Requirement of the Caspase-3/CPP32 Protease Cascade for Apoptotic Death following Cytokine Deprivation in Hematopoietic Cells*

Takayuki Ohta‡, Taisei Kinoshita‡, Mihiko Naito§, Tadashige Nozaki§, Mitsuko Masutani‡, Takashi Tsuura§, and Atsushi Miyajima‡

From the ‡Laboratory of Cellular Biosynthesis and §Laboratory of Biomedical Research, Institute of Molecular and Cellular Bioscience, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113, Japan; ¶Biochemistry Division, National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan

Hematopoietic cytokines transduce cell survival signals, which are distinct from the signals necessary for the stimulation of DNA synthesis. Recently, the Ras and phosphatidylinositol 3-kinase pathways have been shown to play important roles in preventing apoptosis in various cell types, e.g. hematopoietic cells and neuronal cells. Withdrawal of cytokine(s), in turn, results in rapid inactivation of these survival pathways and eventually leads to cell death accompanied by the hallmarks of apoptosis. However, the mechanism of cell death caused by cytokine deprivation has not been fully elucidated. In this study, we demonstrate that caspase-3/CPP32, a member of the caspase/interleukin-1β-converting enzyme family, is activated upon interleukin (IL)-3 deprivation in IL-3-dependent cells as well as IL-2 deprivation in IL-2-dependent cells. In addition, poly(ADP-ribose) polymerase, a cellular substrate for the caspase family proteases, was degraded into apoptotic fragments in both cell lines after cytokine removal. Furthermore, inhibition of a caspase family protease by synthetic peptides suppressed apoptotic death. These results indicate that the activation of a caspase-like protease(s) is required for the progression of apoptosis following cytokine deprivation. However, readdition of IL-3 did not restore the proliferative potential of the cells that survived in the presence of the peptide inhibitor after IL-3 depletion. Therefore, cellular commitment to apoptosis appears to precede the activation of a caspase-like protease(s).

Hematopoietic cells require an appropriate cytokine(s) for their survival; in the absence of cytokines, these cells not only cease proliferation, but also undergo rapid apoptotic death (1). The strict dependence of hematopoietic cells on cytokines is the key to hematopoietic homeostasis. Hematopoietic cells expanded by cytokines produced from activated T cells rapidly return to a normal level upon removal of inflammatory stimulation (2). Cytokines also regulate steady-state hematopoiesis to preserve an adequate population of peripheral hematopoietic cells (2). Accordingly, the strict cytokine dependence for survival is an important mechanism that inhibits hematopoietic hyperplasia, and alterations of the dependence often result in malignant transformation (3, 4). However, until recently, the precise mechanism of intracellular signaling for cell survival has not been well documented.

We previously reported by using truncated mutants of the human granulocyte-macrophage colony-stimulating factor receptor and kinase inhibitors that the Ras signaling pathway is important for hematopoietic cell survival (5). More recently, it was shown that the antiapoptotic effect of the Ras pathway is mediated not only by the Raf/mitogen-activated protein kinase pathway but also by a rapamycin/wortmannin-sensitive pathway in which phosphatidylinositol 3-kinase is involved (6, 7). Furthermore, several lines of evidence clearly demonstrated that the signaling pathway involving PKB/Akt protein kinase is important for survival of various types of cells (8–13). Thus, cytokines regulate survival of hematopoietic cells through the activation of multiple signaling pathways. In contrast, the removal of cytokines rapidly inactivates these cell survival signals and results in massive apoptotic cell death (1, 5).

In addition to cytokine withdrawal, a number of apoptotic signals lead to the commitment of death through several distinct mechanisms (14). For example, ligands for the Fas/tumor necrosis factor-α receptor family (for a review, see Ref. 15) provoke the cellular suicide program, which does not require novel mRNA/protein synthesis. In contrast, γ-radiation-induced apoptosis is likely to involve transcriptional regulation through p53-dependent (16, 17) or IRF1-dependent mechanisms (18). However, after the commitment, there appears to be a general cell killing mechanism that is involved in most types of apoptosis (1, 19). Members of the interleukin-1β-converting enzyme (ICE1; recently referred to as a caspase (20)) family proteases, originally identified as a gene required for the programmed cell death in nematodes (21), mediate various types of apoptotic signals in mammalian cells, and inhibition of these proteases by synthetic peptide inhibitors or certain viral proteins often results in prevention of apoptosis (22–25). To date, at least nine different caspase-related proteases have been identified, and their potential roles in apoptosis have been

* This work was partly supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan, a grant from New Energy and Industrial Technology Development Organization, a grant from Core Research for Evolutional Science and Technology (CREST), and a research grant from the Toray Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Tel., 81-3-5800-3551; Fax, 81-3-5800-3550; E-mail: miyajima@hgc.ims.u-tokyo.ac.jp.

