cells with 100 μl of 10% SDS in 0.01M HCl. Control wells contained medium plus cells (total absorbance) or medium alone (background absorbance). Cell death was calculated as the percentage of MTT inhibition.

DNA fragmentation assay. After IPTG and LOV treatment for 48 h, cells (1×10^6) in the 150-mm plates were harvested and washed with PBS buffer. After addition of 100 μl lysis buffer [1% of NP-40 (Sigma) in 20 mM EDTA; 50 mM Tris-HCl, pH7.5] and mixing well, the cell lysates were centrifuged and the supernatants were collected. The supernatants were incubated with 50 μl of RNase A (20 mg/ml) and 20 μl of SDS (10%) at 56°C for 2 h. Then, 35 μl of proteinase K (20 mg/ml) was added and incubated at 37°C overnight. DNA fragments were precipitated after addition of 150 μl of 10 M NH_4OAc and 1.2 ml of 100% ethanol at −20°C overnight. After centrifuging and drying the DNA pellets, the DNA pellets were resuspended in 15 μl Tris-EDTA buffer and electrophoresed on a 1% agarose gel in TBE buffer at 30V for 8 h. DNA ladder was observed after staining with ethidium bromide solution and exposing to the UV light (7).

CPP32 activity assay. After IPTG and LOV treatment for 48 h, 1×10^6 of cells in the 150-mm plates were harvested and resuspended in 50 μl of chilled cell lysis buffer (ApoAlert™ CPP32 colorimetric assay kit, CLONTECH, CA, USA). The CPP32 activity was determined following CLONTECH's protocol.

Luciferase and β-galactosidase activity assays. The luciferase and β-galactosidase activities were determined by a Dual-light luciferase and β-galactosidase reporter gene assay system (TROPIX, MA, USA) (7).

Flow cytometry. Cells (2×10^5) were seeded into 6-well trays. After IPTG induction, the cells were treated with LOV for 24 h and harvested. The cell pellets were suspended in PBS buffer and fixed with 70% ethanol. After centrifuging and resuspending with 0.8 ml of PBS, cells were stained with 100 μl of propidium iodide (100 μg/ml) and 100 μl RNase (400 μg/ml) solution in the dark for 30 min. The cell suspension (5×10^3 cells) was assayed by the program CellFIT of fluorescence-activated cell sorter (FACScan; Becton, Dickinson Immunocytometry system, CA, USA).

RESULTS

Ha-ras^{Val12} expression in various NIH/3T3 transformants and LOV sensitivity of the cells. Figure 1A shows the pedigree of NIH/3T3 and its derivatives containing the inducible Ha-*ras*^{Val12} oncogene and the constitutively expressed transgenes. The relative Ha-*ras* activities in these cell lines with or without IPTG induction were evaluated. The Ha-*ras* transformants without induction showed diverse levels of basal Ras activity from the highest 7-4 cells (12.3×) to the lowest ribZ4 cells (1.9×) as compared to NIH/3T3 cells (Table 1). These basal Ras activities were due to the leakage of Ha-*ras* transgene expression (14). While IPTG was added, the levels of Ras activity were significantly increased in 7-4 and 2-12 cells. The 7-4-2 cells, a derivative of 7-4 cells, constitutively expressing *bcl-2* gene showed only one half of Ha-*ras* activity in 7-4 cells, indicating that Bcl-2 may suppress Ha-*ras* activity. The ribZ4 cells in which a ribozyme specifically suppressed Ha-*ras*^{Val12} activity showed the minimal increased Ras activity (15). The Ras activity in parental NIH/3T3 cells was the lowest. Ha-*ras* expression levels at the transcriptional and translational stages are consistent with that at the functional level (data not shown). To ascertain whether Ras activity affects cell sensitivity to LOV, we determined the quantitative correlation between these two events. The 50% cell inhibitory concentration (IC_{50} value) of LOV to the cells was used to represent cell sensitivity. The transfectants harboring Ha-*ras* oncogene become more sensitive to LOV as compared with their parental NIH/3T3 cells even under Ha-*ras* uninduced condition (Table 1). It is apparent that the basal Ras activity mentioned above affects the sensitivity of the cells to LOV. As we expected, the Ha-*ras* transformants became more susceptible to LOV while Ha-*ras* was overexpressed by IPTG induction (7-

