BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **245,** 211–215 (1998)
ARTICLE NO. RC988397

Hepatocyte Growth Factor/Scatter Factor Activates the Apoptosis Signaling Pathway by Increasing Caspase-3 Activity in Sarcoma 180 Cells

Naokatu Arakaki, Jamil Ahsan Kazi, Takehiro Kazihara, Tomokazu Ohnishi, and Yasushi Daikuhara[1]

Department of Biochemistry, Kagoshima University Dental School, Kagoshima 890-8544, Japan

Received March 2, 1998

Hepatocyte growth factor, which is now known to be the same protein as scatter factor, induced oligonucleosomal fragmentation of nuclear DNA of Sarcoma 180 cells and increased the activity of caspase-3, a key component in control of the apoptotic cell death pathway to about 2.6 times that in control cells on 48 hr incubation, but did not increase the activity of caspase-1. Both HGF-induced DNA fragmentation and caspase-3 activity were completely inhibited by co-incubation with an inhibitor of caspase-3, Ac-DEVD-H. In contrast, HGF did not affect the expression of the apoptosis suppressors Bcl-2 and Bcl-x. These results indicate that HGF activates the apoptosis signaling pathway by increasing caspase-3 activity in Sarcoma 180 cells.

Hepatocyte growth factor (HGF), which was initially isolated as a potent mitogen for hepatocytes in primary culture (1, 2), is now known to be a broad-spectrum mitogen for a variety of cell types (3) including melanocytes and endothelial and epithelial cells. Moreover, HGF has been found to be the same molecule as "scatter factor" (SF) (4), which dissociates and increases the motility of epithelial cells. HGF also induces branching tubule formation of Madin-Darby canine kidney epithelial cells in a three-dimensional collagen gel matrix (5). *In vivo,* HGF is a potent angiogenic factor (6) and is involved in organ regeneration (reviewed by Goldberg and Rosen (7)) and tumor invasiveness (8, 9). Therefore, HGF is now known to be a multifunctional cytokine.

In addition to its mitogenic activity, HGF has tumor cytotoxic activity, inhibiting growth of some tumor cell lines such as Sarcoma 180 cells (10) and Hep G2 human hepatoblastoma cells (11). Over the past few years, however, although many studies have been focused on the signaling pathways of HGF in cell mitogenesis and in dissociation and scattering of epithelial cells, little is yet known about the mechanism of the signaling pathway for the tumor cytotoxic activity of HGF.

In this study, we found that HGF induces oligonucleosomal fragmentation of nuclear DNA and increases caspase-3 activity, which are biochemical markers of apoptosis in Sarcoma 180 cells, but did not increase their caspase-1 activity. We also showed that HGF did not affect the expressions of Bcl-2 and Bcl-x proteins, which are suppressors of apoptosis. The present results show that activation of the apoptosis signaling pathway by HGF is involved, at least in part, in its tumor cytotoxic activity on Sarcoma 180 cells.

MATERIALS AND METHODS

Reagents. The sources of materials used in this work were as follows: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Research Organics, Inc., Cleveland, OH; acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-H), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-H), Ac-YVAD-α-(4-methyl-coumaryl-7-amide) (Ac-YVAD-MCA), and Ac-DEVD-MCA from the Peptide Institute, Osaka, Japan; Dulbecco's Modified Eagle Medium (DMEM) from Nissui Pharmaceutical Co., Tokyo, Japan; Sarcoma 180 cells from RIKEN Cell Bank, Tsukuba, Japan; proteinase K and RNase A from Boehringer Mannheim GmbH, Mannheim, Germany; dithiothreitol (DTT) from Nacalai Tesque, Kyoto, and (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride

[1] To whom correspondence should be addressed: Department of Biochemistry, Kagoshima University Dental School, 35-1 Sakuragaoka-8, Kagoshima 890-8544, Japan. Fax: +81-99-275-6138. E-mail: daiku@dentc.hal.kagoshima-u.ac.jp.

