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Increased Stability of Nucleophosmin/B23 in Anti-Apoptotic Effect of Ras during Serum Deprivation

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ABSTRACT

We obtained evidence that increased stability of nucleophosmin/B23 is involved in antiapoptotic effect of ras during serum deprivation. Nucleophosmin/B23 in serum-deprived (0% serum) NIH-3T3 cells was found to be highly unstable with a half-life less than 4 h. In contrast, nucleophosmin/B23 in serum-deprived ras-transformed (RAS-3T3) cells was as stable as that in serum-supplemented NIH-3T3 or RAS-3T3 cells. Treatment of RAS-3T3 cells with nucleophosmin/B23 antisense oligomer significantly potentiated the apoptosis induced by serum deprivation. Much less caspase-3 activity was noted in the lysate derived from serum-deprived RAS-3T3 cells compared with that in the lysate of serum-deprived NIH-3T3 cells. Cell permeable caspase-3 inhibitor added in the medium blocked the decrease of nucleophosmin/B23 and apoptosis induced by serum deprivation in NIH-3T3 cells. The inhibitor, on the other hand, promoted significant decrease of nucleolin/C23 in NIH-3T3 cells during serum deprivation. Unlike nucleolin/C23, down-regulation of nucleophosmin/B23 was thus not proliferation-dependent but caspase-3- and apoptosis-dependent. Our results indicate important relationships among ras, nucleophosmin/B23, activation of caspase-3, and induction of apoptosis.

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Uncontrolled cell proliferation is the hallmark of cancer, and tumor cells typically acquire damage to genes that directly regulate cell growth. Molecular oncology has focused on the control of proliferation, in which many oncogenes seem to act. However, there is now compelling evidence that the other side of the equation, the rate of cell death, must be considered as well. Cell suicide has now been recognized as a critical control point. Death by apoptosis is implied to be an active process providing an additional means of precisely regulating cell numbers and biological activities (Kerr et al., 1972; Wyllie, 1980; Wyllie et al., 1980). Equally important is that, unlike simple degeneration, cell death is dependent on active participation of cellular components that can potentially be suppressed. Aberrant cell survival resulting from such inhibition of cell death would be expected to contribute to oncogenesis.

The identification of genes and their products that are involved in response to growth stimuli is essential for understanding normal cell growth and death. During the past decade, numerous regulatory factors that control the balance between a cycling and quiescent state have been identified. These include proto-oncogenes and negative and positive regulatory growth factors. Other proliferation-associated molecules are being studied to determine their potential role in cell growth regulation (Fonagy et al., 1992; Chou and Yung, 1995). Protein nucleophosmin/B23, NO38, or numatrin (Yung et al., 1985; Schmidt-Zachmann et al., 1987) is a major nucleolar phosphoprotein that displays a number of activities. These include a potential role as a positive regulator of cell proliferation. Nucleophosmin/B23 is significantly more abundant in tumor and proliferating cells than in normal resting cells (Feuerstein et al., 1988; Chan et al., 1989). Nucleophosmin/B23 mRNA is 50- and 5-fold higher in Novikoff hepatoma and hypertrophic rat liver, respectively, than in normal rat liver (Chan et al., 1989). We recently demonstrate that nucleophosmin/B23 is transcriptionally down-regulated during retinoic acid (RA)-induced cellular differentiation (Hsu and Yung, 1998) and sodium butyrate-induced apoptosis (Liu and Yung, 1998) of HL-60 leukemia cells. The potentiation of RA-induced differentiation and sodium butyrate-induced apoptosis by nucleophosmin/B23 antisense oligomer implies that nucleophosmin/B23 plays a role in the regulation of nucleolar function for cellular differentiation and apoptosis. Whether the decrease of nucleophosmin/B23 as a result of antisense treatment would render tumor cells more susceptible to chemotherapeutics or induction of

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ABBREVIATIONS: RA, retinoic acid; MAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; PVDF, polyvinylidene difluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MAPK, mitogen-activated protein kinase.

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cellular differentiation or apoptosis becomes a major question to be addressed.

A small set of cellular genes, termed proto-oncogenes, seems to be frequent targets for genetic alterations leading to cancer. To date, the proto-oncogenes that are most often implicated in this process are members of the ras gene family (Lowy and Willumsen, 1993). Ras is a member of a family of GTPases that are essential components of receptor-mediated signaling cascade that regulates cell growth and differentiation. The susceptibility to induced apoptosis of cancer cells may well be an important determinant in the therapeutic response (Dive and Hickman, 1991). It is clear that oncogene and oncosuppressor gene activity can influence the probability of cell death. The first link between ras and apoptosis comes from the work of Wyllie et al. (1987), who show that animal tumors with constitutive expression of c-H-ras oncogene have a remarkably low incidence of spontaneous apoptotic cell death. The oncogenic activation of the ras gene is frequently observed in human cancers (Bos, 1989). Ras oncogene (Adrens et al., 1993; Chou and Yung, 1997) is shown to inhibit apoptosis in a variety of experimental model systems.

In this study, we hypothesized that regulation of nucleophosmin/B23 could be one of the steps in deciding whether the cells are mortal or immortal with respect to their susceptibility to induction of apoptosis. Attempts were therefore made to determine whether and how nucleophosmin/B23 was involved in modulating the response of NIH-3T3 and v-H-ras-transformed cells toward apoptosis in serum deprivation. Our results indicated that nucleophosmin/B23 plays a significant role in mediating antiapoptotic effect of ras.

Experimental Procedures

Chemicals and Antibodies. DL-Dithiothreitol (DTT) and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO). Wright dyes and Trypan Blue were from Sigma, and Giemsa stain was purchased from Aldrich (Milwaukee, WI). Peptide caspase-3 inhibitor (Ac-DEVD-CHO), cell permeable caspase-1 inhibitor (YVAD-CHO-cell-permeable) and caspase-3 inhibitor (DEVD-CHO-cell-permeable) were from Calbiochem (Torrence, CA). Monoclonal antibody (MAb) to nucleophosmin/B23 was kindly provided by Dr. P. K. Chan (Department of Pharmacology, Baylor College of Medicine, Houston, TX). Monoclonal antibody to ras was from Transduction Laboratories (Lexington, KY). MAb to nucleolin/C23 was kindly provided by Dr. N. H. Yeh (Institute of Microbiology and Immunology, National Yang Ming University, Taipei, Taiwan). MAb to β-actin was from Sigma.