The abbreviations used are: ICE, interleukin-1β-converting enzyme; IL, interleukin; PARP, poly(ADP-ribose) polymerase; DEVDCMA, Ac-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide; YVAD, Ac-Tyr-Val-Ala-Asp; YVAD-MCA, Ac-Tyr-Val-Ala-Asp-4-methylcoumaryl-7-amide; Z-VAD, benzylxycarbonyl-Val-Ala-Asp-CH₂OC(O)-2,6-dichlorobenzene; Z-Asp, benzylxycarbonyl-Asp-CH₂OC(O)-2,6-dichlorobenzene; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
examined in various systems (19, 20). However, apoptosis induced by cytokine deprivation has been considered to occur simply due to the inactivation of signaling pathways responsible for cell survival, and its mechanism has not been explored. Moreover, it was suggested that in an IL-2-dependent cytotoxic T cell line, a caspase-like protease is not required for apoptosis caused by IL-2 deprivation (26).

We show in this study that the removal of cytokines (IL-3 and IL-2) leads to the activation of a caspase-like protease that cleaves the specific target sequence of a caspase-3/CPP32-class cysteine protease. We also found that the degradation of the endogenous apoptotic substrate poly(ADP-ribose) polymerase (PARP) occurred following caspase-3 activation. Furthermore, the addition of peptide inhibitors for caspase-like proteases blocked the activation of caspase-3, PARP breakdown, DNA fragmentation, and subsequent cell death, suggesting that the activation of caspase-3 plays an essential role in the process of apoptosis following cytokine deprivation. However, the cells were no longer able to restore their growth potential despite the blockade of all signs of apoptotic death by the inhibitor. Therefore, the commitment to apoptosis following cytokine deprivation appears to precede activation of the caspase-like protease cascade.

EXPERIMENTAL PROCEDURES

Materials—Synthetic peptide-based substrates for caspase-3/CPP32 (Ac-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide; DEVD-MCA) and caspase-1/ICE (Ac-Tyr-Val-Ala-Asp-4-methylcoumaryl-7-amide; YVAD-MCA) were purchased from Peptide Institute, Inc. (Osaka, Japan). Inhibitors for caspase/ICE family proteases (benzyloxycarbonyl-Val-Ala-Asp-Ch-Oc(O)-2,6-dichlorobenzene (Z-Asp)) were synthesized as described previously (25) or purchased from Peptide Institute, Inc. The apoptotic substrate poly(ADP-ribose) polymerase (PARP) was established by immunizing guinea pigs with the C-terminal 99-kDa fragment of the human PARP protein (28), and it recognizes both the full-length (110 kDa) and the two apoptotic fragments (89 and 24 kDa) of both human and mouse PARP proteins.

Cell Culture and the Growth Assay—Murine IL-3-dependent Ba/F3 and IL-2-dependent CTLL-2 cells were used. The human T cell line Jurkat was used as a positive control in most experiments. IL-3-dependent lines were maintained in RPMI 1640 supplemented with 5% fetal calf serum and 2 ng/ml of mouse IL-3 produced in silkworm cells (29). CTLL-2 cells were cultured in RPMI 1640 with 10% fetal calf serum, recombinant mouse IL-2, and 50 µM 2-mercaptoethanol. Jurkat cells were maintained in RPMI 1640 with 10% fetal calf serum. To deplete cytokines, cells were washed twice with cytokine-free medium, and in several experiments appropriate concentrations of caspase-family inhibitors were added to the culture media. Proliferative potential and cell survival were determined by the MTT assay (30) and the trypan blue dye exclusion assay (31) as described previously.