Abbreviations used: Ac-YVAD-H, acetyl-Tyr-Val-Ala-Asp-aldehyde; Ac-DEVD-H, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-MCA, Ac-YVAD-α-(4-methyl-coumaryl-7-amide); Ac-DEVD-MCA; Ac-DEVD-α-(4-methyl-coumaryl-7-amide); *p*-APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride hydrochloride; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; HGF, hepatocyte growth factor; ICE, interleukin 1β-converting enzyme; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PBS, Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline; SDS, sodium dodecyl sulfate; SF, scatter factor; TNF-α, tumor necrosis factor-α.

0006-291X/98 $25.00

(*p*-APMSF) from Wako Pure Chemical Industrials, Osaka. Recombinant human HGF was prepared and purified as described previously (12). Other materials used in this study were described previously (2, 13, 14).

Assay of tumor cytotoxic activity. In this study, the tumor cytotoxic activity of HGF was determined by an MTT assay (15). Sarcoma 180 cells were plated into Nunc 24-well plastic dishes (Roskilde, Denmark) in 0.4 ml of DMEM supplemented with 10% fetal calf serum (FCS) (Irvine Scientific, Santa Ana, CA) and 100 μg/ml of kanamycin sulfate (basal medium) at a density of 2×10^5 cells/ml and cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 hr, the cells were washed once with basal medium and cultured in basal medium with or without HGF for the indicated times. After incubation, the medium was replaced by 400 μl of basal medium containing MTT (0.5 mg/ml). After 30 min, this medium was removed and 400 μl of acidified isopropanol (0.04N HCl in isopropanol) was added to the wells to solubilize formazan crystals. A aliquots (200 μl) of the solutions were transferred to 96-well plates and the absorbance of the MTT formazan products was measured at 570 nm using a reference wavelength of 650 nm.

Preparation of DNA fragments. Cells (2×10^5/ml) were cultured as described above except for use of 10 ml of medium/plate in 8.5 cm plastic plates (Nunc). After incubation, all cells were collected by centrifugation at 500 × g for 3 min, and washed once with cold Mg^{2+}- and Ca^{2+}-free phosphate buffered-saline (PBS). About 5×10^6 cells were lysed in 200 μl of 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA, 0.5% Triton X-100 and 0.1% SDS for 10 min at 4 °C. The lysate was centrifuged at 12,000 × g for 20 min, and the supernatant containing fragmented DNA was treated with RNase A (100 μg/ml) for 60 min at 37 °C, and then digested with proteinase K (100 μg/ml) for 60 min at 37 °C. Fragmented DNA was precipitated with isopropanol, washed with 70% ethanol and dissolved in 50 μl of 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. Then 10 μl samples were subjected to electrophoresis in 1.2% agarose gel containing 0.1 mg/ml ethidium bromide.

Assay of caspase activity. Caspase activities were measured essentially as described by Enari *et al.* (16). Briefly, after incubation of cells (2×10^5/ml) in 8.5 cm plastic plates with or without additives as indicated, all cells were collected, washed as described above and homogenized in 0.5 ml of extraction buffer (10 mM HEPES-KOH, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 100 μM*p*-APMSF, 10 μg/ml of leupeptin, 1 μg/ml of pepstatin A) in a Dounce homogenizer (type A pestle). The cell extracts (80-90 μg of protein) obtained as described above were diluted with 1 ml of reaction buffer (50 mM HEPES-KOH, pH 7.4, 10% sucrose, 0.1% CHAPS, 10 mM DTT, 0.1 mg/ml of ovalbumin), and incubated for 30 min at 30 °C with 10 μM of fluorescent substrates in the presence or absence of 10 μM of a specific inhibitor of caspase-1 or caspase-3. Ac-YVAD-MCA and Ac-DEVD-MCA were used as substrates and Ac-YVAD-H and Ac-DEVD-H as specific inhibitors of caspase-1 and caspase-3, respectively. The fluorescence of the cleaved substrates was determined with a spectrofluorometer (Type 850 Hitachi spectrofluorometer, Hitachi, Tokyo) set at an excitation wavelength of 380 nm and emission wavelength of 460 nm. Specific caspase-1- and caspase-3-like activities were determined by subtracting the values obtained in the presence of inhibitors. One unit of enzyme activity corresponds to the activity that cleaves 1 pmol of the respective substrate at 30 °C in 1 min per mg protein.

