Calcium is involved in several steps of the apoptotic process. In nuclei, endonucleases are presumed to be the main targets of calcium; however, little is known about its role during the cytosolic phase of apoptosis. We used a cell-free system to address this question. Our results show that CaCl₂ triggered nuclear apoptosis (i.e. typical morphological change and DNA fragmentation) at concentrations of 5 mM. This concentration was lowered 10-fold by the co-incubation with cytosolic extracts from nonapoptotic cells. Apoptotic changes induced by the incubation of nuclei with CaCl₂ in the presence of these cytosols were strongly reduced in the presence of an inhibitor of caspase-3 and to a lesser extent by an inhibitor of caspase-1. We also show that calcium-induced apoptosis is affected by protease inhibitors such as N-tosyl-L-phenylalanine chloromethyl ketone, but not by calpain or several lysosomal protease inhibitors. The addition of CaCl₂ to the cell-free system increased a caspase-3 activity in nonapoptotic cytosols as shown by specific antibodies and an enzymatic assay. No activation of a caspase-3-like activity by the addition of cytochrome c was observed in these extracts under similar conditions. The enhanced caspase-3 activity induced by calcium was inhibited by protease inhibitors affecting morphological nuclear apoptosis except for those responsible for the degradation of lamin A. These results suggest that CaCl₂ could trigger, in normal cells, an apoptotic cascade through the activation of cytosolic caspase-3 activity.

Apoptosis is a cell death program originally characterized by specific morphological and biochemical modifications in higher eukaryotic cells (1, 2). These structural changes such as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation, and DNA fragmentation are considered as landmarks of the apoptotic process (3). Although apoptosis plays an important role in the normal physiology of the cell, and in many pathological situations, little is known about the molecular mechanisms involved in the regulation and/or in the execution of this program (4, 5). Specific proteases called caspases and protein members of the proto-oncogene BCL-2 family have been shown to be the key elements of the executive terminal phase of apoptosis (6–8). The caspases involved in apoptosis are generally divided into initiators and executioners (9). The initiators caspases (e.g. caspase-1 or -8) are implicated in the activation of the executioners (e.g. caspase-3 or -6), which in turn are responsible for the terminal phase of apoptosis along with other enzymes such as proteases, nucleases, kinases, and so forth (reviewed in Refs. 9–11). It has recently been proposed that BCL-2-like proteins regulate the activation of caspases through complex interactions with other proteins (7) and/or through the control of ion fluxes across membranes of organelles (12, 13). Indeed, since BCL-2 overexpression has been shown to inhibit apoptosis-associated Ca²⁺ waves in the endoplasmic reticulum or the nuclear membranes, it has been proposed that members of the BCL-2 family could regulate the cellular calcium homeostasis (14–18). Numerous data have shown the involvement of Ca²⁺ homeostasis in apoptosis and in particular the prelethal increase of its intracellular concentration (reviewed by McConkey and Orrenius (19)). Indeed, the addition of Ca²⁺ to isolated nuclei can directly promote apoptotic nuclear changes by a mechanism apparently involving nuclear endonucleases and proteases (20). However, despite the accumulation of data, the link between intracellular calcium homeostasis and the activation of the apoptotic program remains unknown. We have shown, using a cell-free system, that calcium could play an important role in the induction of caspase-3-like activity by regulating the release of cytochrome c from mitochondria, a protein linked to the activation of caspases.1 In the present study, we investigated the direct effect of CaCl₂ on apoptotic-like modifications of purified rat liver nuclei incubated with cytosolic extracts derived from nonapoptotic or apoptotic cells.

EXPERIMENTAL PROCEDURES

Peptide inhibitors of caspase 1 (Ac-YVAD-CHO) or caspase 3 (Ac-DEVD-CHO), calpain inhibitor I (Ac-Leu-Leu-norleucinal) and II (Ac-Leu-Leu-methioninal), h-APF-OH, an inhibitor of the nuclear scaffold protease (NSP)² (20), ac-DEVD-AMC, a fluorescent caspase-3 substrate, and h-FPG-AMC, a fluorescent chymotrypsin-like substrate were obtained from Bachem (France). Leupeptin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride, E64, N₅₆-tosyl-L-lysine chloromethyl ketone (TLCK), and N₅₆-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were from Sigma (St. Quentin, France). A peptide mimicking the apoptotic cleavage site of lamin A (RLVEIDNGKQR) and thus an inhibitor of caspase-6 (21) was synthesized by Genosys (Cambridge, UK). The

