THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 29, Issue of July 22, pp. 18877–18880, 1994
Printed in U.S.A.

A Calcium-dependent Nuclease from Apoptotic Rat Thymocytes Is Homologous with Cyclophilin

RECOMBINANT CYCLOPHILINS A, B, AND C HAVE NUCLEASE ACTIVITY*

(Received for publication, March 14, 1994, and in revised form, May 6, 1994)

Jennifer W. Montague‡§, Marcia L. Gaido§¶, Carole Frye¶, and John A. Cidlowski‡¶‖**

From the Departments of ‡Biochemistry and Biophysics and ¶Physiology and the ‖Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

Apoptosis is an important physiological process that involves the deletion of specific cells in a controlled and timely manner. A biochemical hallmark typifying apoptosis in normal lymphocytes is DNA cleavage caused by a calcium-dependent nuclease. We have previously identified and purified an 18-kDa nuclease (NUC18) from glucocorticoid-treated rat thymocytes whose activity is associated with this apoptotic DNA fragmentation. Partial protein sequencing of pure NUC18 has generated two peptide sequences that have a remarkable similarity to rat cyclophilin A and other members of the cyclophilin family. We report here that recombinant cyclophilins A, B, and C have a calcium/magnesium-dependent nuclease activity with biochemical and pharmacological properties similar to those of NUC18. Our results raise the intriguing possibility that cyclophilin or a cyclophilin-related protein may play a role in lymphocyte apoptosis.

Apoptosis, or programmed cell death, occurs in many physiological situations where selective deletion of cells is required (1, 2). A well studied model of programmed cell death is glucocorticoid-induced apoptosis in thymocytes (3–7). These lymphocytic cells have been extensively characterized morphologically and biochemically during the progression of apoptosis and exhibit chromatin cleavage characteristic of internucleosomal DNA degradation. This type of DNA degradation is one of the biochemical events that is often associated with apoptosis in lymphocytes, but not in all apoptotic cells (8). Activation of the nuclease responsible for this DNA degradation is thought to be the commitment step of these cells to death (6, 9), although it does not appear to be the initial event in other examples of apoptotic death (10, 11). Several reports have demonstrated the existence of an endogenous endonuclease that shows a tight association between internucleosomal DNA degradation and apoptosis in thymocytes (7, 9, 12). This nuclease activity is activated by calcium and magnesium ions and is sensitive to inhibition by zinc ions (9) and aurintricarboxylic acid (13, 14).

Our laboratory has isolated and characterized a nuclease whose activity is associated with the degradation of DNA in rat thymocytes undergoing apoptosis. The activity of this low molecular weight protein, designated NUC18, correlates with the DNA degradation that occurs during apoptosis in these cells (15) and is regulated by several activators of apoptosis in lymphocytes, including glucocorticoids, calcium ionophores (16), and inhibitors of protein synthesis (17). NUC18 enzymatic activity requires calcium and magnesium ions and is inhibited by zinc ions and aurintricarboxylic acid (15). NUC18 has now been purified to homogeneity as judged by silver staining of SDS-polyacrylamide gels, and we show here that NUC18 is remarkably similar to the cyclophilin family of proteins.

Cyclophilins were first identified as the high affinity binding proteins for the immunosuppressant drug cyclosporin A (CsA)[1] (18). Since being identified in mammals, cyclophilin and cyclophilin-related peptides have been isolated from a wide variety of species, including yeast (19), *Drosophila* (20), *Neurospora* (21), and even bacteria (22, 23). Cyclophilin has been found in every tissue type studied to date in humans (19, 24) and exhibits varied cellular localization, including cytoplasmic forms (18) and nuclear forms (24, 25), as well as endoplasmic reticulum-directed (26) and even forms associated with the secretory pathway (27). Cyclophilins have peptidylprolyl *cis-trans*-isomerase activity that is inhibited upon binding to CsA (28, 29). Recent studies, however, have shown that inhibition of cyclophilin isomerase activity by CsA cannot account for the immunosuppressive effect of CsA (30). Although the mechanism of immunosuppression by CsA is thought in part to include inhibition of lymphocyte activation (31), the precise mode of action remains elusive.