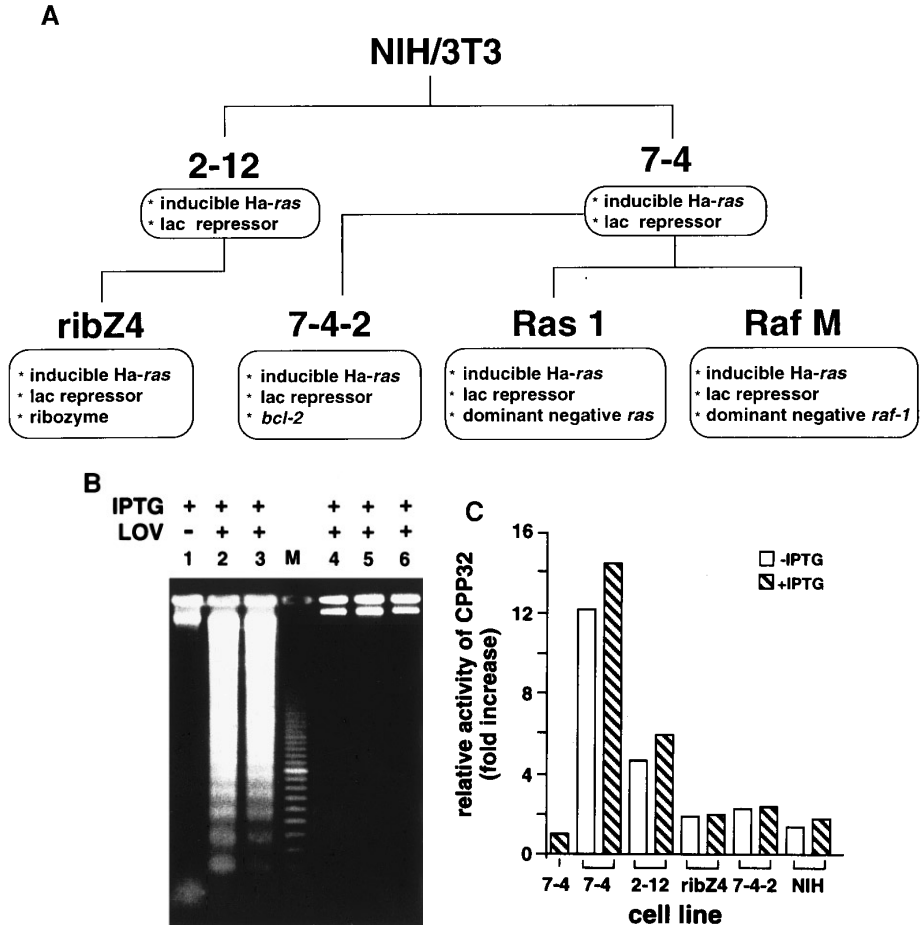

FIG. 1. Pedigree of NIH/3T3 cells and DNA fragmentation as well as CPP32 activity of the Ha-*ras* transformants. (A) The pedigree of NIH/3T3 cells. (B) DNA fragmentation of cultured cells in the presence of IPTG with or without LOV treatment for 48 h was investigated. Lane 1: 7-4 cells as a negative control treated with IPTG only. Lanes 2 to 6: cells with both IPTG and LOV (lane 2: 7-4 cells; lane 3: 2-12 cells; lane 4: ribZ4 cells; lane 5: 7-4-2 cells; lane 6: NIH/3T3 cells. M: 100-bp marker. (C) CPP32 activity of the cells after the same treatment as described above was quantitatively measured. The CPP32 activity was represented by fold increase of the control 7-4 cells with IPTG but without LOV (set as 1-fold). The results of the negative controls such as 2-12, ribZ4, and 7-4-2 cells for DNA fragmentation and CPP32 activity assays were similar to those of 7-4 cells (data not shown).