1 P. Juin, K. Tremblais, N. T. Lecabellec, M. Gregoire, K. Meflah, and F. M. Vallette, submitted for publication.

2 The abbreviations used are: NSP, nuclear scaffold protease; TLCK, N₅₆-tosyl-L-lysine chloromethyl ketone; TPCK, N₅₆-tosyl-L-phenylalanine-chloromethyl ketone; CE, cytosol extract; CCE, control CE; ACE, apoptotic CE; CEB, cell extract buffer; AMC, 7-amido-4-methyl coumarin.

This paper is available on line at http://www.jbc.org
Calciu m Activation of Caspase in Cell-free System

chemicals used in this study unless otherwise specified were obtained from Sigma (St. Quentin, France). Antibodies against cytochrome c were obtained from Pharmingen (Clinisciences, France), anti-pro-caspase-3 were from Transduction Laboratories (Medgene Science, France), and anti-pro-caspase-8 were from Santa Cruz (TEBU, France).

Purification of Nuclei and Obtention of Cytosolic Extracts—Rat liver nuclei were prepared according to Newmeyer and Wilson (22). The rat glioblastoma cell line A15A5 and the human promyelocyte leukemic cells HL60 were grown to confluence at 37 °C and in 5% CO2. Apoptosis was then induced in these cells by a short UV-B treatment (1 min). Most of the cells displayed, after 48 h, characteristic morphological apoptotic changes. The cytosolic extracts (CE), from both control (CCE) or apoptotic cells, were obtained as described in Cotter and Martin (23).

Briefly, the cells were washed twice in phosphate-buffered saline, pH 7.2, the resulting pellet was resuspended in the cell extract buffer (CEB) (50 mM HEPES, pH 7.4, 50 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 10 mM dithiothreitol, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride in order to minimize proteolysis degradation in the extracts. Cells or apoptotic bodies were centrifuged at 3,000 × g for 10 min at 4 °C, and the supernatant was discarded. The pellets were transferred to a 2-ml glass Dounce homogenizer in the remaining CEB. Cells were allowed to swell in a 1:1 dilution in CEB for 30 min on ice. Cells were lysed gently with 30 strokes with a B-type pestle. The cell lysate was then transferred to a 1-ml tube and centrifuged at 15,000 × g for 15 min at 13,000 × g. The cytosol was removed and kept frozen at a concentration of approximately 10 mg/ml at −80 °C.

Analysis of DNA Content and Caspase-3-like Activity—Cell-free reactions (50 μl) were carried out as follows. Cytosols were diluted to the desired concentrations in the reaction mixture which contained 2 × 105 rat liver nuclei, 10 mM HEPES, pH 7.4, 50 mM KCl, 1 mM EGTA, 2 mM MgCl2, 1 mM dithiothreitol, 1 mM ATP, 10 mM phosphocreatine, 50 μM creatine kinase, 10 mM malate, 8 mM succinate, and 250 mM sucrose. Calcium and peptides were then added, and the resulting mixture was incubated for 2 h at 37 °C. At the end of the incubation, a small aliquot of the mix was removed and stained for 10 min with Hoechst 33342 (2.5 μM) at room temperature and examined by fluorescence microscopy (Olympus, BX 60). The rest of the mix was analyzed for internucleosomal degradation of the DNA and caspase-3 activity.

After centrifugation of the samples (15 min, 4000 × g at 4 °C), caspase-3 activity was measured in the supernatant and DNA fragmentation in the pellet. DNA was purified and analyzed on 1.5% agarose gel after staining by ethidium bromide. Caspase-3 activity was measured in the supernatant by following the cleavage of 50 μM peptide Ac-DEVD-AMC.

The fluorescence of the cleaved substrate was determined every 30 min using a spectrophotometer (Fluorolite 1000, Dynatech Laboratories) set at an excitation wavelength of 365 nm and emission wavelength of 465 nm.