In addition to its known immunosuppressive effect, CsA has been shown to have considerable toxic effects on a variety of cell types and tissues (30). Gene disruption experiments in lower eukaryotes have shown that the cyclophilins are essential for the toxic response to CsA (32). Recently, we have shown that the addition of CsA to a mouse thymoma cell line (S49.1) results in the induction of apoptotic death of the cells, determined by morphology and the production of internucleosomally cleaved DNA fragments.[2] We show here that pure recombinant cyclophilins have sequence similarity and immunological cross-reactivity to NUC18 and that these proteins exhibit calcium/magnesium-dependent nuclease activity. These data together suggest a possible role for the cyclophilin family in thymocyte apoptosis.

MATERIALS AND METHODS

Purification and Sequencing of NUC18—The NUC18 protein was purified by M. Gaido from nuclei of rat thymocytes as described previously (15), and the purity of the preparation was judged by detection of a single band after silver staining (33) of the polyacrylamide gel. Gel-purified material was electrophoretically transferred to polyvinylidene

* This work was supported by National Institutes of Health Grant DK 32078. 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.

§ These authors contributed equally to this work.

** To whom correspondence should be addressed: Dept. of Physiology, CB 7545, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-1523; Fax: 919-966-6927.

[1] The abbreviations used are: CsA, cyclosporin A; IPTG, isopropyl-1-thio-β-D-galactoside; HPLC, high pressure liquid chromatography.

[2] F. M. Hughes, Jr., personal communication.

difluoride (Immobilon P, Millipore Corp.) membranes and subjected to automated Edman degradation with an Applied Biosystems 470 Sequencer with on-line HPLC analysis. Initial attempts at Edman degradation revealed a blocked amino terminus. Purified protein was subjected to trypsin digestion, and peptide fragments were purified further by HPLC with a standard C18 column. Derived peptides were then subjected to automated Edman degradation.

Production and Expression of Recombinant Cyclophilin A—The cyclophilin A cDNA was generously provided by Dr. Christopher Walsh (Harvard University, Cambridge, MA), and the bacteria for its expression were kindly donated by Dr. Aziz Sancar (University of North Carolina, Chapel Hill, NC). NM522 bacteria, without or with the cyclophilin A expression vector, were grown to an A_{595} of 0.7 and made 0.2 mM IPTG. Cells were harvested via centrifugation, washed with 20 mM Tris-HCl, pH 8.0, resuspended in 9 ml of 20 mM Tris-HCl, pH 8.0, and passaged twice through a French press at 20,000 p.s.i. Lysates were spun for 25 min at 20,000 × *g*, and supernatants were made 0.4% protamine sulfate. Precipitated DNA was removed by centrifugation for 20 min at 20,000 × *g*. Aliquots of the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

Radioactive Gel Nuclease Assays—The radioactive gel nuclease assays were performed as described previously (15). Briefly, the gels were prepared as for a Laemmli system, but with the addition of 75 μg of calf thymus DNA and 750,000 cpm of [α-^{32}P]dCTP-labeled double-stranded plasmid DNA made by nick translation. SDS was removed by soaking the gel overnight in a buffer of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 2 mM magnesium chloride, followed by three 20-min washes. The activation step involved agitating the gels at 37 °C in fresh buffer with 2 mM calcium chloride (length of activation is noted in figure legends). Gels were dried down and exposed for autoradiography. Areas of ^{32}P-labeled DNA loss demonstrate nuclease activity. Purified cyclophilins A, B, and C were generously provided by Sandoz Laboratories, Basel, Switzerland. Cyclophilin A crystals were generously provided by Dr. Hengming Ke (University of North Carolina, Chapel Hill, NC).

RESULTS

Sequence Similarity and Immunological Cross-reactivity of Purified NUC18 and Rat Cyclophilin A—NUC18 was purified as described previously (15), then subjected to trypsin digestion and HPLC purification of the peptides. Edman degradation was performed to determine the amino acid sequence of two of the trypsin peptides. Peptide 1 of NUC18 (TVVFGK) corresponds to residues 125–130 of rat CypA (HVVFGK), with the exception of the His/Thr mismatch, and peptide 2 corresponds exactly to the first 16 residues of rat CypA (VNPTVFFDITADGEPL) (34). Cyclophilin A is one member of the cyclophilin family of proteins, which have been identified in a variety of species, as well as in diverse tissue types within species (17–26). Cyclophilin A consists of 165 residues, with a corresponding size of approximately 18 kDa (35); cyclophilin B and cyclophilin C are slightly larger, consisting of 208 residues at 21 kDa (26) and 212 residues at 22.8 kDa (36), respectively. The amazing sequence similarity between NUC18 and cyclophilin A, which encompasses the region of highest homology within the cyclophilin family, suggests that NUC18 might be another member of the cyclophilin family. To independently address the issue of homology between NUC18 and cyclophilin, we raised antibodies against the two proteins and examined them for cross-reactivity. Western blot analysis showed that both types of antibodies recognize the purified cyclophilin A protein and an 18-kDa protein from a thymocyte nuclear extract of dexamethasone-treated rats (data not shown). These results suggest the two proteins are immunologically similar. Because NUC18 was purified based on its calcium/magnesium-dependent nuclease activity, which is tightly associated with apoptosis in these cells (12), we examined cyclophilins for potential nuclease activity.