Cells. v-H-ras-transformed and their parental NIH 3T3 cells (Liu et al., 1995) were gifts from Dr. S. F. Yang (Institute of Molecular Biology, Academia Sinica, Taiwan). Cells were grown in DMEM supplemented with 10% calf serum, 2 mM glutamine, 50 U/ml penicillin, 100 μg/ml G418, and 50 μg/ml streptomycin in a 5% CO_2 humidified incubator at 37°C.

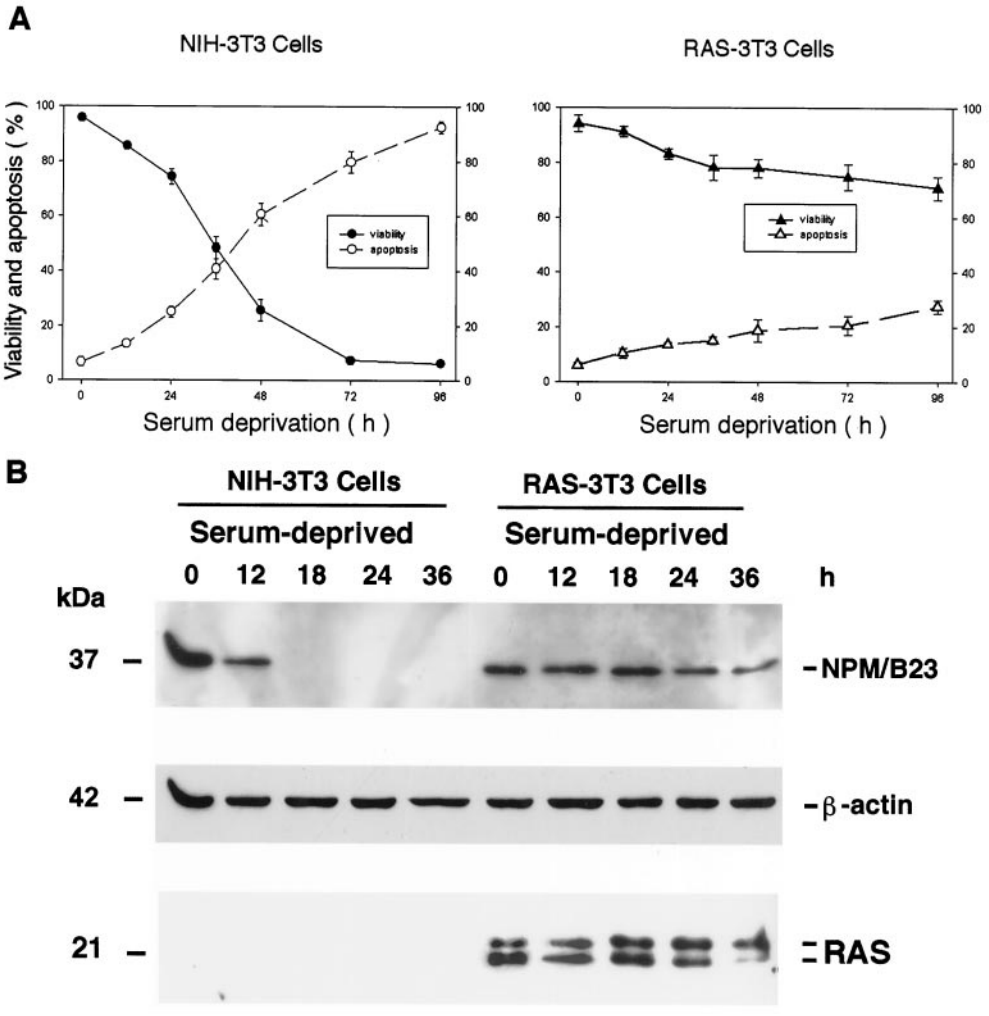

Fig. 1. Nucleophosmin/B23 protein analysis in NIH-3T3 and ras-transformed cells during serum deprivation. A, determination of the levels of viable cells or apoptotic cells (see under *Experimental Procedures*) after NIH-3T3 or ras-transformed (RAS-3T3) cells were under serum deprivation for various times (12–96 h). Points, means (± S.D.) of triplicates. The figure is a representative of the results obtained in at least three independent experiments. B, NIH-3T3 or RAS-3T3 cells were under serum deprivation for various times (12–36 h). Cells were harvested, washed, and the lysates were centrifuged at 12,000*g* for 30 min at 4°C. Total cellular proteins (10 μg) were separated by 10% SDS-PAGE and blotted onto PVDF papers. The blotted papers were incubated with monoclonal anti-nucleophosmin/B23 (NPM/B23), monoclonal anti-ras or monoclonal anti-β-actin antibody for 2 h at room temperature. Immunoreactivity was determined by examination of the enhanced chemiluminescence reaction.

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Induction of Cell Death. v-H-ras-transformed and their parental NIH-3T3 cells (approximately 1.0×10^5) in 35-mm plastic tissue culture dishes were grown in serum-free DMEM and collected at indicated times. To inhibit apoptosis, NIH-3T3 cells were coincubated with 25 μM permeable caspase inhibitor, YVAD-CHO-cell-permeable or DEVD-CHO-cell-permeable in serum-free DMEM.

Assessment of Apoptotic Cells. Morphological assessment of apoptotic cells was performed using the Wright-Giemsa staining method. Cells ($0.5–1.0 \times 10^5$) were prepared on slides in 35-mm, 6-well microculture plates and stained with Wright-Giemsa stain. Morphology of cells was examined under light microscope (400×). Apoptotic cells were identified according to the following criteria: condensed nuclei, blebbing of plasma membrane, and decrease in cell size. Triplicate 200-cell counts were performed.

Cell Cycle Analysis. To estimate the proportion of cells in different phases of cell cycle, cellular DNA contents were measured by flow cytometry. Briefly, cells (5×10^5 cells) were fixed by 70% ethanol (in PBS) in ice for 30 min and then resuspended in PBS containing 40 μg/ml propidium iodide and 0.1 mg/ml RNase (Boehringer-Mannheim, Mannheim, Germany). After a 30-min reaction at 37°C, 2×10^4 cells were analyzed in a FACStar cytofluorometer (Becton-Dickinson, San Jose, CA) equipped with an argon-ion laser at 488 nm.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out according to the protocol of Laemmli (1970).

