Intracellular Calcium Stores Are Not Required for Bcl-2-mediated Protection from Apoptosis*

(Received for publication, July 12, 1996, and in revised form, August 5, 1996)

Jason E. Reynolds§ and Alan Eastman§

From the Department of Pharmacology, Dartmouth Medical School, Hanover, New Hampshire 03755

The ability of Bcl-2 to inhibit cell death is well documented but its mechanism of action remains elusive. Recent reports have suggested that Bcl-2 prevents apoptosis by inhibiting the release of Ca\textsuperscript{2+} from the thapsigargin-sensitive Ca\textsuperscript{2+} store. The mobilization of Ca\textsuperscript{2+} from this store has been implicated as a signal regulating apoptotic cell death induced by glucocorticoids and by interleukin-3 withdrawal. The present study was designed to determine if Bcl-2 would still inhibit apoptosis after depletion of intracellular Ca\textsuperscript{2+} stores. We compared the response of two Chinese hamster ovary cell lines (5AHSmyc and 5A300bcl-2.2) following incubation with the calcium ionophore ionomycin to deplete intracellular Ca\textsuperscript{2+} stores. Continued incubation of 5AHSmyc cells in calcium-free media induced substantial apoptotic DNA fragmentation within 4 h and >95% loss of viability within 48 h. However, 5A300bcl-2.2 cells showed no evidence of DNA fragmentation or loss of viability over the same time period. Intracellular Ca\textsuperscript{2+} was analyzed with the Ca\textsuperscript{2+}-sensitive fluorescent dye INDO-1 and confirmed that ionomycin was capable of releasing Ca\textsuperscript{2+} from intracellular stores in both cell lines. These results show that depletion of intracellular Ca\textsuperscript{2+} stores induces apoptosis and that these Ca\textsuperscript{2+} stores are not required for the protection afforded by Bcl-2.

Apoptotic cell death is characterized morphologically by cell shrinkage, cytoplasmic vacuolization, and nuclear chromatin condensation (1, 2). During apoptosis, specific proteolytic and nucleolytic activities occur prior to loss of membrane integrity (3–5). Disruption of the signals that normally regulate apoptosis contributes to the pathogenesis of many human diseases (6). Despite the significance of apoptosis, its regulation remains poorly understood. Apoptosis is initiated by many insults which activate multiple pathways that converge on a common final pathway, sometimes called the execution phase (7, 8). Bcl-2 inhibits apoptosis induced by multiple insults suggesting that it acts at, or close to, the execution phase. A family of Bcl-2-related proteins has been identified; these proteins form homodimers or heterodimers, and the consequence can be either protection or enhanced apoptosis (9, 10).

The mechanism of action of the Bcl-2 family of proteins remains elusive. It has been suggested that Bcl-2 inhibits apoptosis by acting as an antioxidant (11, 12). However, recent reports have demonstrated that Bcl-2 inhibits apoptosis when oxygen is dramatically reduced, and from insults that are not responsive to other antioxidants (13–15). Hence, Bcl-2 action is far broader than just an antioxidant.

A commonly implicated signal in the execution phase of apoptosis and a potential site for Bcl-2 action is the regulation of intracellular calcium levels. Many reports have suggested that elevations in intracellular free calcium correlate with apoptosis (16–18). In contrast, previous work in our laboratory has suggested that increased intracellular free Ca\textsuperscript{2+} does not correlate with apoptosis (19–22). Furthermore, we have shown that Bcl-2 protects cells from staurosporine-induced apoptosis, yet does not suppress the staurosporine-induced increase in intracellular free Ca\textsuperscript{2+} (21). However, these reports have not ruled out the possibility that mobilization of intracellular Ca\textsuperscript{2+} stores may be a key regulator of apoptosis, nor do they exclude the possibility that regulation of intracellular Ca\textsuperscript{2+} stores may be a target of Bcl-2 function. The thapsigargin-sensitive Ca\textsuperscript{2+} store has been implicated in regulating apoptosis (23, 24). Thapsigargin is a selective inhibitor of the endoplasmic reticulum-associated Ca\textsuperscript{2+}-ATPase which pumps Ca\textsuperscript{2+} against a concentration gradient into the endoplasmic reticulum. Inhibition of this pump by thapsigargin induces a transient increase in cytoplasmic free Ca\textsuperscript{2+} as it leaves the endoplasmic reticulum down its concentration gradient. The mobilization of Ca\textsuperscript{2+} during apoptosis is suggested by the observation that cells induced to undergo apoptosis by incubation with glucocorticoids, hydrogen peroxide, or by withdrawal of interleukin-3, have reduced levels of Ca\textsuperscript{2+} in their thapsigargin-sensitive Ca\textsuperscript{2+} stores (23–25). It has also been shown that Bcl-2 prevents this loss of Ca\textsuperscript{2+} from these stores and blocks thapsigargin-induced apoptosis (23, 25, 26). This has led to the hypothesis that Bcl-2 prevents apoptosis by regulating the homeostasis of intracellular calcium.