Nuclease Activity of Recombinant Cyclophilin A—The first approach we used to evaluate potential nuclease activity of cyclophilin was to introduce and express a cyclophilin A gene into bacterial cells. NM522 cells were transformed with a recombinant human cyclophilin A cDNA that is under control of an IPTG-inducible promoter. The transformed and induced cells expressed a protein in the 17–18-kDa range that was not observed in the extracts from the control NM522 cells (Fig. 1*A*, *lanes 4* and *3*, respectively). The same extract preparations were then subjected to a radioactive gel nuclease assay. A nuclease activated in this assay will digest away the radiolabeled DNA in its immediate vicinity, clearing a small area of radioactivity. Thus, the resulting autoradiograph of the dried gel appears black wherever there is radioactive DNA with "holes" at the site of an activated nuclease. Analysis of the nuclease activity in the extracts revealed that the bacteria containing cyclophilin A exhibit calcium/magnesium-dependent DNA degradation properties in the 17–18-kDa range, while the control bacterial extracts exhibited no detectable nuclease activity under the same conditions (Fig. 1*B*, *lanes 3* and *2*, respectively). For comparison, *lane 1* of Fig. 1*B* shows the relative nuclease activity associated with 1 μg of pure micrococcal nuclease (*MN*). The bacterial proteins themselves provide a negative control by demonstrating that a site cleared of radioactivity does not form simply because a protein is located at that position. Thus, it appears from this genetic approach that the recombinant cyclophilin A protein either has inherent nuclease activity or is capable of activating an endogenous bacterial nuclease of precisely the same molecular weight as cyclophilin after polyacrylamide gel electrophoresis, protein renaturation, and activation.

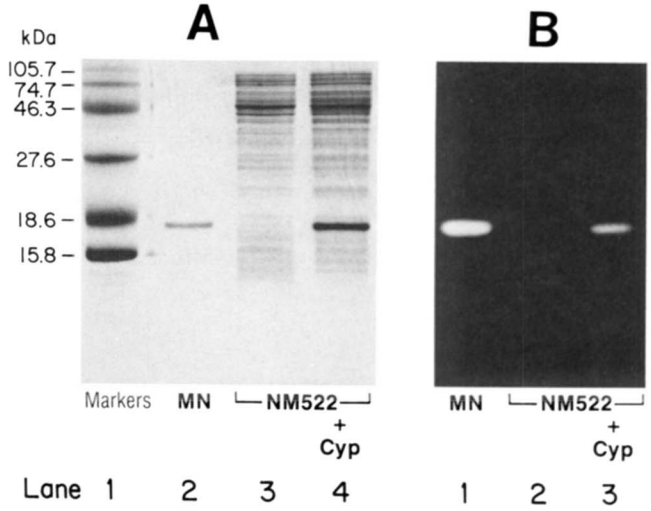

FIG. 1. **Protein staining and nuclease activity of NM522 bacterial cells expressing cyclophilin A.** *A*, Coomassie Blue-stained Laemmli SDS-polyacrylamide gel. *Lane 1*, molecular mass markers; *lane 2*, 1 μg of micrococcal nuclease (*MN*); *lane 3*, 15 μg of protein from IPTG-induced control NM522 cells; *lane 4*, 15 μg of protein from IPTG-induced NM522+CypA cells. *B*, nuclease activity gel. *Lane 1*, 0.5 μg of micrococcal nuclease; *lane 2*, 15 μg of IPTG-induced NM522 protein; *lane 3*, 15 μg of IPTG-induced NM522+CypA. The radioactive gel nuclease assay was performed as described under "Materials and Methods," with a 4-h incubation. The autoradiograph is black where there is radioactively labeled DNA, and the "holes" indicate the site of an active nuclease.