Immunoblot Analysis. Cells were harvested, washed twice in ice-cold PBS, and lysed in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Na_2HPO_4, 100 mM NaCl, 20 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 30 mg/ml DNase, and 30 mg/ml RNase). The lysate was boiled in SDS sample buffer (62.5 mM Tris, pH 6.8, 5% β-mercaptoethanol, 10% glycerol, 2.0% SDS, 0.001% bromphenol blue) and fractionated by 10% SDS polyacrylamide gel electrophoresis. Coomassie blue-stained SDS-PAGE was examined by densitometry scanning to ascertain that there were virtually equal amounts of cellular proteins in each lane. The separated proteins in SDS-PAGE were electrotransferred to Hybond-PVDF membrane (Amersham Pharmacia Biotech, Aylesbury, UK), which was next soaked in a blocking solution [5% (w/v) nonfat milk in TBST buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% (v/v) Tween-20)] for 1 h at room temperature. The soaked PVDF membrane was then incubated with MAb against nucleophosmin/B23 [diluted 1:2000 in 3% (w/v) nonfat milk in TBST] for 2 h at room temperature, washed with TBST buffer three times for 15 min each, incubated at room temperature for 1 h in horse-radish peroxidase-conjugated goat anti-mouse IgG antibody (diluted 1:2000 in TBST buffer), and finally washed three times with TBST for 15 min each. Immunoreactivity was determined by examination of the enhanced chemiluminescence reaction (ECL, Amersham).

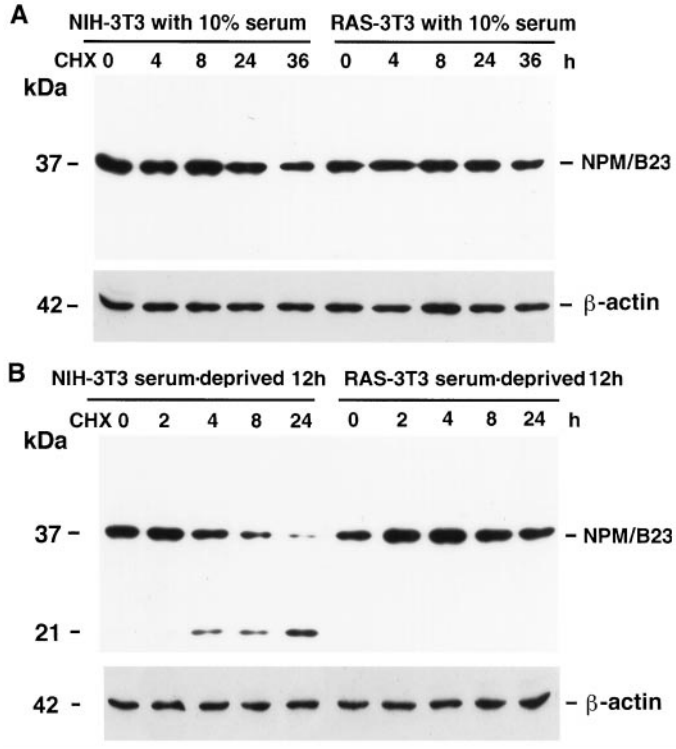

Fig. 2. Nucleophosmin/B23 protein is more stable in ras-transformed cells than nucleophosmin/B23 in NIH-3T3 cells during serum deprivation. NIH-3T3 or ras-transformed (RAS-3T3) cells were supplemented with 10% serum (Fig. 2A) or serum-deprived (Fig. 2B) for 12 h. Cycloheximide (CHX; 25 μg/ml) was added to the serum-supplemented or serum-deprived medium and the cells were further incubated for various times (2–36 h). Cells were harvested, washed, and the lysates were centrifuged at 12,000*g* for 30 min at 4°C. Total cellular proteins (10 μg) were separated by 10% SDS-PAGE and blotted onto PVDF papers. The blotted papers were incubated with monoclonal anti-nucleophosmin/B23 (NPM/B23) or monoclonal anti-β-actin antibody for 2 h at room temperature. Immunoreactivity was determined by examination of the enhanced chemiluminescence reaction.

Oligonucleotides. The phosphorothioate analogs of deoxyoligonucleotides corresponding to nucleotides −2~18 of the nucleophosmin/B23 cDNA were synthesized in the reverse (5′−GCT ACC TTC TAA GCT ACC TG−3′) and the antisense (5′ −GTC CAT CGA ATC TTC CAT CG−3′) orientations (ASIA Company Ltd., Wilsonville, OR). These synthetic deoxyoligonucleotides composed the 5′-region of the nucleophosmin/B23 cDNA including the translation initiation codon.

Transfection with Antisense Nucleophosmin/B23 Oligonucleotides. RAS-3T3 cells were seeded at a density of 3×10^5 per well in 1.0 ml of serum-deprived DMEM. Nucleophosmin/B23 antisense or reverse oligonucleotide (20 μM) and 6 μg of lipofectamine reagents in serum-deprived DMEM were mixed gently and incubated for 45 min at room temperature. The mixture was then added to RAS-3T3 cells and incubated in serum-deprived medium for 24 to 96 h at 37°C in a CO_2 incubator.

Measurement of Caspase Activity. The activities of caspases were measured according to the methods described (Wang et al., 1998) with a slight modification. Briefly, cells harvested at desired times were washed with PBS and cell lysates were prepared by repeated freezing and thawing of cells in extraction buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA. Caspase-1 or caspase-3 was allowed to react in cell lysates containing 40 μg of protein with 200 μM caspase colorimetric substrate Ac-YVAD-pNA or Ac-DEVD-pNA, respectively, in the absence or presence of specific inhibitor (Ac-YVAD-CHO for caspase-1 or Ac-DEVD-CHO for caspase-3) at 37°C for 6 h in caspase assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, and 10% glycerol). The amount of pNA released was estimated by measuring the absorbance at 405 nm, and the relative activity was calculated.

In Vitro Translation of Nucleophosmin/B23 and the Cleavage Assay. Full-length nucleophosmin/B23 was in vitro-translated from the plasmid pCR3-B23 by using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI). The cleavage assay of in vitro-translated nucleophosmin/B23 was performed in the buffer used to measure caspase-3 activity (see above). In vitro-translated nucleophosmin/B23 was incubated for 4 h at 37°C with 5 or 10 units of active recombinant caspase-3 or 10 μg of lysate prepared from serum-deprived apoptotic HeLa cells in the presence or absence of 25 μM caspase-3 inhibitor (Ac-DEVD-CHO). Proteins were then separated by 10% SDS-PAGE. Full-length nucleophosmin/B23 and its degradation fragment were detected by autoradiography with Fuji MacBAS1500 PhosphorImager.