Measurement of the Caspase-3 Activity—Growing or cytokine-depleted cells (1 x 10⁶) were harvested and lysed in lysis buffer composed of 10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, and 5 mM dithiotreitol. Insoluble materials were removed by centrifugation (40,000 rpm, 20 min, 4 °C), and each cell lysate was then incubated with 20 µM concentration of the fluorogenic substrate (YVAD-MCA or DEVD-MCA) in ICE buffer (20 mM HEPES-KOH (pH 7.4), 10% glycerol, and 2 mM dithiothreitol) for 60 min at 37 °C. The 7-amino-4-methylcoumarin released from the fluoropeptides was measured with excitation at 380 nm and emission at 460 nm using a fluorescence spectrophotometer (model F2000; Hitachi, Tokyo, Japan).

Analysis of PARP Degradation—Degradation of the endogenous apoptotic substrate (32) PARP after cytokine withdrawal was analyzed by Western blotting. Control or cytokine-depleted cells (6 x 10⁶) were washed twice with phosphate-buffered saline and lysed with 20 µl of 1 x Laemmli’s sample buffer. Equal amounts of each sample were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Hybond ECL; Amersham Corp.). PARP protein was then probed with anti-human PARP antibody (Amersham).

DNA Fragmentation Analysis—Low molecular weight chromosomal DNA was purified according to the protocol described elsewhere (5). Briefly, 6 x 10⁶ cells were washed with phosphate-buffered saline and lysed with 200 µl of lysis buffer (10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.2% Triton X-100). Samples were incubated on ice for 10 min, and insoluble materials were removed by centrifugation (15,000 rpm, 10 min). The supernatant was transferred to a new tube, and the nucleic acid fraction was purified by extraction with phenol/chloroform/isoamyl-alcohol (25:24:1) twice followed by ethanol precipitation. The precipitate was dissolved in 20 µl of TE/RNase (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 2 µg/ml RNase A) and incubated for 30 min at 37 °C. Equal amounts (10 µl) of each sample were separated by 1.5% agarose gel electrophoresis, and DNA was visualized by ethidium bromide staining.

Cell Cycle Analysis—Ba/F3 cells were deprived of IL-3 in the presence of Z-VAD for 24 h. Then one half of the cells were fixed with 70% ethanol, and the other half were washed twice with phosphate-buffered saline and IL-3 was added. After a 16-h incubation with IL-3, cells were fixed with 70% ethanol. As a positive control, IL-3 was added to the cells deprived of IL-3 for 6 h and incubated for 16 h. As a negative control, Ba/F3 cells deprived of IL-3 for 24 h were fixed. All fixed cells were stained with PI/RNase solution (3.2 mM sodium citrate, 50 µg/ml propidium iodide, 500 ng/ml RNase A, and 0.1% Triton X-100) for 30 min at room temperature. After staining, cells were resuspended and analyzed by a FACScan.

RESULTS

Cytokine Depletion Up-regulates Caspase-like Activity—To examine whether caspase-like activity increases following cytokine deprivation in IL-3- and IL-2-dependent cell lines, we analyzed enzymatic activities of the caspase family proteases using fluorogenic peptide substrates (YVAD-MCA and DEVD-MCA). Cell lysates from growing or cytokine-depleted cells were incubated with substrates, and increases in fluorescence due to enzymatic cleavage of the peptides were measured with a fluorometer. As a positive control, anti-Fas antibody-stimulated Jurkat cells were used, since Fas-induced apoptosis is known to require activation of caspase-family proteases (22–24). In IL-3-dependent (Ba/F3 and 32D) and IL-2-dependent (CTLL-2) cells, withdrawal of the cytokine resulted in increases

![Figure 1](https://example.com/fig1.png)
in 7-amino-4-methylcoumarin fluorescence, indicative of the activation of caspase-3/CPP32 (Fig. 1). Up-regulation of the caspase-3 activity was detected as soon as 5 h post-depletion and kept increasing up to 18–24 h in both cell lines. The caspase-3 activation in CTL2-2 cells was somehow slower than that in IL-3-dependent cell lines, consistent with the slower kinetics of cell death in CTL2-2 cells upon IL-2 deprivation (data not shown). The pattern of the caspase-3 activation was similar to that observed in Jurkat cells treated with anti-Fas antibody, CH11 (500 ng/ml). These results suggest that caspase-3 is activated in cytokine-dependent cells after cytokine withdrawal. We also tested caspase-1 activity in these cell lines using another substrate (YVAD-MCA). However, we did not detect any activity that reproducibly cleaved this substrate (see "Discussion").