Immunoblot analysis. After incubation of the cells (2×10^5/ml) in 8.5 cm plastic plates with or without HGF, they were collected, washed as described above and lysed in 10 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 % sodium deoxycholic acid, 100 μM *p*-APMSF, 20 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 mM vanadate and 100 mM NaF, for 30 min at 4 °C. Cell extracts (50-60 μg of protein) were treated with Laemmli's SDS-PAGE sample buffer (17), resolved by SDS-PAGE, and subjected to Western blotting analysis as described previously (14). Membranes were probed with anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Bcl-x (Transduction Laboratories, Lexington, KY) polyclonal antibodies, and located with an AURORA Western Blot kit (ICN Biomedicals, Costa Mesa, CA) according to the manufacturer's instructions.

Other methods. Protein was determined with BCA Protein Assay Reagent (Pierce, Rockford, IL). Student's *t*-test for unpaired samples to compare the mean values of groups was done according to Snedecor and Cochran (18).

RESULTS AND DISCUSSION

As shown in Figure 1-A, HGF caused dose-dependent reduction in the viability of Sarcoma 180 cells, assessed by an MTT assay, as described by Shima *et al.* (10) and on incubation for 48 hr, 30 ng/ml HGF decreased the viability about 50%. Because many cytotoxic factors including tumor necrosis factor-α (TNF-α) (19) have been shown to induce apoptotic cell death of target cells, we examined whether HGF activates the apoptosis signaling pathway in Sarcoma 180 cells. As shown in Fig.1-B, HGF (30 ng/ml) evidently induced oligonucleosomal fragmentation of nuclear DNA, which is a biochemical marker of apoptosis, after incubation for 48 hr, but not 24 hr.

Recent studies have shown that the interleukin 1β-converting enzyme (ICE) family of proteases, now designated as caspases, are key components in control of the apoptotic cell death pathway and that these enzymes are activated during apoptosis by various apoptotic stimuli (20). To confirm that HGF activates the apoptosis signaling pathway in the cells, we next examined whether HGF activated these enzymes. In this study, we measured caspase-1 and caspase-3 activities, because these enzymes have been shown to be activated by Fas-mediated apoptosis (16) and other apoptosis stimuli (21). As shown in Table 1, HGF increased caspase-3 activity to about 2.6 times the control value on 48 hr incubation, but did not increase caspase-1 activity. Moreover, it did not increase caspase-3 activity significantly after 24 hr incubation, consistent with our finding that it did not induce DNA fragmentation on 24 hr incubation (Fig.1-A). Increase in caspase-3 activity (to about 2.3 times the control value) was observed in the cytosol from cells incubated for 72 hr with HGF, but with no increase in caspase-1 activity (data not shown). Table 1 also shows that HGF-induced increase in caspase-3 activity was completely inhibited by co-incubation with a specific inhibitor of caspase-3, Ac-DEVD-H (100 μM) (16). This inhibitor was effective at 20 μM and maximally effective at 100-150 μM (data not shown). This inhibitor also completely inhibited HGF-induced DNA fragmentation (Fig. 1-C). However, the caspase-3 inhibitor restored HGF-suppressed growth of Sarcoma 180 cells only partially (Fig. 2).

Bcl-2 and Bcl-x are known to function upstream of caspases and protect cells from apoptosis induced by many death-inducing signals (20, 22). Because the ratio