4 and 2-12 cells). The ribZ4 cells with low level of basal Ras activity showed slight increase of LOV sensitivity. While LOV was added, it decreased Ha-*ras* activity in the cells by blocking isoprenylation of Ras. However, the relative levels of Ha-*ras* activities among the cells were unchanged (Table 1). In general, we found the higher Ras activity in the cells the more susceptible of the cells to LOV challenge. These results demonstrate that the level of Ras activity closely correlates with LOV sensitivity of the cells.

Ha-ras overexpression proceeded with LOV challenge triggering cell apoptosis. As shown in Fig. 1B, DNA fragmentation was detected in 7-4 and 2-12 cells with higher Ha-*ras* activity (lane 2 and 3) after LOV treatment. In contrast, the cells with lower Ha-*ras* activity (ribZ4, 7-4-2 and NIH/3T3 cells) showed no DNA fragmentation (Fig. 1B, lane 4, 5, 6). These data clearly show that LOV accompanied with overexpressed Ha-*ras* could trigger the fibroblasts undergoing apoptosis. Ha-*ras* overexpression without LOV did not induce apoptosis in all of the Ha-*ras* transformants, indicating that Ha-*ras* activation required LOV challenge to induce cell apoptosis (only the data of 7-4 cells was shown; Fig. 1B, lane 1).

CPP32 (caspase-3) is an important mediator of apoptosis. The evaluation of CPP32 activity in LOV-treated or -untreated cells was conducted in the presence or absence of IPTG induction, and the results were consistent with the data of DNA fragmentation analysis. That is, the group (7-4 and 2-12 cells) with higher Ras activity showed higher CPP32 activity (at least 3.3 fold of untreated 7-4 cells) after LOV treatment (Table 1 and Fig. 1C). The group (ribZ4, 7-4-2 and NIH/3T3 cells) with lower Ras activity showed lower CPP32 activity (Table 1 and Fig. 1C). Both the level of Ras activity and cell sensitivity to LOV correlate with the severity of LOV induced apoptosis. This correlation was further demonstrated by 7-4-2 cells in which overex-

TABLE 1

The Ha-Ras Activity and Susceptibility of Ha-*ras* Transformed Cells to LOV

Cell line	Relative Ras activity[a] (fold increase)			Cell susceptibility[b] (IC_{50} of LOV; μM)	
	−IPTG	+IPTG	+IPTG/LOV	−IPTG	+IPTG
NIH/3T3	1.0[c]	1.2	0.7	171.4	173.9
ribZ4	1.9	2.0	1.1	146.2	122.2
2-12	6.6	13.3	7.2	59.2	34.0
7-4	12.3	22.4	10.3	26.5	12.6
7-4-2	4.7	5.5	3.6	136.1	129.8

Note. For the experimental procedures see "Materials and Methods."
[a] These data are the mean of three independent experimental results.
[b] These data are the mean of tetraplicates.
[c] The Ha-*ras* activity of NIH/3T3 cells without IPTG and LOV treatment was set as 1.

pressed Bcl-2 might lower CPP32 activity (Fig. 1C), prevent DNA fragmentation (Fig. 1B, lane 5) and finally caused the reversion of the drug sensitivity to LOV through suppressing Ha-*ras* activity (Table 1).