Western Blot Analysis and Quantification of Priccase-3 and -8—Western blots were performed as described in Chatel et al. (25) using first antibodies diluted 1:100 for anti-procaspase-8 and cytochrome c and 1:1000 for anti-procaspase-3. The antibodies bound to Immobilon-P (Millipore, France) were detected by enhanced chemiluminescence (Amersham, France) and the amounts of procaspases after scanning with a Bio-Rad Gel Program (Signal Analytics, Vienna, VA).

RESULTS

Morphological Modifications and DNA Fragmentation Induced by CaCl2 in Isolated Rat Liver Nuclei: Potentiation by Nonapoptotic Cytosolic Extracts—Rat liver nuclei (2 × 107) were incubated in CEB (see “Experimental Procedures”) for a fixed period of time in the presence of increasing amounts of CaCl2 (from 0 to 5 mM), in the absence or in the presence of nonapoptotic cytosolic extracts (100 μg of CCE). After a 2-h incubation and in the absence of CCE, the intranucleosomal degradation of DNA was observed only in nuclei incubated with 5 mM CaCl2 (Fig. 1). This is in agreement with a previous report (20). The addition of + CCE of non-CCE from the rat glioblastoma cell line A15A5 lowered considerably the amount of CaCl2 required to trigger an apoptotic-like DNA fragmentation, since the intranucleosomal degradation was then observed with concentrations of CaCl2 as low as 0.5 mM (Fig. 1).

A similar effect was observed with cytosols derived from different sources such as the human HL60 or K562 cells and the rat pheochromocytoma cell line PC12 (data not shown).

We also studied the apoptotic morphological events occurring in the nuclei after incubation with calcium. Little or no morphological changes were observed in nuclei incubated in the absence of CCE with concentrations of up to 1 mM CaCl2. At higher concentrations (5 mM CaCl2), the nuclei displayed the typical apoptotic changes in the chromatin structure appearing highly condensed at the nuclear periphery (data not shown).

In the presence of CCE (100 μg) and in the absence of added CaCl2, rat liver nuclei exhibited a normal pattern, and their nuclear envelope remained intact upon co-incubation with CCE (Fig. 2A). The addition of CaCl2 to the CCE triggered nuclear morphological changes. After identical periods of incubation, structural changes exhibited by nuclei were enhanced with the addition of increasing concentrations of CaCl2 to the cytosolic extracts. Chromatin condensed progressively at calcium concentrations as low as 0.05 mM (Fig. 2B), to eventually either cluster against the nuclear periphery or display the characteristic half-moon features observed at higher concentrations (i.e. 0.5 mM CaCl2) (Fig. 2C). At concentrations equal or superior to 1 mM CaCl2, the nuclei were shrinking and the chromatin started to collapse into high density structures (Fig. 2D). These morphological changes were highly reminiscent to those observed in apoptotic cells in vitro or in vivo (26–28).

These changes were observed after a 2-h incubation at 37 °C, but it should be noted that the addition of CaCl2, even at the lower concentrations (i.e. 0.2 mM CaCl2), resulted to the dramatic final condensation of chromatin and degradation of DNA but after much longer periods of incubation (data not shown). Under these conditions, the addition of ZnCl2 (1 mM) abolished the nuclear apoptosis even in the presence of 5 mM CaCl2 (data not shown). The addition of CCE appeared thus to potentiate, both morphologically and biochemically, the calcium-induced apoptosis in the cell-free system. It should be noted that the total (free and bound) calcium concentration in CCEs was always below 170 μM (data not shown) and thus could not account for the enhanced apoptotic effect observed in the presence of cytosols.