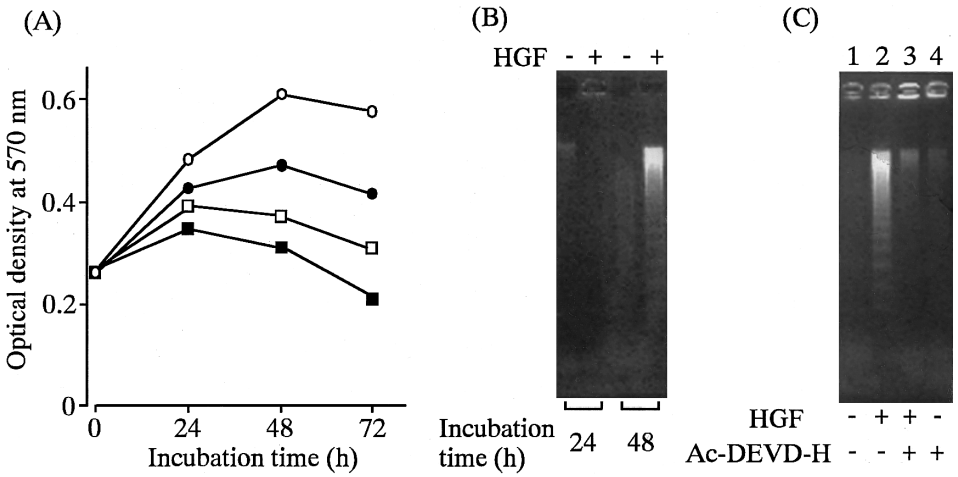

FIG. 1. Effects of HGF on Sarcoma 180 cells. (A) Suppression of cell viability by HGF. Cells (2×10^5/ml) were incubated with or without HGF for 24, 48, and 72 hr. Cell viability was determined by MTT assay as described in "MATERIALS AND METHODS". ○, control; ●, 3 ng/ml HGF; □, 10 ng/ml HGF; ■, 30 ng/ml HGF. Experiments were repeated four times and typical data are shown. (B) Induction of DNA fragmentation by HGF. Cells (2×10^5/ml) were incubated with or without HGF (30 ng/ml) for 24 and 48 hr. The fragmented DNA was separated, and analyzed by agarose gel electrophoresis as described in "MATERIALS AND METHODS". (C) Effect of the caspase-3 inhibitor Ac-DEVD-H on HGF-induced DNA fragmentation. Cells (2×10^5/ml) were incubated with or without HGF (30 ng/ml) in the presence or absence of Ac-DEVD-H (100 μM) for 48 hr. The fragmented DNA was separated, and analyzed as in B.

of antagonists (Bcl-2 and Bcl-x) to agonists (caspases) may dictate whether a cell will respond to apoptotic stimuli (23), we next examined the effect of HGF on the expressions of these proteins in Sarcoma 180 cells. The expressions of these proteins were not affected by HGF (Fig. 3).

The present results clearly show that HGF activates the apoptosis signaling pathway by increasing caspase-3 activity without affecting caspase-1 activity or the expressions of the apoptosis suppressors Bcl-2 and Bcl-x in Sarcoma 180 cells. However, our results also suggest the existence of other pathways, besides that for induction of apoptosis, for the tumor cytotoxic activity of HGF, because the HGF-suppressed growth of the cells was only partly restored when the apoptosis signaling pathway was completely inhibited by a caspase-3 inhibitor, as assessed by DNA fragmentation and caspase-3 activity (Fig. 2). In this regard, it is noteworthy that we recently found that N-acetyl cysteine, a precursor of glutathione and an intracellular free-radical scavenger, almost completely prevented HGF-induced growth suppression and activation of the apoptosis signaling pathway in Sarcoma 180 cells, suggesting that HGF induces oxidative stress in the cells and this is a cause of the tumor cytotoxic activity of HGF (Arakaki, *et al.* unpublished observations). We also found that HGF enhances expression of the cyclin-dependent kinase inhibitor p21/Waf1/Cip1 within 3 hr in the cells (Arakaki, *et al.* unpublished observations). Thus, HGF seems to activate diverse signaling pathways involved in growth suppression of Sarcoma 180 cells.

Recently, Enari *et al.* (16) reported that Fas activa-

TABLE 1

Activities of Caspase-1 and Caspase-3 in HGF-Treated Sarcoma 180 Cells

Incubation time (h)	Caspase-1 activity (unit)		Caspase-3 activity (unit)	
	Control	+ HGF	Control	+ HGF
0	16.0 ± 1.4		11.3 ± 1.7	
24	17.9 ± 2.9	19.6 ± 2.8	13.1 ± 1.8	15.4 ± 3.5
48	22.8 ± 4.3	26.4 ± 5.1	16.7 ± 1.3	44.2 ± 4.8**
48 + Ac-DEVD-H[a]			9.4 ± 1.0	10.5 ± 2.9

Note. Cells (2×10^5/ml) were incubated with or without HGF (30 ng/ml) for the indicated times. Cell extracts were prepared and caspase activities were measured as described under MATERIALS AND METHODS. Values are means ± SDs for three separate experiments.