In the present study, we examined the ability of Bcl-2 to inhibit apoptosis following depletion of intracellular calcium stores using the calcium ionophore, ionomycin. We show that treatment of Chinese hamster ovary (CHO) cells with ionomycin effectively depleted the thapsigargin-sensitive calcium store. This emptying of intracellular calcium stores also induced apoptosis. Overexpression of Bcl-2 inhibited this apoptosis without affecting the ability of either thapsigargin or ionomycin to mobilize stored calcium. These results demonstrate that, while depletion of intracellular calcium stores may occur during apoptosis, the ability of Bcl-2 to inhibit apoptosis is independent of intracellular calcium.

* This work was supported in part by National Institutes of Health Grant CA50224. Analysis of intracellular calcium was performed in the Herbert C. Englert Cell Analysis Laboratory, supported in part by Cancer Center Core Grant CA23108. 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.

† Supported by a Pharmaceutical Research and Manufacturers Association Medical Student fellowship.

§ To whom correspondence and reprint requests should be addressed. Tel.: 603-650-1501; Fax: 603-650-1129.

1 The abbreviations used are: CHO, Chinese hamster ovary; AM, acetoxymethyl ester; BSA, bovine serum albumin; MEM, minimal essential medium.
Experimental Procedures

Chemicals—Ionomycin and thapsigargin were purchased from Calbiochem (La Jolla, CA). Each chemical was dissolved in dimethyl sulfoxide and stored as either a 5 mM or 150 μM stock solution, respectively. INDO-1 AM was purchased from Molecular Probes, Inc. (Eugene, OR). Pluronic F127, EGTA, bovine serum albumin, and non-essential amino acids were purchased from Sigma. α-MEM, S-MEM, fetal bovine serum, horse serum, and penicillin/streptomycin were purchased from Life Technologies, Inc. (Grand Island, NY).

Cell Culture—The CHO cell lines 5AHSmyc and 5A300bcl-2.2 were cultured as described previously (27). Briefly, the 5AHSmyc cells are CHO cells which express a cDNA encoding the human c-Myc proto-oncogene; expression of this gene is controlled by a Drosophila heat shock promoter (28). We have previously shown that unless the cells are subjected to heat, no c-Myc protein can be detected (27). The 5A300bcl-2.2 cells were generated by transfecting 5AHSmyc cells with a cDNA encoding the human bcl-2 gene (29). Both cell lines were grown in α-MEM supplemented with 2.5% fetal bovine serum, 2.5% horse serum, and penicillin/streptomycin.

Intracellular Calcium—Intracellular calcium was measured using a Meridian Ultima laser confocal microscope (Okemos, MI) and the calcium-sensitive fluorescent dye INDO-1 (30). CHO cells were grown overnight on a glass coverslip, then loaded with INDO-1 AM (12.5 μM) in serum-free S-MEM containing 0.025% Pluronic F127 and 0.5% BSA for 30–60 min at 37 °C. The cells were then washed three times with serum-free S-MEM (which contains no calcium) and maintained in S-MEM during analysis. INDO-1 was excited at 355 nm and emission intensities measured at 405 and 480 nm using a pinhole aperture setting of 1600 μM. The ratio of 405/480 nm fluorescence is proportional to the amount of intracellular free calcium. Over the analysis period (5 min), ionomycin and thapsigargin were added sequentially to the cells and the corresponding change in 405/480 nm ratio was measured. For each experiment, there were between 4 and 9 cells/field.