Influence of Protease Inhibitors and Free Radicals Scavengers on the Cytosolic Potentiation and Calcium-induced Apoptosis—We found that either trypsin or heat pretreatment of CCEs abolished the potentiation of the calcium-induced apoptosis by cytosolic extracts (data not shown). We thus postulated that proteinaceous components were involved in this process. Apoptosis was initiated in the cell-free system by the incubation of rat liver nuclei with 0.5 mM CaCl2 and 100 μg of CCE in the presence of inhibitors of caspases such as ac-YPAD-
CHO (100 μM), an inhibitor of caspase-1, or ac-DEVD-CHO (100 μM), an inhibitor of caspase-3-like proteases. As shown in Fig. 3B, in the absence of these inhibitors, the addition of CaCl₂ resulted, after 2 h, in an almost complete disintegration of rat liver nuclei. The addition of 100 μM ac-YVAD-CHO also affected this process, but the changes occurred more slowly and were not as dramatic as those observed in calcium-treated nuclei (Fig. 3C). The addition of 100 μM ac-DEVD-CHO, on the other hand, completely prevented the onset of apoptosis (Fig. 3D). Since calpain was shown to be involved in the apoptotic process (11), calpain inhibitors I and II were added to the reaction mixture with CCE and calcium. However, no effects of these inhibitors were observed on calcium-induced apoptosis in the cell-free system (data not shown). On the contrary, the addition of the radical scavenger GSH, as well as the inhibitor of the NSP (h-AFP-oh) inhibited the morphological changes observed upon the addition of CaCl₂ and CCE (data not shown). Next, we examined the effect of these inhibitors on the DNA integrity and found, in agreement with morphological observations, that E64, a calpain inhibitor, had no effect on the DNA fragmentation. On the other hand, GSH protected the DNA structure in the presence of CCE and calcium. However, the correlation between biochemical and morphological changes was not absolute, because the fragmentation of the DNA was unaffected by the addition of ac-YVAD-CHO (Fig. 4). On the other hand, the addition of 100 μM ac-DEVĐD-CHO inhibited the DNA intranucleosomal degradation (Fig. 4).

Induction of a DEVDase Activity in Nonapoptotic Cytosols—Both morphological and DNA analyses suggested that caspase-like activities were involved in the CCE plus calcium-induced apoptosis in the cell-free system. We examined the effect of calcium on the caspase activity present in our extracts using the cleavage of the peptide DEVD-AMC as an index of caspase-3-like activity. CCE (100 μg) displayed low levels of caspase-3-like activity. As shown in Fig. 5, a 3-fold increase of a DEVDase activity was observed upon the addition of 0.5 mM CaCl₂. As this increase was observed both in the absence and in the presence of nuclei, we ruled out the contribution of a nuclear protease to this activity (data not shown). Under these conditions, we found no YVADase activity both in absence and in presence of CaCl₂ (data not shown). The calcium induction of the cytosolic DEVDase appeared to be specific of nonapoptotic cytosols as the addition of CaCl₂ had no effect on the caspase-3-like activity present in ACE at protein concentrations of 25 μg (Fig. 5) and higher (data not shown).

Several reports have shown that cytochrome c also induced a caspase-3-like activity in normal cytosols (29–32). As illustrated in Fig. 6A, no or little cytochrome c was found in CCEs derived from HL60 or A15A5 cells, whereas HL60 ACE exhibited large amounts of this protein. We compared the kinetics of induction of the DEVDase activity by calcium and cytochrome c in 40 μg of ACE at 37 °C (Fig. 6B). Under these conditions no activation of DEVDase activity was observed in the presence 2 μM bovine cytochrome c and 1 mM dATP (Fig. 6B). On the other hand, the addition of 0.5 mM CaCl₂ produced a rapid and important increase of the caspase-3-like activity, which reached a plateau after 120 min (Fig. 6B). We have verified the specificity of the DEVDase activation using a chymotrypsin substrate (GGF-AMC). No significant difference in the chymotrypsin activity was observed between control CCEs and cytochrome c/dATP or CaCl₂-treated CCEs (Fig. 6C).
Caspase-3—we used several protease inhibitors to study the nature of the caspase involved in this activation as well as the possible involvement of other proteases. The DEVDase activated in CCE in the presence of CaCl₂ was slightly inhibited by an inhibitor of caspase-1 and completely abolished by the addition of an inhibitor of caspase-3 (Fig. 7). Since, as previously shown by McConkey (20), lamins are cleaved during calcium-induced apoptosis, a peptide mimicking the cleavage site of lamin A (21) was used to test the involvement of caspase-6 in the calcium-induced DEVDase. The caspase-3-like activity was not affected by this peptide, thus ruling out the involvement of caspase-6/Mch2 in this process. Other protease inhibitors such as TPCK and TLCK inhibited the DEVDase activity but with different affinities. TPCK was a more powerful inhibitor than TLCK (data not shown), suggesting the involvement of a chymotrypsin-like protease in this process. Calpain inhibitors such as E64 or calpain inhibitors I and II had no affect on this activity (Fig. 7). Other inhibitors of the calcium-induced morphological changes such as GSH or NSP did not affect the onset of the caspase-3-like activity (Fig. 7), suggesting that they acted downstream of this activation.