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Results

More Drastic Decrease of Nucleophosmin/B23 in NIH-3T3 Than in Ras-Transformed 3T3 Cells during the Apoptosis Induced by Serum Deprivation. The morphology and the survival of v-H-ras-transformed (RAS-3T3) and their parental NIH 3T3 cells under serum deprivation (0% serum) were examined (Fig. 1A). After 48 to 96 h of serum deprivation, about 60 to 92% of NIH 3T3 cells exhibited highly condensed nuclei and decrease in cell size (Fig. 1A). These features are the characteristics of apoptotic cells (Wyllie et al., 1980). Also concomitant with the increase in the percentage of NIH 3T3 cells exhibiting morphological features of apoptosis, there was a decrease in cell viability as determined by Trypan Blue exclusion ability (Fig. 1A). In contrast, RAS-3T3 cells were resistant to serum deprivation (Fig. 1A). Lower percentage (~28%) of RAS-3T3 cells exhibited morphological characteristics of apoptosis 96 h after serum deprivation (Fig. 1A). The steady-state nucleophosmin/B23 protein level in these cells during serum deprivation (12–36 h) was also determined (Fig. 1B). Nucleophosmin/B23 decreased in both NIH-3T3 and RAS-3T3 cells during serum deprivation (Fig. 1B). However, it decreased much more drastically in NIH-3T3 cells than in RAS-3T3 cells (Fig. 1B). In parallel, serum deprivation resulted in little change in the protein amount of ras in RAS-3T3 cells. Ras was barely detected in NIH-3T3 cells (Fig. 1B).

To compare the stability of nucleophosmin/B23 protein in NIH-3T3 and RAS-3T3 cells during serum deprivation, the half-life of nucleophosmin/B23 was measured by incubating serum-deprived (for 12 h) cells with cycloheximide (5 μg/ml) for up to 24 h. Nucleophosmin/B23 in NIH-3T3 and RAS-3T3 cells supplemented with serum was stable in both and its half-life was longer than 24 h (Fig. 2A). Nucleophosmin/B23 in serum-deprived NIH-3T3 cells, however, was found to be highly unstable, with a half-life of less than 4 h. Degradation product of nucleophosmin/B23 was detected at about 21 kDa in serum-deprived NIH-3T3 cells treated with cycloheximide for 4 to 24 h. In contrast, nucleophosmin/B23 in serum-deprived RAS-3T3 cells was as stable as that in serum-supplemented NIH-3T3 or RAS-3T3 cells (Fig. 2B).

Potentiation of Apoptosis in Ras-Transformed Cells by Nucleophosmin/B23 Antisense Oligomer. Antisense oligonucleotide was next used to examine the role of nucleophosmin/B23 in apoptosis. Western blot analysis (Fig. 3A) showed that there was a significant decrease in the level of nucleophosmin/B23 in RAS-3T3 cells treated with nucleophosmin/B23 anti-