[a] Cells (2×10^5/ml) were incubated with or without HGF (30 ng/ml) in the presence of 100 μM Ac-DEVD-H (a caspase-3 inhibitor) for 48 hr and then caspase-3 activity was measured.

** $P < 0.001$ vs. control (without HGF).

tion of mouse WR19L transformant cells induced transient ICE (now called caspase-1)-like activity with a peak at 10 min and then rapid decrease after 20 min, whereas ICE/caspase-1, CPP32 (now called caspase-3)-like activity gradually accumulated in the cytosol of the cells after 60 min. Their results also indicated that ICE/caspase-1 functions upstream of CPP32/caspase-3, although it is not clear whether the ICE/caspase-1-like protease directly activates the CPP32/caspase-3-like protease during Fas-induced apoptosis. Then, we measured the caspase activities in cytosolic extracts of cells incubated for various times (10, 60, 120, 180, and 360 min) with 30 ng/ml HGF. However, neither the caspase-1 nor the caspase-3 activity increased during the period of incubation with HGF tested (data not shown). Therefore, caspase-1 is probably not involved in activation of the apoptosis signaling pathway by HGF. The lack of a major role of ICE in apoptosis has also been demonstrated using ICE-deficient mice, in which apoptosis occurs essentially normally (24), and in spontaneously apoptotic osteosarcoma cells (25), in which CPP32, but not ICE, has been shown to be important for the initiation of apoptotic cell death. However, further detailed time course studies on the effect of HGF on caspase-1 activity may be needed to exclude the involvement of caspase-1 in the HGF-activated apoptosis signaling pathway.

HGF exerts stimulatory or inhibitory effects on cell growth. Although the mechanisms of these effects are not yet clear, the *in vitro* system described here should be useful as a model for investigating how these different actions of HGF are regulated.

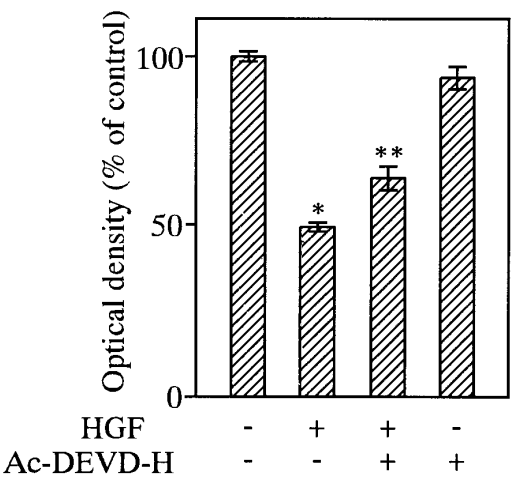

FIG. 2. Effect of the caspase-3 inhibitor Ac-DEVD-H on HGF-suppressed growth of Sarcoma 180 cells. Cells (2×10^5/ml) were incubated with or without HGF (30 ng/ml) in the presence or absence of Ac-DEVD-H (100 μM) for 48 hr. Cell viability was determined by MTT assay as described in Fig.1-A. Values are means ± SDs from four different experiments. *$P < 0.001$ *vs.* control (without HGF), **$P < 0.02$ *vs.* HGF alone.

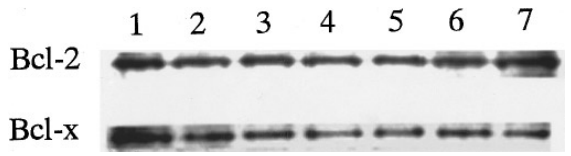

FIG. 3. Effects of HGF on the expressions of Bcl-2 and Bcl-x in Sarcoma 180 cells. Cells (2×10^5/ml) were incubated with (lanes 3, 5, and 7) or without (lanes 1, 2, 4, and 6) HGF (30 ng/ml) for 0 hr (lane 1), 7 hr (lanes 2 and 3), 24 hr (lanes 4 and 5), and 48 hr (lanes 6 and 7). Cell extracts were prepared, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, probed with anti-Bcl-2 (0.2 μg/ml) or anti-Bcl-x (0.2 μg/ml) polyclonal antibodies, and examined with an AURORA Western Blot kit as described in "MATERIALS AND METHODS".