We have used antibodies to detect procaspase-3 and pro-caspase-8 in CCE incubated with increasing concentrations of CaCl₂. The Western blot in Fig. 8 reveals a disappearance of the procaspase-3 upon incubation with CaCl₂ which parallels the activation of a DEVDase. Conversely, pro-caspase-8, an initiator caspase linked to Fas or tumor necrosis factor apoptotic pathways (33), was not affected under these conditions. The latter result suggests that caspase-3 is responsible at least partially for the DEVDase activity induced by calcium in CCEs.

**DISCUSSION**

Since apoptosis does not always require protein synthesis, elements involved in this process have to be already present in nonapoptotic cells. Our data show that calcium could induce apoptotic-like changes in isolated nuclei and that these changes were potentiated by the addition of nonapoptotic cytosols. Different CCEs were analyzed derived either from human hematopoietic cell lines (HL60 and K562) or from cells derived from rat nervous tissues (A15A5 and PC12). We have also shown that addition of calcium to these extracts activated a DEVD cleaving protease. As caspase-3 inhibitors blocked the calcium-induced apoptosis in the presence of CCEs, proteases belonging to this family were likely to be involved in this potentiation. Inhibition of the NSP or the addition of GSH blocked the calcium-induced nuclear apoptosis in the presence of cytosol but not the DEVDase activity. These results suggest that an antioxidant process as well as a resident mechanism of nuclear degradation involved in nuclear apoptosis functions downstream of cytosolic caspase activation. Recently, Liu et al. (34) isolated a heterodimeric protein called DFF (DNA fragmentation factor) which could provide a link between activation of caspase-3 and DNA fragmentation. This factor, which is not regulated by calcium, induces DNA fragmentation in HeLa cells after cleavage by caspase-3.
The DEVDase activity appears to be rapidly induced by calcium at very low concentrations of CCEs. Under the same conditions, cytochrome c and dATP had little effect on DEVDase activation. However, cytochrome c activation of DEVDase can be observed with higher concentrations of CCEs, indicating different thresholds of activation for calcium and cytochrome c, which probably thus rely on different mechanisms. Of all the noncaspase agents, only TPCK appears to specifically and efficiently inhibit the calcium induced DEVDase activity (Fig. 7). Recently, TPCK has been shown to block the conversion of the inactive 32-kDa caspase-3 precursor into the mature caspase-3 (35), thus providing an explanation of its inhibitory effect on apoptosis reported in several studies (11). It is noteworthy that, under our conditions, no increase in a chymotrypsin activity was observed (Fig. 6), ruling out a general proteolytic activation by calcium which in turn would lead to an nonspecific activation of a DEVDase activity.

The calcium induced DEVDase activity was attributed to caspase-3, as no YVADase (caspase-1-like) activity was found, the amount of procaspase-3 was specifically affected by calcium (Fig. 8) and as the inhibition of caspase-6 did not interfere with this activity (Fig. 7). The nature of this activation remains to be established, in particular the intervention of other proteases (including other caspases) and/or cytoplasmic components in this process. Even if the calcium concentrations used in our study are elevated compared with resting intracellular calcium concentrations, one could postulate that, under certain circumstances, the release of calcium from intracellular organelles could lead to high local cytosolic concentration which could activate a caspase-3-like protease. Our observation could thus be relevant in certain physiopathological situations. In agreement with this hypothesis, it has recently been shown that the glutamate-induced apoptosis of cerebellar granule neurons, which is characterized by a sustained intracellular rise of Ca2+, is mediated by a post-translational activation of a caspase-3-like protease (36).

Acknowledgments—We thank Dr. J. L. Orsonneau (Laboratoire de Biochimie Générale, CHR de Nantes) for the quantification of cytosolic calcium. We thank Dr. J. Menanteau (U419 INSERM) for fruitful discussions throughout this study.