Calcium Depletion and DNA Fragmentation—5AHSmyc and 5A300bcl-2.2 cells were grown overnight in complete α-MEM. To deplete the intracellular calcium stores, the cells were washed with S-MEM, then incubated in S-MEM containing 10 μM ionomycin and 500 μM EGTA for 30 min at 37 °C. The cells were then washed with S-MEM prior to incubating for an additional 1–6 h in S-MEM supplemented with alanine (5 g/liter), aspartate (10 g/liter), glutamate (15 g/liter), proline (8 g/liter), and 0.1% BSA. At selected times, 10^6 cells were harvested by scraping, and DNA integrity was analyzed by agarose gel electrophoresis method detailed elsewhere (31, 32). Briefly, the cells were added directly to the wells of a 2% agarose gel where they were lysed and digested with ribonuclease A and proteinase K. The gel was electrophoresed for 16 h and the DNA was visualized with ethidium bromide. High molecular weight DNA remains near the top of the gel while smaller fragments down to 180 base pairs in length are resolved in the gel.

Cell Viability Assay—3–5 × 10^5 cells were plated in 6-well dishes and allowed to attach overnight. The cells were then depleted of intracellular calcium as described above. Following calcium depletion, the cells were washed and incubated for 6–48 h in S-MEM supplemented with amino acids and BSA as above. At harvest, the cells floating in the media were collected, the adherent cells were trypsinized, and all of the cells were pooled, pelleted, and resuspended in 1 ml of phosphate-buffered saline. A 10-μl aliquot was assayed for viability as determined by the ability of the cells to exclude trypan blue.

Results

Analysis of Intracellular Ca^{2+} Stores in CHO Cells—The calcium ionophore, ionomycin, has been shown to release Ca^{2+} from the thapsigargin-sensitive intracellular stores in thymic lymphocytes and mouse lymphoma cells (24, 33). However, its ability to deplete the thapsigargin-sensitive Ca^{2+} stores in CHO cells has not been studied. Therefore, ionomycin (10 μM) and thapsigargin (150 nM) were added sequentially to 5AHSmyc and 5A300bcl-2.2 cells and the resulting change in intracellular Ca^{2+} monitored using a laser confocal imaging system with the Ca^{2+}-sensitive fluorescent probe INDO-1; the ratio of emission intensities at 405 and 480 nm is proportional to intracellular Ca^{2+}. Upon addition of thapsigargin to 5AHSmyc cells, there was a rise in intracellular free Ca^{2+} that peaked after 2 min and declined to baseline again by 5 min (Fig. 1A).
Subsequent incubation with ionomycin caused only a small transient increase in intracellular free Ca\(^{2+}\), probably from mitochondrial stores that are also known to be depleted by ionomycin (34). Hence, the bulk of the intracellular Ca\(^{2+}\) stores were sensitive to thapsigargin. When the order of addition was reversed, ionomycin caused a much more rapid increase in intracellular free Ca\(^{2+}\) that declined to baseline within 2 min (Fig. 1B). This rapid recovery observed with ionomycin treatment is explained by the additional permeation of the cytoplasmic membrane which facilitates rapid exit of the released Ca\(^{2+}\). When thapsigargin was added after ionomycin, no additional release of Ca\(^{2+}\) was observed demonstrating that ionomycin effectively depleted the thapsigargin-sensitive store.