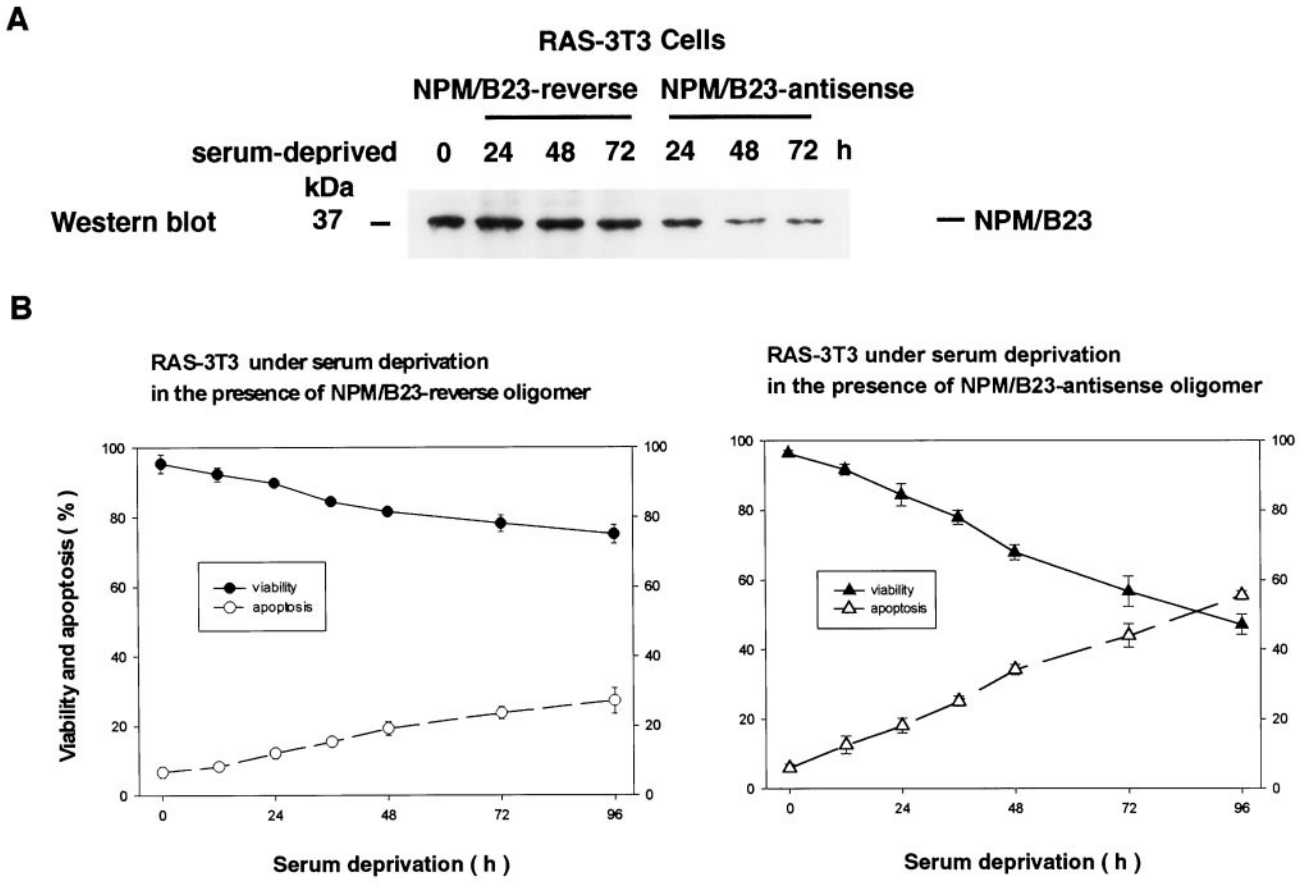

Fig. 3. Potentiation of apoptosis in ras-transformed cells by nucleophosmin/B23 antisense oligomer. A, Ras-transformed (RAS-3T3) cells were under serum deprivation in the presence of nucleophosmin/B23 reverse (NPM/B23-reverse) or antisense (NPM/B23-antisense) oligomer for various times (24–72 h). Cells were harvested, washed, and the lysates were centrifuged at 12,000*g* for 30 min at 4°C. Total cellular proteins (10 μg) were separated by 10% SDS-PAGE and blotted onto PVDF papers. The blotted papers were incubated with monoclonal anti-nucleophosmin/B23 (NPM/B23) antibody for 2 h at room temperature. Immunoreactivity was determined using the enhanced chemiluminescence reaction. B, determination of the levels of viable or apoptotic cells (see *Experimental Procedures*) after RAS-3T3 cells were under serum deprivation in the presence of nucleophosmin/B23 reverse (NPM/B23-reverse) or antisense (NPM/B23-antisense) oligomer for various times (12–96 h). Points, means (±S.D.) of triplicates. The figure is representative of the results obtained in at least three independent experiments.

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sense oligomer during serum deprivation for 24 to 72 h (Fig. 3A). In control cells, the nucleophosmin/B23 reverse oligomer-transfected RAS-3T3 cells, 12 to 28% exhibited morphological features of apoptosis after 12 to 96 h of serum deprivation (Fig. 3B). On the other hand, down-regulation of nucleophosmin/B23 increased susceptibility of RAS-3T3 cells to apoptosis induced by serum deprivation (Fig. 3). Between 17 and 58% of RAS-3T3 cells transfected with nucleophosmin/B23 antisense oligomer became apoptotic after 12 to 96 h of serum deprivation (Fig. 3B). Nucleophosmin/B23 reverse or antisense oligomer alone caused virtually no effect on apoptosis in serum-supplemented RAS-3T3 cells.

To provide more definite evidence regarding the sensitivity of RAS-3T3 cells treated with nucleophosmin/B23 reverse or antisense oligomer to induction of apoptosis, flow cytometry was used to identify apoptotic cells. Peak with less than G_1 DNA-content was observed in nucleophosmin/B23 antisense oligomer-treated RAS-3T3 cells after 48 to 96 h of serum deprivation. Little or virtually no apoptotic peak was detected in nucleophosmin/B23 reverse oligomer-transfected RAS-3T3 cells during serum deprivation (Fig. 4).

Blockage of Serum Deprivation Induced Apoptosis by Caspase-3 but not Caspase-1 Inhibitor in NIH-3T3 Cells. Recent work demonstrates that a family of cysteinyl aspartate-specific proteases, the caspases, is activated during apoptosis and is necessary for several processes within the apoptotic pathway (Alnemri et al., 1996; Wood and Newcomb, 1999). The caspase activity of NIH-3T3 and RAS-3T3 cells during serum deprivation was thus determined in vitro. A marked increase in caspase-3 activity was detected in the lysate derived from NIH-3T3 cells that had been serum-deprived for 18–36 h (Fig. 5). The caspase-3 specific inhibitor, Ac-DEVD-CHO, added in the lysate, reduced the caspase activity to a very low level. In contrast, much less caspase-3 activity was noted in the lysate derived from serum-deprived RAS-3T3 cells (Fig. 5). Caspase-1 activity was not detected in the lysates derived from either serum-deprived NIH-3T3 or RAS-3T3 cells (data not shown). To elucidate whether or not activated caspase-1 or caspase-3 participated in the process of apoptosis induced by serum deprivation in NIH-3T3 cells, the effect of the respective cell-permeable inhibitors on inhibition of apoptosis was examined. After 48 to 72 h of serum deprivation, 60 to 80% of NIH-3T3 cells exhibited the features of highly condensed nuclei and decrease in cell size, which are characteristic of apoptosis (Fig. 6A). When DEVD-CHO (25 μM), the cell permeable caspase-3 inhibitor, was added in the medium, lower percentages (<20%) of cells exhibited morphological characteristic of apoptosis after 24 to 72 h of serum deprivation (Fig. 6B), whereas the cell permeable caspase-1 inhibitor YVAD-CHO had little effect on apoptosis induced by serum deprivation (Fig. 6C). However, inhibition of growth could still be observed in NIH-3T3 cells during serum deprivation in the presence of caspase-1 or caspase-3 inhibitor (data not shown).

During serum deprivation in the absence of caspase inhibitor or in the presence of 25 μM cell-permeable caspase-1 inhibitor, nucleophosmin/B23 decreased significantly (Fig. 7A). In the presence of 25 μM cell-permeable caspase-3 in-