REFERENCES

1. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) Cell 88, 347–354
2. Kerr, J., Wylie, A., and Currie, A. (1972) Br. J. Cancer 26, 239–257
3. Vaux, D. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 786–789
4. Vaux, D. L., and Stasser, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2239–2244
5. Korsmeyer, S. J. (1996) Trends Genet. 11, 101–105
6. Reed, J. C. (1994) J. Cell Biol. 124, 1–6
7. Reed, J. C. (1997) Nature 387, 773–776
8. Kroemer, G. (1997) Nat. Med. 3, 614–620
9. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 445–446
10. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) Cell 87, 171
11. Sukharreva, S. A., Pleshakova, O. V., and Sadovnikov, V. B. (1997) Cell Death Differ. 4, 457–462
12. Antonsson, B., Conti, P., Caviat, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.-M., Mazi, G., Maudrell, K., Gamble, F., Sadoul, R., and Martinou, J.-C. (1997) Science 277, 370–372
13. Muchmore, S. W., Sattler, M., Liang, H., Meadows, R. P., Harlan, J. E., Yoon, H. S., Nettesheim, D., Chang, B. S., Thompson, C. B., Wong, S.-L., Shi-Chung, N., and Fesik, S. W. (1996) Nature 381, 335–341
14. Bailey, G., Miyashita, T., Williamson, J. R., and Reed, J. C. (1993) J. Biol. Chem. 268, 166–1669
15. He, H., Lam, M., McCormick, T. S., and Distelhorst, C. W. (1997) J. Cell Biol. 138, 1219–1228
16. Lam, M., Dubynak, G., Chen, L., Nunez, G., Miesfeld, R. L., and Distelhorst, C. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6569–6573
17. Marin, M. C., Fernandez, A., Bick, R. C., Brishay, S., Buja, M. L., Snuggs, M., McConkey, D. J., von Endtschach, C. A., Keating, M. J., and McDonnell, T. J. (1996) Oncogene 12, 2259–2266
18. Murphy, A. N., Bredesen, D. E., Cortopassi, G., Wang, E., and Fiskum, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9893–9898
19. McConkey, D. J., and Orrenius, S. (1997) Biochem. Biophys. Res. Commun. 239, 357–366
20. McConkey, D. J. (1996) J. Biol. Chem. 271, 22298–222406
21. Takahashi, A., Alnemri, E. S., Ladizhick, Y. A., Fernandes-Alnemri, T., Litwack, G., Moor, R. D., G. B., Poirier, G. G., Kaufmann, S. H., and Earnshaw, W. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8395–8400
22. Newmeyer, D. D., and Wilson, K. L. (1991) Methods Cell Biol. 36, 607–624
23. Cotter, T. G., and Martin, S. J. (1996) Techniques in Apoptosis: A User’s Guide.

*M. Pelletier and F. M. Vallette, manuscript in preparation.*
Calcium Activation of Caspase in Cell-free System

24. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Goreau, Y., Griffin, P. R., Labelle, M., Lazebnick, 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

25. Chatel, J. M., Grassi, J., Frobert, Y., Massoulie, J., and Vallette, F. M. (1993) *Proc. Natl Acad. Sci. U. S. A.* 90, 2476–2480

26. Tounekti, O., Belehadek, J., and Mir, L. M. (1995) *Exp. Cell Res.* 217, 506–515

27. Sun, D. Y., Jiang, S., Zheng, L.-M., Ojcius, D. M., and Young, J. (1994) *J. Exp. Med.* 179, 559–568

28. Earnshaw, W. C. (1995) *Trends Cell Biol.* 5, 217–220

29. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* 275, 1129–1132

30. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) *Science* 275, 1129–1132

31. Liu, X., Kim Naekyung, C., Yang, J., Jemmerson, R., and Wang, X. (1996) *Cell* 86, 147–157

32. Kluck, R. M., Martin, S. J., Hoffman, B. M., Zhou, J. S., Green, D. R., and Newmeyer, D. D. (1997) *J. Exp. Med.* 185, 4639–4649

33. Nagata, S. (1997) *Cell* 88, 355–365

34. Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) *Cell* 89, 175–184

35. Han, Z., Hendrickson, E. A., Brenner, T. A., and Wyche, J. H. (1997) *J. Biol. Chem.* 272, 13432–13436

36. Du, Y., Bales, K. R., Dodel, R. C., Hamilton-Byrd, E., Horn, J. W., Czilli, D. L., Simmons, L. K., Binhui, N., and Paul, S. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 11657–11662