Bacterially Expressed Recombinant Cyclophilin A Nuclease Activity Is Blocked by Zinc and Aurintricarboxylic Acid—To further evaluate the nuclease activity associated with recombinant cyclophilin and its relationship to NUC18, we studied the sensitivity of this "cyclophilin A nuclease activity" to two chemicals known to inhibit both pure NUC18 nuclease activity (15) and apoptosis in these cells: zinc (37) and aurintricarboxylic acid (13). Fig. 2 shows autoradiographs derived from nuclease activity gels comparing micrococcal nuclease (*MN*, *lane 1*) to NM522 bacterial extract (*lane 2*) and crude recombinant cyclophilin A from NM522 bacterial extract (*lane 3*). *Panel A* of Fig. 2 shows that no nuclease activity was detected from any

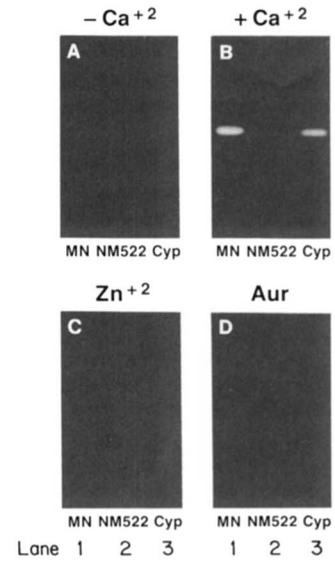

FIG. 2. **Sensitivity of NM522-expressed cyclophilin A nuclease activity to zinc ions and aurintricarboxylic acid.** Radioactive gel nuclease assays were performed as described under "Materials and Methods" and activated for 4 h. Each of the four gels contain 0.5 μg of micrococcal nuclease (*lane 1*), 1 μg of NM522 protein (*lane 2*), and 1 μg NM522+CypA protein (*lane 3*). The gels in *A* and *B* show nuclease activity in the presence and absence of Ca^{2+}. Gels labeled Zn^{+2} (*C*) or *Aur* (*D*) were treated for 1 h with either 2 mM $ZnCl_2$ or 1 mM aurintricarboxylic acid prior to being placed in calcium activating solution.

sample in the absence of added calcium. Calcium addition in *panel B* resulted in discernible nuclease activity from both micrococcal nuclease and crude protein lysates of bacteria expressing recombinant cyclophilin A, but not from extracts of non-cyclophilin expressing bacteria. The nuclease activity inherent to micrococcal nuclease was partially inhibited by zinc (*panel C*). This known inhibitor of apoptosis clearly abolished the activity derived from samples of bacteria transformed with the cyclophilin A expression plasmid. Similarly, aurintricarboxylic acid blocked nuclease activity associated with both micrococcal nuclease and cyclophilin A (*panel D*). Thus the nuclease activity of bacterially expressed cyclophilin A displays the same sensitivity to inhibitors observed previously for NUC18 purified from rat thymocyte nuclei (15).

Purified Recombinant Cyclophilins A, B, and C Exhibit Nuclease Activity in the Radioactive Gel Assay—Since the genetic approach taken thus far to assess the nuclease activity of cyclophilin did not fully exclude the unlikely possibility that the induced cyclophilin was activating an endogenous bacterial nuclease, we obtained and evaluated samples of purified human recombinant cyclophilins A, B, and C. According to the limits of the silver stain detection, there are no contaminating proteins in any of the purified preparations (Fig. 3*A*). As described previously, cyclophilin B (21 kDa) (26) and cyclophilin C (22.8 kDa) (36) are larger than cyclophilin A (18 kDa) (35). The difference in sizes between the proteins demonstrates further that nuclease activity is inherent to cyclophilin A and other members of the cyclophilin family. The nuclease activity observed in each lane of the radioactive gel assay corresponds exactly to the size of the protein in that lane (Fig. 3*B*). Contaminating proteins of a different molecular weight that are too scarce to be visualized with silver staining can be observed in the catalytic radioactive gel nuclease activity. For example, a higher molecular weight protein in the micrococcal nuclease lanes was barely discernible by silver staining, but was revealed by its enzymatic activity in the radioactive nuclease gel assay. This situation was not observed in any of the cyclophilin lanes.