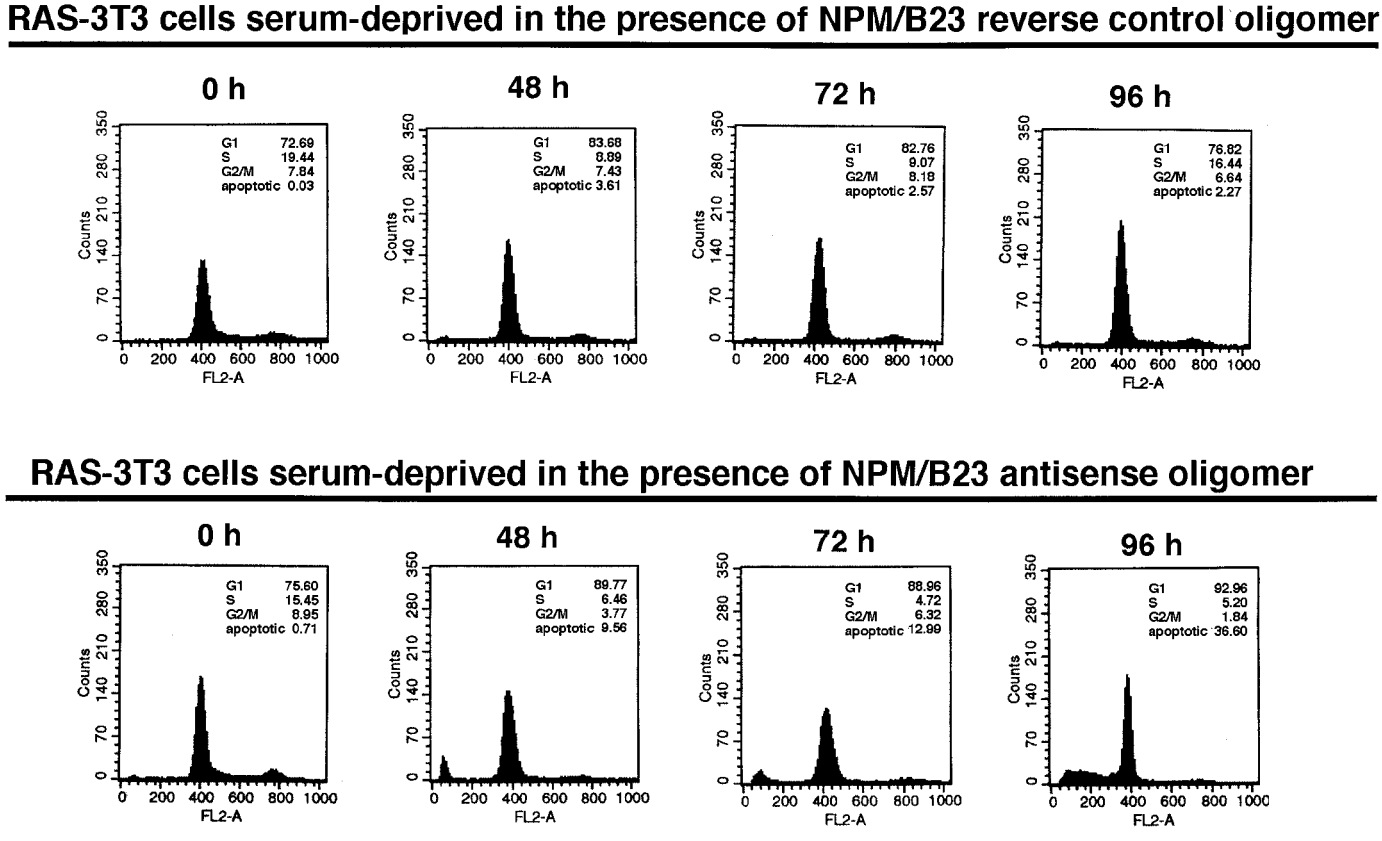

Fig. 4. The appearance of the apoptotic cells with less than G_1 DNA content. Ras-transformed (RAS-3T3) cells were under serum deprivation in the presence of nucleophosmin/B23 reverse (NPM/B23-reverse) or antisense (NPM/B23-antisense) oligomer for various times (48–96 h). Cells were harvested and were sampled for flow cytometric analysis. The figure is a representative of the results obtained in at least three independent experiments.

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hibitor, on the other hand, virtually no or little decrease of nucleophosmin/B23 protein level was observed during serum deprivation (Fig. 7A). When a lower concentration (10 μM) of caspase-3 inhibitor was used, there was similar but less inhibition of nucleophosmin/B23 cleavage (data not shown). It is reported that the stability of nucleolin/C23 is cell proliferation-dependent (Chen et al., 1991). In parallel to nucleophosmin/B23, the nucleolin/C23 protein level was also determined during serum deprivation in the absence or presence of caspase inhibitors. The results showed that nucleolin/C23 decreased significantly in NIH-3T3 cells during serum deprivation in the absence or presence of cell-permeable caspase-3 or caspase-1 inhibitor (Fig. 7B). Furthermore, cleavage of in vitro-translated nucleophosmin/B23 could be seen with the addition of caspase-3 (5 or 10 U) or apoptotic cellular extract (10 μg of lysate) (Fig. 8). The caspase-3 inhibitor (Ac-DEVD-CHO) was able to block such cleavage reactions (Fig. 8).

Discussion

Our previous study (Chou and Yung, 1997) and the present study provide evidence that parental NIH-3T3 cells lose via-