The involvement of WAF1, cell cycle, and NF-κB in LOV-induced apoptosis. To determine what downstream factors mediate the susceptibility of the Ha-*ras* transformed cells to LOV, we evaluated WAF1 activity and its relationship with LOV sensitivity. As shown in Table 1 and Fig. 2A, the Ha-*ras* transformants with higher basal Ha-*ras* activity showed lower WAF1 activity as compared to lower Ha-*ras* activity of their parental NIH/3T3 cells. While Ha-*ras* activity was increased by IPTG, the levels of WAF1 activity was inversely decreased as shown in 2-12, 7-4 and 7-4-2 cells. This inverse correlation between Ha-*ras* and WAF1 was further confirmed by ribZ4 cells in which Ha-*ras* was suppressed by the ribozyme and WAF1 activity was accordingly rebounded. LOV treatment significantly increased WAF1 activities of 7-4 and 2-12 cells (about 3.3 and 4.0 fold increase, respectively, as compared with that of only IPTG induced condition), whereas the WAF1 activity of ribZ4 cells like NIH/3T3 cells was not affected by LOV. Our data demonstrate that WAF1 activity is inversely regulated by Ha-*ras*, therefore, may involve in the sensitivity of the cells to LOV. Moreover, Bcl-2 overexpression has no evident effect on WAF1 activity as shown in 7-4-2 cells.

Will altered WAF1 activity disrupt the normal cell cycle and lead the cells to death? Flow cytometry was conducted to analyze the cell population during the cell cycle progression with or without LOV. As shown in Fig. 2B, the 2-12 and 7-4 cells (with high Ha-*ras* activity and low WAF1 activity) have larger S phase population, representing faster cell cycle progression. While LOV was added to the cells, the major cells population with higher Ha-*ras* expression shifted to G_0/G_1 phase (Fig. 2B, 7-4 and 2-12 cells). The cell cycle of NIH/3T3 and ribZ4 cells (with low Ha-*ras* activity and high WAF1 activity) was less affected by LOV (Fig. 2B). Our data indicate that LOV-sensitive cells have a higher tendency to accumulate in G_0/G_1 phase.

Figure 2C shows that regardless of the differential levels of Ha-*ras* expression, the NF-κB activities in all the Ha-*ras* transfectants are about two fold higher than those of the parental NIH/3T3 cells. Ha-*ras* activation by IPTG further increased NF-κB activity, in contrast, LOV treatment decreased NF-κB activity. However, a quantitative correlation between NF-κB and Ras activities in those Ha-*ras* transformants could not be established, indicating that other factors accompanied with NF-κB activity may be responsible for LOV-induced apoptosis.

The involvement of Ras and Raf-1 in the sensitivity of the cells to LOV was confirmed by the blockage of dominant negative Ras^{Asn17} and $Raf\text{-}1^{CB4}$. To ascertain the involvement of Ha-*ras* oncogene as well as its downstrean Raf-1 pathway in LOV-induced apoptosis, the Ha-*ras* transformants overexpressing either dominant negative Ras^{Asn17} (Ras 1) or $Raf\text{-}1^{CB4}$ (Raf M) was investigated. As compared with parental 7-4 cells, Ras 1 and Raf M mutants showed suppressed Ras activity (at least 3 fold decrease) with or without IPTG induction, indicating that in these mutants Ras signalling was indeed blocked at either Ras or Raf-1 level (Table 2). Our data further demonstrated the resistance of Ras 1 and Raf M to LOV was increased at least 3.9 fold as compared with that of their parental 7-4 cells (Table 2). These data provide further evidence that Ras/Raf-1 signalling pathway involves in LOV-induced apoptosis.