The activation of caspase-3 during the apoptotic process after cytokine depletion might result in the proteolytic degradation of a cellular substrate for caspase-3. Since PARP is an endogenous caspase-3 substrate (32), we examined PARP degradation in Ba/F3 cells by Western blotting analysis using anti-PARP polyclonal antibody (Fig. 2). In control Jurkat cells, stimulation with anti-Fas antibody yielded two apoptotic fragments (89 and 24 kDa), and the original 110-kDa protein decreased during the apoptotic process. Similarly, in IL-3-depleted Ba/F3 cells, the 110-kDa PARP gradually disappeared during the process of apoptosis, and levels of 89- and 24-kDa fragments increased. PARP breakdown was detectable from 6–9 h post-depletion, and most of the 110-kDa PARP had disappeared at 24 h, suggesting that degradation of PARP follows the caspase-3 activation. To verify the generality of these observations, PARP degradation was examined in IL-2-dependent CTL2-2 cells, and we found that IL-2 deprivation also resulted in massive degradation of the PARP protein.

Caspase Family Inhibitors Block Apoptosis—The above results clearly indicated that caspase-3 is activated in response to cytokine deprivation. We next examined whether the activation of caspase-3 is required for the apoptotic process following cytokine depletion or if the proteolytic activity found in dying cell lysates is solely a consequence of dynamic changes in cellular conditions due to cell death. To distinguish between these two possibilities, cytokine-dependent cells were treated with several caspase family inhibitors (Z-VAD, Z-Asp, and YVAD) after cytokine removal. Upon IL-3 deprivation, most of the control Ba/F3 cells died; viability of the cells declined to less than 10% at 24 h after IL-3 deprivation. In contrast, the Z-VAD-treated cells were resistant to IL-3 deprivation, and the apparent viability of the cells was more than 80% at the same time point (Fig. 3), and nearly 50% of cells were still alive even after 3 days incubation without IL-3. The effect of Z-VAD was dose-dependent, and the effective concentration for death suppression was similar to that necessary to inhibit Fas-induced apoptosis in Fas-transfected Ba/F3 cells (Fig. 4A). Another inhibitor, Z-Asp (25), also suppressed apoptosis in a dose-dependent manner, although to a lesser degree. To exclude the possibility that the effects of inhibitors observed in Ba/F3 cells were specific for this cell line, we repeated the same experiment using IL-3-dependent 32D cells as well as IL-2-dependent CTL2-2 cells (Fig. 4B). Although the levels of inhibition in these cells were variable from cell to cell, results were fundamentally reproducible in both cell types. One inhibitor (YVAD) was less effective in inhibiting apoptosis than Z-VAD or Z-Asp in all cell lines tested. However, since YVAD inhibited the caspase-3 activity in vitro at high concentrations (data not shown), the inability of this inhibitor to suppress apoptosis was probably due to inefficient permeability through the cell membrane.

Commitment to Apoptosis Precedes Caspase Family Protease Activation—We further characterized Ba/F3 cells of which survival is maintained by Z-VAD in the absence of IL-3. As shown in Fig. 5A, Z-VAD blocked the fragmentation of chromosomal DNA, which is an early event of apoptosis, while YVAD did not. We also noted that condensation and fragmentation of nuclei were prevented by Z-VAD (data not shown). Furthermore, Z-VAD inhibited the activation of caspase-3 as well as the subsequent PARP breakdown (Fig. 5B and C). Z-Asp also blocked PARP degradation to a lesser extent, correlated with its weak anti-apoptotic potential in Ba/F3 cells. These results indicate that inhibitors are able to block apoptosis at an early stage in the process.