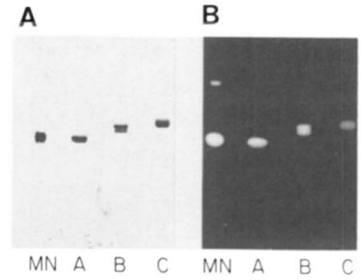

FIG. 3. **Silver stain and radioactive gel nuclease assay with purified human recombinant cyclophilins.** Each lane contains 3 μg of pure protein. *Panel A*, silver stain (33) of micrococcal nuclease and purified cyclophilins A, B, and C (*lanes MN, A, B,* and *C*, respectively). *Panel B*, radioactive gel nuclease assay with micrococcal nuclease and the purified cyclophilin samples. The radioactive gel nuclease assay was performed as described under "Materials and Methods," with a 3-h activation.

To more rigorously extend these analyses, nuclease assays were performed on resolubilized crystals of cyclophilin A. The purity of the prepared sample of resolubilized crystals is shown in Fig. 4. According to the silver stain analysis, no contaminating proteins are present, although we sometimes observe cyclophilin A as an apparent dimer even under denaturing conditions. Thus, resolubilized crystals of cyclophilin A have the capacity for calcium/magnesium-dependent DNA degradation. It is important to note that calcium/magnesium-dependent nuclease activity by cyclophilins has also been also observed in non-denaturing conditions with purified plasmid and resolubilized cyclophilin A crystals, as well as pure recombinant cyclophilin proteins A, B, and C.[3]

DISCUSSION

Cyclophilins have been identified in numerous organisms (13–22) and display varied subcellular localization, including the nucleus (17, 23–26). This family of proteins are well characterized peptidylprolyl *cis-trans*-isomerases that bind to the immunosuppressive drug CsA (27, 28). The striking sequence and structural similarity between cyclophilin and the newly identified 18-kDa nuclease (NUC18) isolated from purified apoptotic rat thymocyte nuclei (15) suggest that NUC18 may represent a nuclear member of the cyclophilin family. NUC18 was purified based on its ability to degrade DNA, and its immunological similarity to cyclophilin A prompted us to evaluate cyclophilins for nuclease activity. We have shown that purified forms of cyclophilin are indeed capable of degrading DNA with kinetics sufficient to account for the DNA fragmentation observed during glucocorticoid-induced apoptosis in thymocytes. Additionally, the nuclease activity from cyclophilin is inhibited by compounds that block both DNA degradation and apoptosis in thymocytes. This novel finding demonstrates that cyclophilins can interact with DNA, as well as with proteins, and suggests that cyclophilins could play a role in thymocyte apoptosis.

Although nuclease activation appears to be a committed step to apoptosis in certain cell types, such as lymphocytes, there are other examples of apoptosis in which DNA degradation and calcium-requiring nuclease activation are not the early events of programmed cell death (10, 38, 39). Often in such instances, chromatin degradation of some sort is a late event that is probably important in facilitating nucleotide recycling and preventing autoimmune responses. Whether the NUC18 or cyclophilin proteins are participating in these examples of apoptosis remains to be determined.

The binding of CsA to cyclophilin inhibits the isomerase activity of cyclophilin, but this effect is not sufficient to account

[3] J. W. Montague and J. A. Cidlowski, unpublished data.

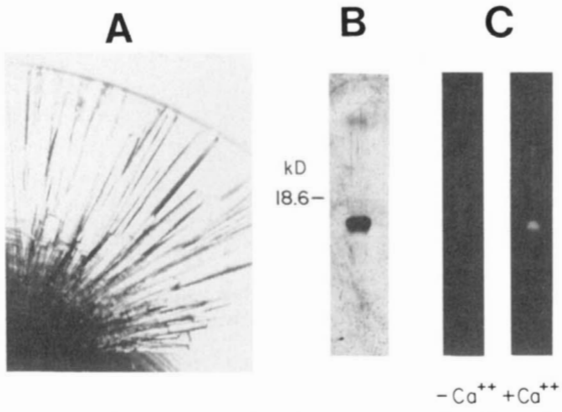

FIG. 4. **Cyclophilin A crystals, silver stain, and radioactive gel nuclease assays with resolubilized CypA crystals.** *A*, picture of CypA crystals similar to those used in the nuclease assay experiments in this figure. The cyclophilin A crystals were prepared by and obtained from Hengming Ke (University of North Carolina, Chapel Hill, NC) (40). The picture of the crystals was taken at 10× magnification with a Nikon microscope and a Nikon FX35-A camera. *B*, silver stain (33) showing the purity of 5 μg of the resolubilized CypA crystals. Crystals were removed from the dialysis wells, transferred to Eppendorf tubes, and spun at 7,000 rpm for 25 min to pellet. Pellets were resuspended in 20 mM Tris, pH 8.0, by sonicating and vortexing. Concentration of protein was determined at A_{280}, with 0.5 units equaling 1 mg/ml protein. *C*, radioactive gel nuclease assays with 5 μg of resolubilized CypA crystals in the absence and presence of calcium ions during a 4-h activation, as described under "Materials and Methods."