When the 5A300bcl-2.2 cells were incubated with thapsigargin or ionomycin, similar results were obtained as in the 5AHSmyc cells, although the magnitude of Ca\(^{2+}\) release was slightly higher in each case (Fig. 1, C and D). As observed in the 5AHSmyc cells, pretreatment of Bcl-2-expressing cells with ionomycin abolished the thapsigargin-induced increase in intracellular free Ca\(^{2+}\). Furthermore, incubation of these cells with ionomycin following thapsigargin also showed the expected depletion of mitochondrial Ca\(^{2+}\) stores. These results confirm that ionomycin can be used to deplete both mitochondrial and endoplasmic reticulum Ca\(^{2+}\) stores in CHO cells, and thereby defines a model in which we can examine the effect of completely depleting intracellular Ca\(^{2+}\), both free and stored, on the ability of Bcl-2 to inhibit apoptosis.

**DNA Digestion**—Having shown that ionomycin can be used to deplete the intracellular Ca\(^{2+}\) stores in CHO cells, we next determined the effect of depleted Ca\(^{2+}\) on the induction of apoptosis. One feature commonly associated with apoptosis is the digestion of genomic DNA into oligonucleosome-sized fragments. Following depletion of intracellular Ca\(^{2+}\), cells were incubated in Ca\(^{2+}\)-free medium for up to 6 h and DNA integrity was assessed (Fig. 2A). In 5AHSmyc cells, DNA fragmentation was clearly visible by 3 h, and maximum by 4 h. This DNA fragmentation was dependent upon depletion in intracellular Ca\(^{2+}\) stores, as incubation for the same time period in Ca\(^{2+}\)-free medium but without prior Ca\(^{2+}\) depletion did not result in DNA fragmentation (data not shown). In contrast, depletion of intracellular Ca\(^{2+}\) in 5A300bcl-2.2 cells showed no DNA fragmentation during the 6-h time course. These results demonstrate that depletion of intracellular Ca\(^{2+}\) is sufficient to induce apoptosis in CHO cells and that Bcl-2 can prevent DNA digestion even after these stores have been depleted.

Following depletion of intracellular Ca\(^{2+}\), the cells were incubated in Ca\(^{2+}\)-free medium to prevent refilling of the stores after removal of ionomycin. When the cells were returned to Ca\(^{2+}\)-containing α-MEM immediately after the depletion protocol, DNA digestion was prevented (Fig. 2B). Readdition of Ca\(^{2+}\) after 3 h in Ca\(^{2+}\)-free medium also prevented any further DNA digestion. These results suggest that the continued incubation in Ca\(^{2+}\)-free media is required to induce DNA digestion, presumably by preventing the reuptake of Ca\(^{2+}\).

**Long-term Protection by Bcl-2**—To determine if the ability of Bcl-2 to prevent DNA digestion at 6 h would translate into long term protection of the cells, we measured trypan blue exclusion for 48 h following depletion of intracellular Ca\(^{2+}\) (Fig. 3). The 5AHSmyc cells retained membrane integrity at 6 h even though their DNA was extensively fragmented; this is consistent with the definition of apoptosis in that DNA digestion occurs before loss of membrane integrity. However, by 12 h about 15% of the cells had lost membrane integrity, and continued incubation in Ca\(^{2+}\)-free medium resulted in dramatic loss of membrane integrity with less than 10% of the cells viable by 48 h. For comparison, 5AHSmyc cells were incubated in Ca\(^{2+}\)-free medium for up to 48 h but without the initial Ca\(^{2+}\) depletion. Under these circumstances, the cells did not begin to lose membrane integrity until 24 h, but by 48 h they also showed less than 10% viability. In each of these conditions, the medium contained 0.1% BSA, but lacked serum to avoid any potential contribution from serum Ca\(^{2+}\). To assess the effect of serum withdrawal on viability, 5AHSmyc cells were incubated in Ca\(^{2+}\)-containing medium. This condition also resulted in loss of membrane integrity by 24 h, although about 50% remained viable by 48 h. These results show that loss of cell viability over
short time periods resulted from Ca\(^{2+}\) depletion, but at longer time periods, loss of survival factors in serum also contributed significantly to cell death.

The 5A300bcl-2.2 cells were incubated under identical conditions as the 5AHSmyc cells. The cells remained >90% viable for at least 48 h under all three incubation conditions; that is, Bcl-2 protected the cells from Ca\(^{2+}\) depletion and subsequent growth in the absence of Ca\(^{2+}\) and serum. Hence, these results show that Ca\(^{2+}\) is not required for Bcl-2 to provide long-term protection.