Since the morphology of surviving cells was apparently the same as that of normal cells, we examined whether the surviving cells are able to proliferate in response to readministration of IL-3. After appropriate incubation periods with Z-VAD (6, 12, and 24 h) in the absence of IL-3, Z-VAD was removed, and IL-3 was added back to the culture media. Thereafter, the proliferative potential of the cells was monitored by the MTT assay (Fig. 6). When the cells were treated with Z-VAD for 6 h, most cells proliferated in response to IL-3 in a manner similar to the control, while some cells lost the ability to grow after 12 h of incubation without IL-3 in the presence of Z-VAD. After 24 h, when 80% of the cells were still able to exclude trypan blue (Fig. 3), the cells were no longer able to respond to IL-3 readdition and eventually underwent apoptotic death. This indicated that although Z-VAD blocks all of the features of apoptotic death tested, including DNA fragmentation, caspase-3...
activation, PARP breakdown, and disintegration of the cell membrane, the cells undergo commitment to death if deprived of the appropriate cytokine for 24 h. Next, we analyzed cell cycle status to see whether cells are arrested in a particular cell cycle stage. As shown in Fig. 7, surviving cells after 24 h of depletion in the presence of Z-VAD are arrested in G1 (74.4%) as well as G2/M (16.7%), and few cells are found to be at S phase (8.9%). Readdition of IL-3 hardly promoted cells to enter S phase, indicating that cells surviving in the presence of Z-VAD for 24 h without IL-3 are no longer progressing to S phase by IL-3 readdition. Consistent with this result, IL-3 failed to induce c-Myc expression in these cells (data not shown).

We then attempted to determine the time when cells irreversibly commit to die after IL-3 withdrawal (Fig. 8). In this experiment, the cells were first deprived of IL-3 for several hours as indicated in the Fig. 8, and then IL-3 or inhibitors (Z-VAD and Z-Asp) were added to the culture media. The cell viability after 24 h of incubation was then measured by the dye exclusion assay. The addition of both IL-3 and inhibitors before first –5 h of IL-3 deprivation effectively rescued the cells from apoptosis; while the recovery rate markedly declined from 8 through 12 h, suggesting that the cellular commitment to apoptosis occurs around this time.

**DISCUSSION**

Growth-promoting or survival cytokines activate the signal transduction pathways responsible for cell survival (5). In turn, withdrawal of these cytokines results in rapid inactivation of these pathways. Apoptosis following cytokine deprivation usually includes chromosomal DNA breakdown and fragmentation of the nucleus (1). Such characteristics are commonly observed in most other types of apoptosis in which various death proteins are involved (19, 33–35). Nevertheless, apoptosis induced by cytokine depletion has been thought to take place simply by
shutting down the survival signaling pathways upon cytokine removal, and its molecular mechanism remains unknown. In this study, we have demonstrated that cytokine withdrawal induces the activation of a death protease cascade. Determination of enzymatic activity of caspase-3/CPP32 using a fluorescent peptide substrate (DEVD-MCA) showed that caspase-3 was activated by cytokine depletion in both IL-3- and IL-2-dependent cell lines. In cells cultured with caspase family inhibitors (Z-VAD or Z-Asp), no activation or very little activation of caspase-3 was detected. In contrast, YVAD, which was not anti-apoptotic in Ba/F3 cells, was unable to block the activation of caspase-3 in this cell line. Furthermore, we found that PARP was degraded during the apoptotic process induced by cytokine deprivation (Fig. 2). We therefore conclude that the common death protease cascade is activated after cytokine deprivation. Enari et al. (22) reported that caspase-1/ICE-like activity (which cleaves YVAD-MCA) increased prior to the activation of caspase-3 upon Fas stimulation. We tested whether the caspase-1-like activity was up-regulated before caspase-3 activation. However, its activity in cytokine-dependent cells used in this study was hardly detectable or fluctuated mark-

**Fig. 6.** Proliferative potentials of cells surviving in the presence of Z-VAD without IL-3. Ba/F3 cells were cultured in the presence of Z-VAD (400 µg/ml) for 6, 12, or 24 h without IL-3. At each time point, the cells were washed with factor-free medium to remove the inhibitor, and IL-3 was then readded to the media. Twenty-four hours later, proliferative responses to various concentrations of IL-3 were examined by the MTT assay. Control growing cells were incubated without IL-3 for 6 h and then restimulated with IL-3 to elicit a maximal response. Note that all of the cells lost their capability to show the proliferative response after 24 h of incubation without IL-3.