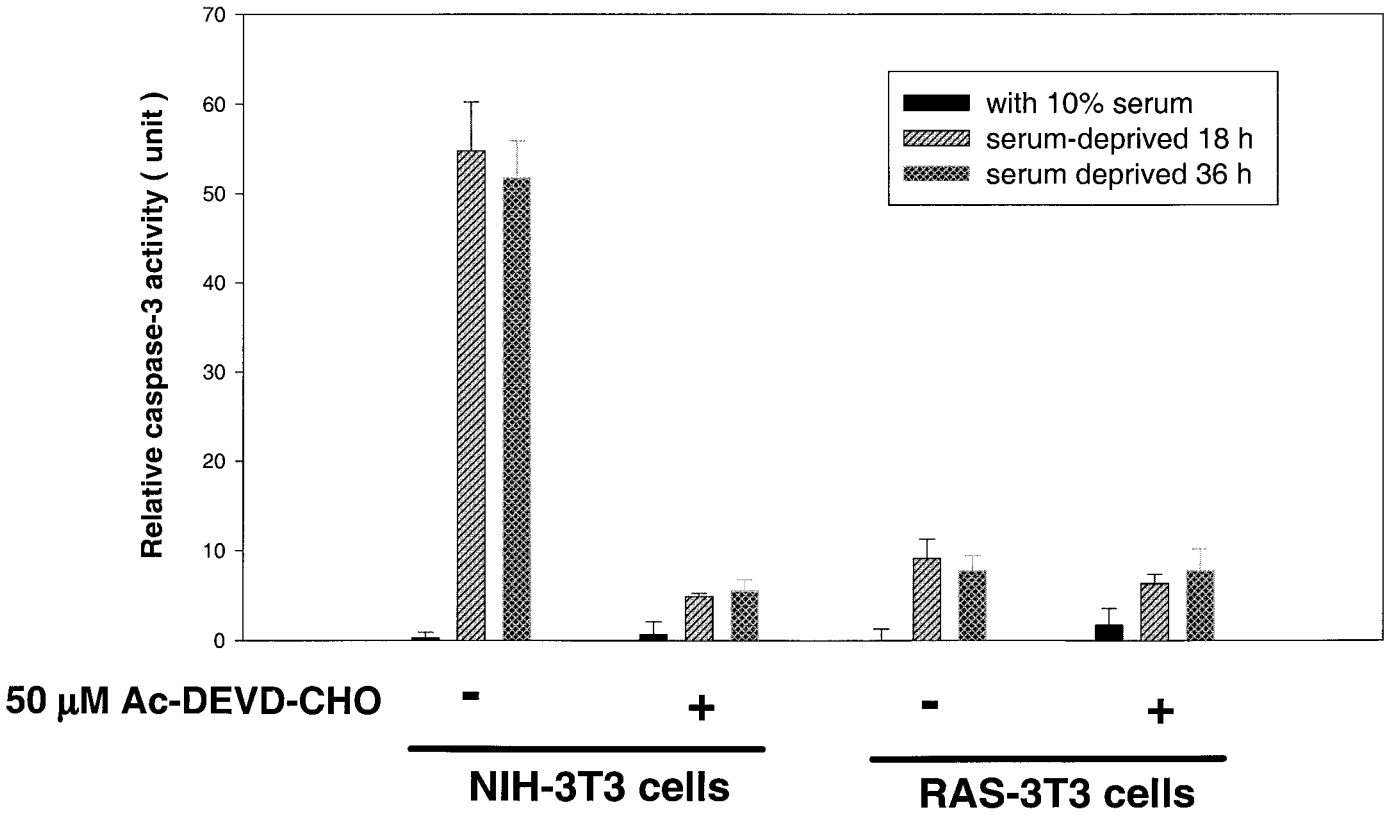

Fig. 5. Less activation of caspase-3 in ras-transformed cells compared with NIH-3T3 cells during serum deprivation. NIH-3T3 or ras-transformed (RAS-3T3) cells were supplemented with serum or serum-deprived for 18 or 36 h. Cells were harvested, washed, and the lysates were centrifuged at 12,000*g* for 30 min at 4°C. Caspase-3 activity was carried out in the absence or presence of caspase-3 inhibitor (Ac-DEVD-CHO) as described under *Experimental Procedures*. Columns, means (± S.D.) of triplicates. The figure is a representative of the results obtained in at least three independent experiments.

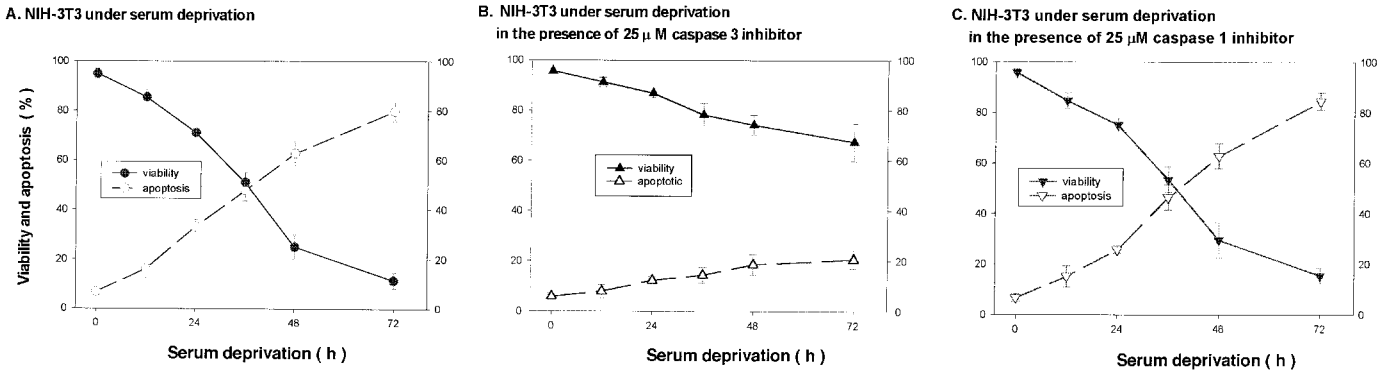

Fig. 6. Caspase-3 but not caspase-1 inhibitor blocks the apoptosis induced by serum deprivation in NIH-3T3 cells. Determination of the levels of viable or apoptotic cells (see *Experimental Procedures*) after NIH-3T3 cells were under serum deprivation in the absence (A) or presence of cell-permeable caspase-3 (B) or caspase-1 (C) inhibitor for various times (12–72 h). Points, means (± S.D.) of triplicates. The figure is a representative of the results obtained in at least three independent experiments.

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bility in serum deprivation. The loss of viability is associated with the appearance of cells that exhibit features characteristic of apoptotic cells (nuclear condensation and cell shrinkage). Interestingly, NIH-3T3 cells that are transformed with the oncogene v-H-ras are much less sensitive to such apoptotic stimuli in serum deprivation. Therefore, the v-H-ras oncogene, with its known growth promoting activity, seems to counteract apoptotic stimuli. This is also supported by flow cytometric analysis of serum-deprived cells. Although the parental NIH-3T3 cells react with the appearance of apoptotic peak after 48 of serum deprivation, no such peak of apoptosis is observed in v-H-ras transformed cells (Chou and Yung, 1997). Studies on such responses of cells (transformed and parental) to apoptosis stimuli may provide a molecular and mechanistic background for introduction of new therapeutic strategies to actively intervene in specific pathways, and favorably tip the balance of growth and apoptosis in the treatment of human tumors.

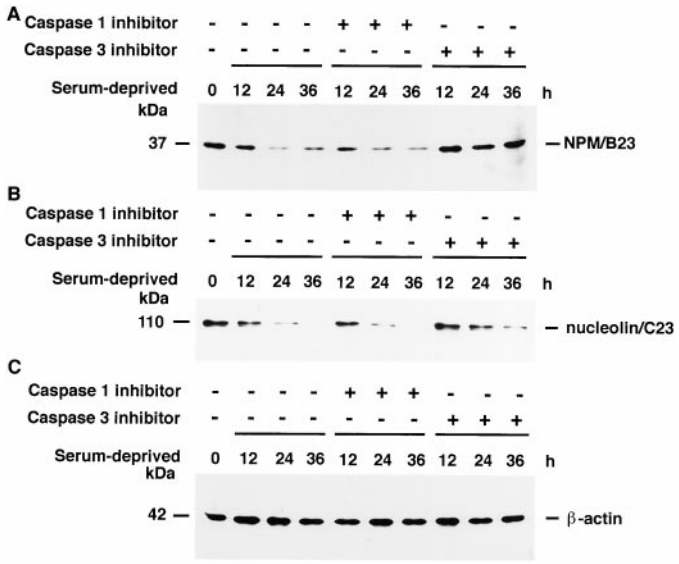

Fig. 7. Caspase-3 but not caspase-1 inhibitor blocks the decrease of nucleophosmin/B23 induced by serum deprivation in NIH-3T3 cells. NIH-3T3 cells were under serum deprivation in the absence or presence of cell-permeable caspase-1 or caspase-3 inhibitor for various times (12–36 h). Cells were harvested, washed, and the lysates were centrifuged at 12,000*g* for 30 min at 4°C. Total cellular proteins (10 μg) were separated by 10% SDS-PAGE and blotted onto PVDF papers. The blotted papers were incubated with monoclonal anti-nucleophosmin/B23 (NPM/B23) (A), monoclonal anti-nucleolin/C23 (B) or monoclonal anti-β-actin antibody (C) for 2 h at room temperature. Immunoreactivity was determined by examination of the enhanced chemiluminescence reaction.