DISCUSSION

To study the effect of Ha-*ras* in the cells, we manipulated Ha-*ras* activities using the following approaches: 1) to activate the inducible Ha-ras^{Val12} transgene by IPTG; 2) to suppress Ha-ras^{Val12} activity by either *ras* specific ribozyme or the dominant negative Ras^{Asn17}; 3) to sup-

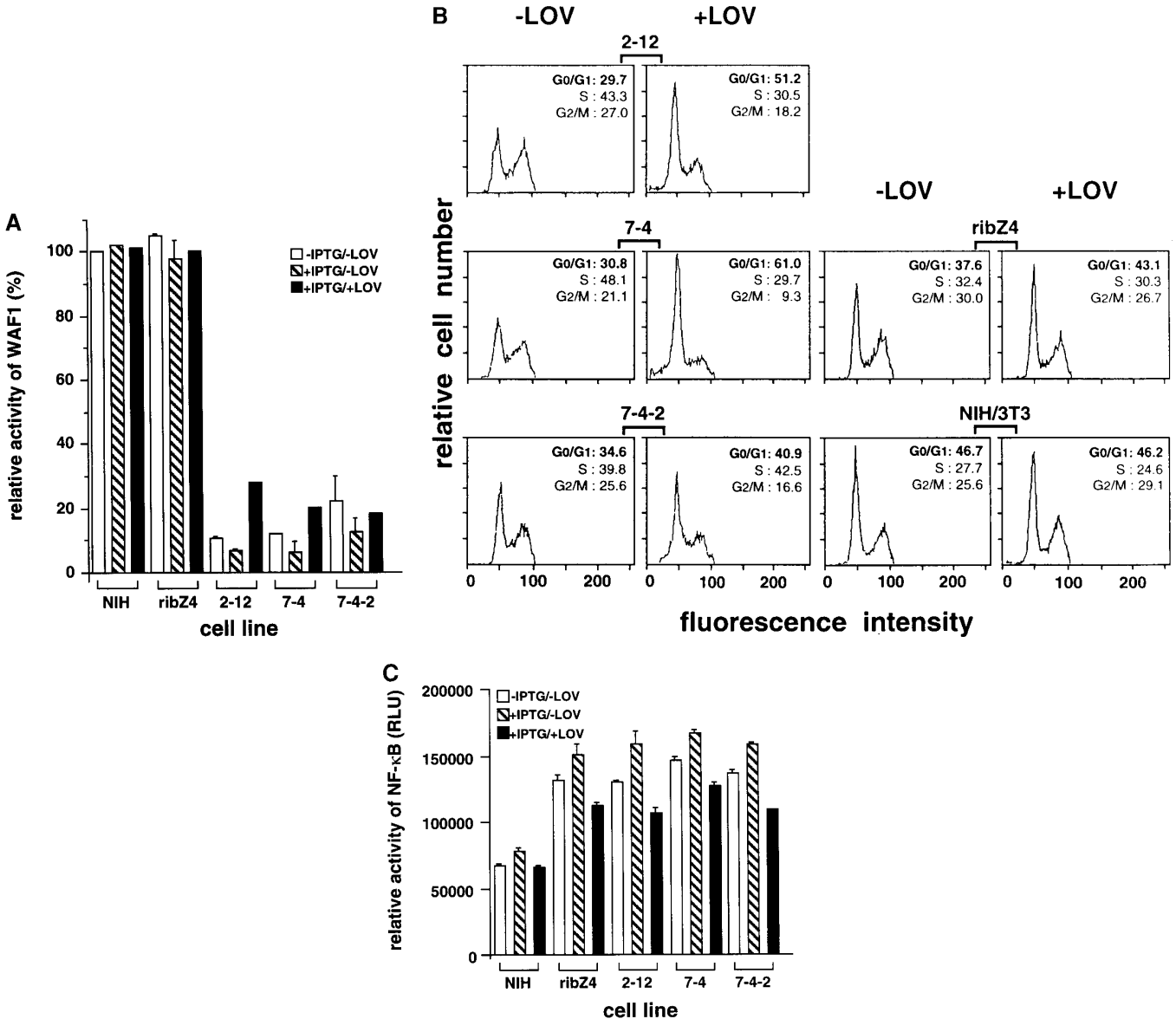

FIG. 2. WAF1, cell cycle, and NF-κB activity of the cells by luciferase activity and flow cytometric analysis. (A) WAF1 activity was analyzed by cotransfecting with WAF1 reporter DNA WWP-Luc (3 μg/dish) and β-galactosidase DNA pSG5lacZ (0.5 μg/dish; as the internal control) by Lipofectin method. The cells were then cultured in α-MEM containing 10% calf serum overnight. After addition of LOV, the cells were incubated for 24 h and harvested. WAF1 activity represented by light signal from the luciferase enzyme in the extract was measured by a luminometer (Lumat, LB 9501, Germany). The WAF1 activity of NIH/3T3 cells without IPTG and LOV treatment was set as 100%. (B) Cell cycle of the Ha-*ras* transformants representing the percentage of cell distribution in each phase is shown in the panels. These data are representative of three independent experiments with similar results. (C) NF-κB activity was analyzed by cotransfecting the cells in 60-mm plates with NF-κB reporter DNA pNFκB-Luc (3 μg/dish) and internal control β-galactosidase reporter DNA pSG5lacZ (0.5 μg/dish). After addition of LOV, the cells were further incubated for 24 h and harvested. NF-κB activity represented by light signal from the luciferase enzyme was measured. RLU: relative luciferase unit.

press Raf-1 activity by the dominant negative Raf-1^{CB4}. All the results clearly demonstrate that the activation levels of Ha-*ras*^{Val12} determined the sensitivity of the cells to LOV and induced cells to death. Chen *et al.* addressed the same conclusion that oncogene activation (such as *src*, *ras* and *raf*) can enhance apoptosis induced by serum-withdrawal or anticancer drugs (12, 20).

It is known that tumor cells with rapid dividing cycle are normally more sensitive to anticancer drug treatments (12). In this study, the LOV-sensitive Ha-*ras* transformants also showed higher proliferation potential by [^{3}H]-thymidine incorporation rate analysis. In contrast, LOV-resistant NIH/3T3 cells showed the lowest [^{3}H]-thymidine incorporation rate (data not shown).

TABLE 2