DISCUSSION

The work presented here provides evidence that depletion of intracellular Ca\(^{2+}\) stores induces apoptosis in CHO cells. This is in agreement with previously published work showing that thapsigargin induces apoptosis in a variety of cell types (26, 35, 36). However, the previous work was performed in the presence of extracellular Ca\(^{2+}\), hence apoptosis could have been induced by the resulting influx of Ca\(^{2+}\). In the present study, no influx of Ca\(^{2+}\) could occur, so it must be the absence of Ca\(^{2+}\) that is responsible for inducing apoptosis. A similar conclusion can be drawn from the observation that calcium chelators can induce apoptosis (22). A decrease in intracellular Ca\(^{2+}\) has also been suggested as a cause of apoptosis following growth factor withdrawal, while incubation with ionomycin prevented this Ca\(^{2+}\) decrease and protected the cells (37). On the other hand, increases in Ca\(^{2+}\) have frequently been associated with apoptosis, and in some circumstances, it has been possible to protect cells by preventing this increase (38, 39). In some instances, preventing the increase in intracellular free Ca\(^{2+}\) does not protect cells (20), while there are other cases in which no increase in intracellular free Ca\(^{2+}\) was observed (19).

Much of the interest in intracellular Ca\(^{2+}\) has arisen from the suggestion that DNA is degraded by a Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease (16, 40, 41). However, the fact that DNA digestion does not correlate with the intracellular Ca\(^{2+}\) level, and that DNA digestion occurs in the complete absence of intracellular Ca\(^{2+}\), seriously questions the role of such an endonuclease in apoptosis. Furthermore, recent experiments that reconstitute apoptosis in vitro have found that Ca\(^{2+}\) is not required for DNA digestion (42, 43). It is possible that other endonucleases are involved under different circumstances. However, even former proponents of the Ca\(^{2+}\)-dependent endonuclease have now suggested that Ca\(^{2+}\) may only be involved in the induction phase of some apoptotic signaling pathways, and may not act as a direct mediator of DNA digestion or at any other step in the execution phase (44).

Although increases in intracellular free Ca\(^{2+}\) are not required for apoptosis, changes in Ca\(^{2+}\) compartmentalization could still be important. This possibility led to investigation of the ability of Bcl-2 to regulate intracellular Ca\(^{2+}\) stores. Specifically, thapsigargin-induced apoptosis can be inhibited by Bcl-2, and it was suggested that this is due to inhibition of capacitative Ca\(^{2+}\) entry at the cytoplasmic membrane (26). It has also been reported that Bcl-2 prevents depletion of thapsigargin-sensitive Ca\(^{2+}\) stores during apoptosis (29). Those results suggested that Bcl-2 regulates apoptosis by regulating intracellular Ca\(^{2+}\) stores. To directly test this hypothesis, we decided to deplete intracellular Ca\(^{2+}\) stores and assess whether Bcl-2 would no longer be able to protect cells. The experimental protocol required that intracellular free Ca\(^{2+}\) also be depleted to ensure that the Ca\(^{2+}\) stores were not refilled. Hence, these experiments effectively questioned whether Bcl-2 could still protect cells in the absence of both free and stored Ca\(^{2+}\).

Initially, we confirmed that ionomycin depleted the thapsigargin-sensitive Ca\(^{2+}\) store in CHO cells as has been established for other cells (24, 30). Additionally, we confirmed that Bcl-2 did not block the ability of either thapsigargin or ionomycin to deplete intracellular Ca\(^{2+}\) stores. Since ionomycin has been reported to deplete both endoplasmic reticulum and mitochondrial Ca\(^{2+}\) (34), we conclude that Bcl-2 does not inhibit the release of Ca\(^{2+}\) from either of these stores. As discussed above, this depletion of intracellular Ca\(^{2+}\) induced apoptosis. The important observation made here is that Bcl-2 still prevented apoptosis in the absence of intracellular Ca\(^{2+}\) stores. Accordingly, we conclude that the protective action of Bcl-2 is independent of changes in intracellular Ca\(^{2+}\) stores.