**Fig. 7.** Cell cycle analysis after IL-3 readdition. Ba/F3 cells were deprived of IL-3 in the presence of Z-VAD for 24 h (C), and then IL-3 was added back. Sixteen hours after the IL-3 addition, cells were stained with PI and analyzed by FACSscan (D). As a positive control (A), Ba/F3 was depleted of IL-3 for 6 h, and the cell cycle was analyzed at 16 h after IL-3 readdition. D shows the cells after 24 h of IL-3 deprivation.

**Fig. 8.** Kinetics of the commitment to apoptosis after IL-3 deprivation. Ba/F3 cells were deprived of IL-3 and incubated for various periods. Then either IL-3 or Z-VAD (400 µg/ml) was added to the culture media, and the viability of the cells was measured after 24 h of incubation.
edly from assay to assay. Probably, other members of the caspase family proteases, such as TX/ICH-2 (caspase-4) (36) that have similar substrate preferences are involved in cytokine deprivation-induced apoptosis rather than caspase-1 itself. It would be important to identify such proteases that is activated before caspase-3 activation. Vasilakos et al. (26) previously reported that the caspase-like protease is not involved in IL-2-deprived-induced cell death. However, we observed caspase-3 activation and PARP degradation in CTL2-2 cells following IL-2 deprivation (Figs. 1C and 2). In addition, treatment of CTL2-2 with the peptide inhibitors after IL-2 removal significantly inhibited apoptosis, extending our conclusion about IL-3-dependent cells to IL-2-dependent cells.

The activation of caspase-3 appears to be indispensable for the apoptotic process, since the addition of membrane-permeable caspase family inhibitors to IL-3-deprived Ba/F3 cells markedly inhibited apoptosis. Moreover, other cell lines, IL-3-dependent 32D cells and IL-2-dependent CTL2-2 cells, were also rescued from apoptosis by the same inhibitors after cytokine deprivation, although the level of inhibition was less than that in Ba/F3 cells. These results indicate that caspase family proteases are critically involved in the process of apoptosis following cytokine deprivation.

Kinetic analyses shown in Fig. 6 demonstrate that the activation of caspase-3 occurs after irreversible commitment to cell death. Even when the apparent viability of cells was maintained >80% (24 h postdepletion) by Z-VAD, readministration of IL-3 (simultaneous removal of Z-VAD) did not restore the proliferative capability and did not allow the progression from death. Even when the apparent viability of cells was maintained (24 h postdepletion) by Z-VAD, readministration of IL-3 did not restore the proliferative capability and did not allow the progression from death. Even when the apparent viability of cells was maintained.

Death Protease Activation upon Cytokine Withdrawal

Acknowledgments—We thank Drs. N. Fujita, T. Mashima, and Y. Ito for helpful comments regarding this work. Also, we are grateful to Dr. S. Yonehara for providing the anti-Fas antibody and Dr. H. Kawai (Kirin Brewery Co. Ltd.) for synthesizing Z-VAD.