ACKNOWLEDGMENTS

We thank Miss Yoko Yasuda for secretarial assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

1. Nakamura, T., Nawa, K., Ichihara, A., Kaige, N., and Nishino, T. (1987) *FEBS Lett.* **224,** 311–316.
2. Gohda, E., Tsubouchi, H., Nakayama, H., Hirono, S., Kakiyama, O., Takahashi, K., Miyazaki, H., Hashimoto, S., and Daikuhara, Y. (1988) *J. Clin. Invest.* **81,** 414–419.
3. Rubin, J. S., Chan, A. M.-L., Bottaro, D. P., Burgess, W. H., Taylor, W. G., Cech, A. C., Hirschfield, D. W., Wong, T., Miki, T., Finch, P. W., and Aaronson, S. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 415–419.
4. Weidner, K. M., Arakaki, N., Hartmann, G., Vandekerckhove, J., Weingart, S., Rieder, H., Fonatsch, C., Tsubouchi, H., Hashida, T., Daikuhara, Y., and Birchmeier, W. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7001–7005.
5. Montesano, R., Matsumoto, K., Nakamura, T., and Orci, L. (1991) *Cell* **67,** 901–908.
6. Bussolino, F., DiRenzo, M. F., Zicho, M., Bocchietto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagnone, L., Coffer, A., Marchisio, P., and Comoglio, P. M. (1992) *J. Cell Biol.* **119,** 629–641.
7. Goldberg, I. D., and Rosen, E. M. (Eds.) (1993) Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the c-Met Receptor, pp. 1–397, Birkhauser Verlag, Basel.
8. Weidner, K. M., Behrens, J., Vandekerckhove, J., and Birchmeier, W. (1990) *J. Cell Biol.* **111,** 2097–2108.
9. Giordano, S., Zhen, Z., Medico, E., Gaudino, G., Galimi, F., and Comoglio, P. M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 649–653.
10. Shima, N., Nagao, M., Ogaki, F., Tsuda, E., Murakami, A., and Higashio, K. (1991) *Biochem. Biophys. Res. Commun.* **180,** 1151–1158.
11. Miyazaki, M., Gohda, E., Tsuboi, S., Tsubouchi, H., Daikuhara, Y., Namba, M., and Yamamoto, I. (1992) *Cell Biol. Int. Rep.* **16,** 145–154.
12. Yoshiyama, Y., Arakaki, N., Naka, D., Takahashi, K., Hirono, S., Kondo, T., Nakayama, H., Gohda, E., Kitamura, N., Tsubouchi, H., Ishii, T., Hishida, T., and Daikuhara, Y. (1991) *Biochem. Biophys. Res. Commun.* **175,** 660–667.
13. Tamura, M., Arakaki, N., Tsubouchi, H., Takada, H., and Daikuhara, Y. (1993) *J. Biol. Chem.* **268,** 8140–8145.
14. Arakaki, N., Kawakami, S., Nakamura, O., Ohnishi, T., Miya-

zaki, H., Ishii, T., Tsubouchi, H., and Daikuhara, Y. (1995) *Hepatology* **22,** 1728–1734.

15. Buttke, T. M., McCubrey, J. A., and Owen, T. C. (1993) *J. Immunol. Methods* **157,** 233–240.
16. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) *Nature* **380,** 723–726.
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
18. Snedecor, G. W., and Cochran, W. G. (1967) Statistical Methods. 6th ed., pp. 1–593, Iowa State Univ. Press, Ames, IA.
19. Laster, S. M., Good, J. G., and Gooding, L. R. (1988) *J. Immunol.* **141,** 2629–2634.
20. Golstein, P. (1997) *Science* **275,** 1081–1082.
21. Duan, H., Chinnaiyan, A. M., Hudson, P. L., Wing, J. P., He, W., and Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 1621–1625.
22. Kroemer, G. (1997) *Nature Med.* **3,** 614–620.
23. Oltvai, Z. N., and Korsmeyer, S. J. (1994) *Cell* **79,** 189–192.
24. Li, P., Allen, H., Banerjiee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., Towne, E., Tracey, D., Wardwell, S., Wei, F-Y., Wong, W., Kamen, R., and Seshadri, T. (1995) *Cell* **80,** 401–411.
25. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. O., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T-T., Yu, V. L., and Miller, D. K. (1995) *Nature* **376,** 37–43.