The Ha-Ras Activity and Susceptibility of Dominant Negative Mutants to LOV

Cell line	Relative Ras activity[a] (fold increase)			Cell susceptibility[b] (IC_{50} of LOV; μM)	
	−IPTG	+IPTG	+IPTG/LOV	−IPTG	+IPTG
7-4	12.3	22.4	10.3	25.9	12.1
Ras 1	2.6	6.1	1.8	113.4	85.7
Raf M	4.1	6.5	2.3	102.1	78.1

Note. For the experimental procedures see "Materials and Methods."

[a] These data are the mean of three independent experimental results. The Ha-*ras* activity of NIH/3T3 cells without IPTG and LOV treatment was set as 1.

[b] These data are the mean of tetraplicates.

The mechanism that controls the switch between cell proliferation and death initiated by Ha-*ras* has rarely been explored.

To unveil why the cells expressing active Ha-*ras* are growing faster and susceptible to death, we concentrated on investigating cell cycle regulating factors, because they direct the cell towards proliferation, growth arrest and apoptosis. Among them are cyclins, cyclin-dependent kinases (CDKs) and associated cyclin inhibitors. WAF1, a major component of the cyclin inhibitors, not only causes growth arrest in G_0/G_1 phase through inhibition of CDKs (21), but also inhibits DNA replication by blocking the proliferating cell nuclear antigen (PCNA) to activate DNA polymerase δ (22). Many reports have demonstrated that WAF1 can be upregulated via a p53-dependent or p53-independent way and is involved in the apoptosis program of the cells (23). We previously showed that WAF1 activation did not require p53 (Chang *et al.*, submitted). The importance of the inverse relationship between Ras signalling and WAF1 activity was demonstrated by that the LOV-sensitive cells (7-4 and 2-12 cells) with lower WAF1 activity showed higher population in S phase, and subsequent LOV treatment increased WAF1 activity and shifted the major population from S to G_0/G_1 phase. Moreover, the cells with higher population in G_0/G_1 phase are more susceptible to LOV (such as 7-4 cells, Fig. 2B). These findings are consistent with Sekiguchis' observations that overexpression of WAF1 resulted in cell cycle arrest in G_1 phase, and cell death (24). Ha-*ras* overexpression may alter the intrinsic sensitivity of the transformants to LOV through aberrant expression of WAF1. The inverse correlation between WAF1 and Ha-*ras* and the relationship between WAF1 involved cell cycle control and apoptosis were verified by Ras 1 and Raf M dominant negative mutant cell lines (data not shown).