The previous reports that Ca\(^{2+}\) was depleted from thapsigargin-sensitive stores during apoptosis (23, 25, 26) can presumably be explained as a consequence rather than a cause of apoptosis. Hence, Bcl-2 prevented the depletion of these stores as a consequence of its ability to prevent apoptosis. The question of how Bcl-2 functions remains elusive. In the Introduction, we explained why Bcl-2 is unlikely to act as a general antioxidant as was previously suggested (11, 12). Bcl-2 has also been reported to function by influencing intracellular trafficking of the p53 tumor suppressor protein (45), yet Bcl-2 protects cells that are mutant for p53 such as the CHO cells used here. Recently, it has been shown that Bcl-2 inhibits the activation of interleukin 1β converting enzyme-like proteases that are essential components of the execution phase of apoptosis (46), yet this only adds to the list of events inhibited by Bcl-2 and does not imply any direct interaction. In related experiments, we have shown that Bcl-2 prevents intracellular acidification that always occurs during apoptosis (21). Intracel-
Bcl-2 Prevents Apoptosis in the Absence of Calcium

lular pH is known to be regulated by extracellular signals and intracellular protein kinase cascades that also prevent apoptosis. We have reported that the retinoblastoma susceptibility protein Rb is dephosphorylated during apoptosis reflecting an imbalance between these same protein kinases and their related protein phosphatases (47). Subsequently, we have shown that Bcl-2 prevents the dephosphorylation of Rb, and that this occurs upstream of activation of interleukin 1β converting enzyme-like proteases, thereby confirming the existence of events between Bcl-2 and the activation of interleukin 1β converting enzyme-like proteases. Other work has suggested that Bcl-2 action might be mediated through its interaction with protein Rb is dephosphorylated during apoptosis reflecting an imbalance between these same protein kinases and their re-