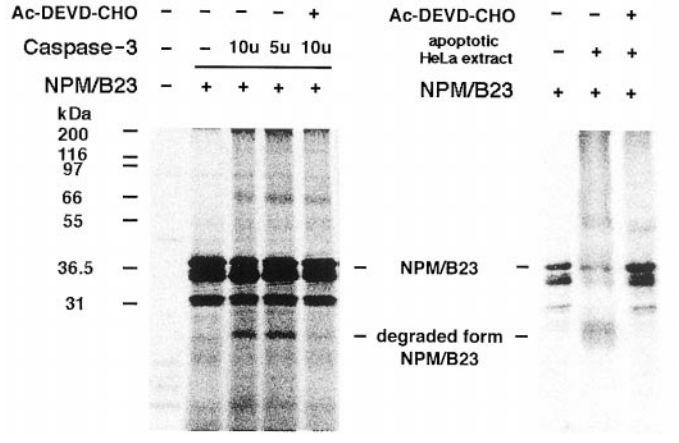

Fig. 8. Cleavage of nucleophosmin/B23 by caspase-3. The in vitro-translated nucleophosmin/B23 (NPM/B23) was incubated for 4 h at 37°C with 5 or 10 U of active recombinant caspase-3 (left) or 10 μg of lysate prepared from serum-deprived apoptotic HeLa cells (right) in the presence or absence of 25 μM caspase-3 inhibitor (Ac-DEVD-CHO). Proteins were then separated by 10% SDS-PAGE. Full-length nucleophosmin/B23 and its degradation fragment were detected by autoradiography.

One important difference between cancer and normal cells is the hyperactivity and the pleomorphism of nucleoli (Busch et al., 1963). Nucleoli in cancer cells undergo extreme variations in size, shape, fine structure, and cytochemical composition (Bernhard and Granboulan, 1968). Although rRNA transcription, processing, and ribosome assembly have been established as major functions of the nucleolus, recent studies suggest that the nucleolus participates in many other aspects of gene expression as well (Pederson, 1998). New results indicate that biosyntheses of signal recognition particle RNA and telomerase RNA involve a nucleolar stage (Pederson, 1998) and the nucleolus is critical to cellular aging (Johnson et al., 1998). A number of studies, including our own, indicate that nucleophosmin/B23, one of the major nucleolar phosphoproteins, plays a role in increased nucleolar activity that is necessary for cell proliferation (Feuerstein and Mond, 1987; Feuerstein et al., 1988; Yung et al., 1990). It is then important for us to have shown recently that nucleophosmin/B23 is one of the key elements in the regulation of nucleolar function for cellular differentiation and apoptosis (Hsu and Yung, 1998; Liu and Yung, 1998). In the present study, we have established that there is a strong relationship between nucleophosmin/B23 and the antiapoptotic effect of ras. Nucleophosmin/B23 in v-H-ras transformed cells is significantly more stable than that in parental NIH-3T3 cells during serum deprivation. v-H-ras transformed cells, having maintained a higher level of nucleophosmin/B23 during serum deprivation, are more "immortal" (i.e., less susceptible to apoptotic death). Nucleophosmin/B23 antisense oligomer, on the other hand, makes ras-transformed cells responsive to induction of apoptosis. The nucleophosmin/B23 gene is thus suggested to have a functional role in growth control, and its regulation may be closely associated with the susceptibility of tumor cells to induction of apoptosis.

Caspases play a major part in the demise of cells that have been triggered to undergo apoptosis (Nicholson and Thornberry, 1997). The data presented here indicate that caspase-3 but not caspase-1 activity is present in NIH-3T3 cells triggered to undergo apoptosis by serum deprivation. RAS-3T3 cells are less sensitive to induction of apoptosis by serum deprivation. Less caspase-3 activity is noted in the lysate derived from serum-deprived RAS-3T3 cells. Treatment of NIH-3T3 cells with cell permeable caspase-3 inhibitor prevents the induction of apoptosis and the decrease of nucleophosmin/B23 during serum deprivation. Nucleophosmin/B23, which is able to be cleaved by caspase-3 in vitro, may be a direct substrate of caspase-3. Taken together, our results indicate important relationships among regulation of nucleophosmin/B23, activation of caspase-3, and induction of apoptosis. Nucleolin/C23 and nucleophosmin/B23 are major nucleolar proteins of exponentially growing eukaryotic cells. Nucleolin/C23 fluctuates in parallel to DNA synthesis. Increased stability of nucleolin/C23 molecule in actively dividing cells is presumably by inhibition of its self-cleaving activity (Chen et al., 1991). Our present study indicates that,

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unlike nucleolin/C23, down-regulation of the nucleophosmin/B23 molecule is not proliferation-dependent but caspase-3 and apoptosis-dependent.

The resistance to apoptosis induction and inhibition of nucleophosmin/B23 cleavage shown by v-H-ras transformed cells can be attributed to ras function, because pretreatment with mevastatin, an agent known to be able to deactivate the ras function, results in recurrence of apoptosis induction (Chou and Yung, 1997) and cleavage of nucleophosmin/B23 (C. C. C., B. Y. M. Y., unpublished observations) in serum deprivation. Mevastatin can promote induction of apoptosis and decrease of nucleophosmin/B23 in HeLa cells in serum deprivation (C. C. C., B. Y. M. Y., unpublished observations). Patterson et al. (1995) show that lovastatin, another ras deactivator, induces apoptosis and down-regulation of nucleophosmin/B23 in T-lymphoblasts. Furthermore, it is shown that NIH-3T3 cells are sensitized to undergo apoptosis by inactivation of MAPK pathway and elevation of caspase-3 activity (Jan et al., 1999). Similarly, we observe that addition of MAPK inhibitor could also result in recurrence of sensitivity of RAS-3T3 cells to induction of apoptosis and cleavage of nucleophosmin/B23 in serum deprivation (C. C. C., B. Y. M. Y., unpublished observations). We speculate that ras, through activation of the MAPK pathway, down-regulates caspase-3 activity, and thus the cellular protein level of nucleophosmin/B23 is maintained. The detailed mechanism or transduction cascade involved in nucleophosmin/B23-mediated antiapoptotic effect of ras is under current investigation using dominant-negative genes in MAPK pathway.

In conclusion, our results provide evidence that nucleophosmin/B23 is importantly related to ras and caspase-3 in antiapoptosis.

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