Fas/Fas-L on the cell surface can be enhanced by many death signals and induces downstream CPP32 (caspase-3) activation (25, 26). However, in LOV-treated cells, CPP32 was activated without disturbing Fas/Fas-L expression levels (data not shown), indicating that Ha-*ras*-mediated and LOV-induced apoptosis is through a Fas/Fas-L-independent signalling pathway. Moreover, Marcelli observed the activation of caspase-7 during LOV-induced apoptosis of the prostate cancer cell line LNCaP (27). Differently, we provide the first evidence for the involvement of CPP32 in LOV-induced apoptosis of transformed fibroblasts. Bcl-2 functions upstream of the caspase signalling and prevents cells from apoptosis through inhibition of the caspase activity (28, 29). Similar results were also demonstrated by LOV-treated 7-4-2 cells in which Bcl-2 suppressed CPP32 activity and prevented cell apoptosis (Fig. 1B and C). Moreover, both Ras and Bcl-2 can associated with Raf-1, therefore, it is possible that a competition between Ras and Bcl-2 for limiting amounts of Raf-1(30). Since Ras/Raf-1 pathway is required for cell apoptosis and Bcl-2/Raf-1 association can prevent it (31). It is likely that in 7-4-2 cells, Bcl-2 overexpression decreased Ras activity and blocked apoptosis by binding with Raf-1 (Table 1). Based on our results, we hypothesize that Bcl-2 may have the following two functions: 1) inhibition of the CPP32 activity; and 2) suppression of the Ras/Raf-1-dependent apoptosis by binding with Raf-1. The investigation whether Bcl-2 binds to Raf-1 and decreases Ras/Raf-1 activity is underway. We and other investigators have shown that Raf-1/MAPK pathway is not only required for promoting cell growth but also required for serum deprivation induced apoptosis (6, 7). In this study, we demonstrate that Raf-1/MAPK pathway is also required for LOV triggered apoptosis. Determination of the fate of the cells evidently depends on other exogenous and/or endogenous factors (12).

Although NF-κB functions as a transcription factor that prevents cells from death, however, some reports claim that it may play the opposite role (32, 33). We previously demonstrated that Ha-*ras* could activate NF-κB, and subsequent TNF-α treatment further enhanced its activity (Chang *et al.*, submitted). In contrast, LOV treatment of Ha-*ras* activated cells sup-

pressed NF-κB activity in this study. Despite the opposite responses of NF-κB to different stimuli in both studies, all the cells underwent apoptosis, implying that the status of NF-κB does not seem to be able to determine the cell fate in our system (34).

In conclusion, oncogenic Ha-*ras* may sensitize the cells to LOV by a Raf-1-dependent manner and the possible mechanism is through suppressing Ha-*ras* activity and increasing WAF1 activity, which subsequently arrests cells in G_0/G_1 phase, activates caspase pathway, and finally triggers apoptosis. It is noteworthy that the system we developed can be used for screening Ha-*ras* oncogene related anticancer agents which have maximal potential for inducing tumor cell apoptosis and minimal side effect to normal cells. Recently, a farnesyltransferase inhibitor L-744,832 has been demonstrated to be able to induce tumor regression in transgenic mice harboring multiple oncogenic mutations by mediating alterations in both cell cycle control and apoptosis (35). Similarly, the application of this study on Ha-*ras* initiated tumorigenesis in animal model seems promising and remains to be determined.

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