REFERENCES
1. Allen, P. D., Bustin, S. A., and Newland, A. C. (1993) Blood Rev. 7, 63–73
2. Arii, K., Lee, F., Miyajima, A., Miyatake, S., Ariai, N., and Yokota, T. (1990) Biochem. J. 270, 783–836
3. Hunter, T. (1997) Cell 88, 333–346
4. Anderson, M. W., Reynolds, S. H., You, M., and Maronpot, R. M. (1992) Environ. Health Perspect. 98, 13–24
5. Kinosita, T., Yokota, T., Ariai, K., and Miyajima, A. (1995) EMBO J. 14, 266–275
6. Andrea, K., Pablos, R., Eugen, U., Christopher, G., Paul, C., Julian, D., and Gerard, E. (1997) Nature 385, 544–548
7. Kinosita, T., Shirouzu, M., Kaniwa, A., Hashimoto, K., Yokoyama, S., and Miyajima, A. (1997) Oncogene 14, in press
8. Yao, R., and Cooper, G. M. (1995) Science 267, 2003–2006
9. Dulek, D., Dutta, R. D., Franke, H., and Birnbaum, M. J. Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Science 275, 661–665
10. Kaufmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Cofer, P., Downward, J., and Evan, G. (1997) Nature 385, 544–548
11. Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellaocca, A., Tischll, P. N., and Hay, N. (1997) Genes Dev. 11, 701–713
12. Kulik, G., Klippel, A., and Deppert, W. J. (1997) Mol. Cell. Biol. 17, 1595–1606
13. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H., and Downward, J. (1997) EMBO J. 16, 2783–2789
14. Hale, A. J., Smith, C. A., Sutherland, L. C., Stoneman, V. E., Longthorne, V. L., Culhane, A. C., and Williams, G. T. (1996) Eur. J. Biochem. 236, 1–26
15. Nagata, S., and Golstein, P. (1995) Science 269, 1449–1456
16. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993) Nature 362, 847–849
17. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. (1993) Nature 362, 849–852
18. Tamura, T., Ishihara, M., Lapphier, M. S., Tanaka, N., Dohi, I., Aizawa, S., Matsuyama, T., Mak, T. W., Taki, S., and Taniguchi, T. (1995) Nature 376, 596–599
19. Kumar, S. (1995) Trends Biochem. Sci. 20, 198–202
20. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) Cell 87, 171
21. Hengartner, M. O., and Horvitz, H. R. (1994) Curr. Opin. Genet. Dev. 4, 581–586
22. Enari, M., Hug, H., and Nagata, S. (1995) Nature 375, 78–81
23. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Garreau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raja, S., Smeal, T. M., Yamin, T.-Y., Yu, V. L., and Miller, D. K. (1995) Nature 376, 37–43
24. Tewari, M., and Dixit, V. M. (1995) J. Biol. Chem. 270, 3255–3260
25. Mashima, T., Naito, M., Kataoka, S., Kawai, H., and Tsuruo, T. (1995) Biochem. Biophys. Res. Commun. 209, 967–971
26. Vasilakos, J. P., Ghayur, T., Carroll, R. T., Giegel, D. A., Saunders, D. A., Saunders, J. M., Quintal, L., Keane, K. M., and Shivers, B. D. (1995) J. Immunol. 155, 3433–3442
27. Yonehara, S., Ishii, A., and Yonehara, M. (1989) J. Exp. Med. 169, 1747–1756
28. Ikemizu, M., Noguchi, S., Yamasaki, N., Sugihara, T., and Miwa, M. (1990) Biochem. Biophys. Res. Commun. 163, 739–745
29. Miyajima, A., Schreurs, J., Otsu, K., Kondo, A., and Maeda, S. (1987) Gene (Amst.) 68, 273–281
30. Meisner, T. (1993) J. Immunol. Methods 55, 55–63
31. Kinosita, T., Yokota, T., Ariai, K., and Miyajima, A. (1995) Oncogene 10, 2207–2212
32. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Nature 371, 346–347
33. Warronick, J. D., Calnan, B., Ngo, V., and Winoto, A. (1994) Nature 367, 277–281
34. Liu, Z. G., Smith, S. W., McLaughlin, K. A., Schwartz, L. M., and Osborne, B. A. (1994) Nature 367, 281–284
35. Ju, S. T., Panka, D. J. C., Hui, H., Rittering, R., el-Khatib, M., Sherr, D. H., Stanger, B. Z., and Marshak-Rothstein, A. (1995) Nature 373, 444–448
36. Faucheu, C., Duan, A. C., Blanchet, A. M., Miossec, C., Herve, F., Collard-Dutilleul, V., Gu, Y., Aldape, R. A., Lippke, J. A., Rocher, C., Su, M. S.-S., Livingston, D. J., Herend, T., and Lalanne, J.-L. (1995) EMBO J. 14, 1914–1922
37. Muzio, M., Chinnaiyan, A. M., Khocheki, F. C., O’Rourke, K., Shevchenko, A., Ni, J., Seffiedi, C., Breit, J. D., Zhang, M., Gentz, R., Mann, K., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
38. Ishida, Y., Agata, Y., Shibahara, K., and Honjo, T. (1992) EMBO J. 11, 3887–3895
39. Whetton, A. D., Bazil, G. W., and Dexter, T. M. (1984) EMBO J. 3, 409–413

2 T. Ohta, unpublished observations.