REFERENCES
1. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
2. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) Int. Rev. Cytol. 68, 251–296
3. Wyllie, A. H. (1980) Nature 284, 555–556
4. Eastman, A., and Barry, M. A. (1992) Cancer Invest. 10, 229–240
5. Martin, S. J., and Green, D. R. (1995) Cell 82, 348–352
6. Thompson, C. B. (1995) Science 269, 1456–1462
7. Eastman, A. (1993) Toxicol. Applied Pharmacol. 121, 160–164
8. Earnshaw, W. C. (1995) Trends Cell Biol. 5, 217–220
9. Distelhorst, C. W., Lam, M., and McCormick, T. S. (1996) Oncogene 12, 2051–2055
10. Lam, M., Dubyk, G., and Distelhorst, C. W. (1993) Mol. Endocr. 7, 686–693
11. Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L., and Korsmeyer, S. J. (1993) Cell 79, 189–192
12. Sedin, L. W., Olsvik, Z. N., Yang, E., Wang, K., Buis, L. H., and Korsmeyer, S. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7834–7838
13. Hockenbery, D. M., Olsvik, Z. N., Yin, X.-M., Milliman, C. L., and Korsmeyer, S. J. (1993) Cell 75, 241–251
14. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T., and Bredesen, D. E. (1993) Science 259, 1274–1277
15. Muschel, R. J., Bernhard, E. J., Garza, L., McKenna, W. G., and Koch, C. J. (1995) Cancer Res. 55, 995–998
16. Shinmura, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1995) Nature 374, 811–813
17. Jacobson, M. D., and Raff, M. C. (1995) Nature 374, 814–816
18. McConkey, D. J., Hartzell, P., Duddy, S. K., Hakanson, H., and Orrenius, S. (1988) Science 242, 256–259
19. Jones, D. P., McConkey, D. J., Nicotera, P., and Orrenius, S. (1989) J. Biol. Chem. 264, 6396–6403
20. Martikainen, P., Kyriakos, N., Tucker, R. W., and Isaacs, J. T. (1991) Cancer Res. 51, 4693–4700
21. Barry, M. A., Reynolds, J. E., and Eastman, A. (1993) Cancer Res. 53, 2349–2357
22. Li, J., and Eastman, A. (1995) J. Biol. Chem. 270, 3203–3211
23. Reynolds, J. E., Li, J., Craig, R. W., and Eastman, A. (1996) Exp. Cell Res. 225, 430–436
24. Kluck, R. M., McDougall, C. A., Harmon, B. V., and Halliday, J. W. (1994) Biochim. Biophys. Acta 1223, 247–254
25. Baffy, G., Miyashita, T., Williamson, J. R., and Reed, J. C. (1993) J. Biol. Chem. 268, 6511–6519
26. Reynolds, J. E., Yang, T., Qian, L., Jenkinson, J. D., Zhou, P., Eastman, A., and Craig, R. W. (1994) Cancer Res. 54, 6348–6352
27. Distelhorst, C. W., Lam, M., and McCormick, T. S. (1996) Oncogene 12, 8051–8055
28. Burm, F. M., Gwinn, K. A., and Kingston, R. E. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5414–5418
29. Bissonette, R. P., Echeverri, F., Mahboubi, A., and Green, D. R. (1992) Nature 359, 552–554
30. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
31. Barry, M. A. and Eastman, A. (1993) Arch. Biochem. Biophys. 300, 440–450
32. Eastman, A. (1995) Methods Cell Biol. 46, 41–55
33. Mason, M. J., and Grinstein, S. (1993) Biochem. J. 296, 33–39
34. St. Bird, G. J., Obie, J. F., and Putney, J. W., Jr. (1992) J. Biol. Chem. 267, 18382–18386
35. Kaneko, Y., and Tsulamato, A. (1994) Cancer Lett. 97, 147–155
36. Jiang, S., Chow, S. C., Nicotera, P., and Orrenius, S. (1994) Exp. Cell Res. 212, 84–92
37. Rodriguez-Tarduey, G., Collins, M., and Lopez-Rivas, A. (1990) EMBO J. 9, 2997–3002
38. McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H., and Orrenius, S. (1989) Arch. Biochem. Biophys. 269, 365–370
39. McConkey, D. J., Chow, S. C., Orrenius, S., and Jondal, M. (1990) FASEB J. 4, 2681–2684
40. Nknokova, L. V., Nelipovich, P. A., and Umasky, S. R. (1992) Biochim. Biophys. Acta 699, 281–289
41. Cohen, J. J., and Duke, R. C. (1984) J. Immunol. 132, 38–42
42. Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G., and Earnshaw, W. C. (1993) J. Cell Biol. 123, 7–22
43. Newmeyer, D. D., Farschon, D. M., and Reed, J. C. (1994) Cell 79, 353–364
44. Zhivotovsky, B., Nicotera, P., Bellomo, G., Hanson, K., and Orrenius, S. (1993) Exp. Cell Res. 207, 163–170
45. Ryan, J. J., Prochownik, E., Gottleib, C. A., Apel, I. J., Merino, R., Nunez, G., and Clarke, M. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5878–5882
46. Chinnaiyan, A. M., Orth, K., O'Rourke, K., Duan, H., Poirier, G. G., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4573–4576
47. Morana, S., Wolf, C. M., Li, J., Reynolds, J. E., Brown, M. K., and Eastman, A. (1996) J. Biol. Chem. 271, 18263–18271
48. Fernandez-Sarabia, M. J., and Bischoff, J. R. (1993) Nature 366, 274–275
49. Wang, H.-G., Miyashita, T., Takayama, S., Sato, T., Torjoke, T., Krajewski, S., Tanaka, S., Howey, L., Troppmair, J., Rupp, U. R., and Reed, J. C. (1994) Oncogene 9, 2751–2756
50. Eastman, A. (1995) Semin. Cancer Biol. 6, 45–52
51. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1326–1331
52. Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q., and Hilbrook, N. J. (1996) J. Biol. Chem. 271, 4138–4142

2 C. M. Wolf, J. M. Reynolds, S. J. Morana, and A. Eastman, submitted for publication.