for the degree of immunosuppression exhibited by CsA. Recent research has demonstrated an additional role for cyclophilin as part of a cyclophilin/CsA complex that can bind to and inhibit the serine/threonine phosphatase calcineurin (31). Inhibition of calcineurin results in the prevention of lymphocyte activation that is characteristic of CsA-induced immunosuppression. Interestingly, we have shown that CsA can stimulate calcium/magnesium-dependent nuclease activity of cyclophilins A, B, and C under native conditions.[3] Furthermore, we have shown that CsA can stimulate apoptosis in S49.1 thymoma cells, based on morphology and DNA fragmentation.[2] Thus, part of the observed immunosuppressive effect of CsA may occur via nuclease activation of cyclophilins A, B, and C. In addition, the well documented toxic effects of CsA (30, 32) may be related to its stimulation of cyclophilin nuclease activity.

In summary, we report that a nuclease whose activity is temporally associated with apoptosis in thymocytes, NUC18, has sequence and structural similarities with the cyclophilin family of proteins. Furthermore, pure recombinant cyclophilins A, B, and C have inherent calcium/magnesium-dependent nuclease activity that is pharmacologically indistinguishable from NUC18. Direct analysis as to the role of this cyclophilin nuclease activity in thymocyte apoptosis is currently under investigation.

Acknowledgment—We thank Dr. David Klapper (University of North Carolina, Chapel Hill, NC) for assistance in trypsin digestion, HPLC purification, and sequencing of our purified NUC18 protein.

REFERENCES

1. Kerr, J., Wyllie, A., and Currie, A. (1972) *Br. J. Cancer* **26,** 239–257
2. Arends, M., and Wyllie, A. (1991) *Int. Rev. Exp. Pathol.* **32,** 223–251
3. Wyllie, A. (1980) *Nature* **284,** 555–556
4. Cohen, J. (1993) *Chest* **103,** 99S–101S
5. Wyllie, A., Morris, R., Smith, A., and Dunlop, D. (1984) *J. Pathol.* **142,** 67–77
6. Compton, M., and Cidlowski, J. (1992) *Trends Endocrinol. Metab.* **3,** 17–23
7. Schwartzman, R., and Cidlowski, J. (1993) *Endocrinology* **133,** 591–599
8. Ellis, R., Yuan, J., and Horvitz, H. (1991) *Annu. Rev. Cell Biol.* **7,** 663–698
9. Cohen, J., and Duke, R. (1984) *J. Immunol.* **132,** 38–42
10. Oberhammer, F., Fritsch, G., Pavelka, M., Froschl, G., Tiefenbacher, R., Purchio, T., and Schulte-Hermann, R. (1992) *Toxic Lett.* **64/65,** 701–704
11. Oberhammer, F., Wilson, J., Dive, C., Morris, I., Hickman, J., Wakeling, A., Walker, P., and Sikorska, M. (1993) *EMBO J.* **12,** 3679–3684
12. Nikonova, L., Beletsky, I., and Umansky, S. (1993) *Eur. J. Biochem.* **215,** 893–901
13. Hallick, R., Chelm, B., Gray, P., and Orozco, E. (1977) *Nucleic Acids Res.* **4,** 3055–3064
14. McConkey, D., Hartzell, P., Nicotera, P., and Orrenius, S. (1989) *FASEB J.* **3,** 1843–1849
15. Gaido, M., and Cidlowski, J. (1991) *J. Biol. Chem.* **266,** 18580–18585
16. Caron-Leslie, L., and Cidlowski, J. (1991) *Mol. Endocrinol.* **5,** 1169–1179
17. Caron-Leslie, L., and Cidlowski, J. (1994) *Endocrine* **2,** 47–52
18. Handschumacher, R., Harding, M., Rice, J., and Drugge, R. (1984) *Science* **226,** 544–546
19. Koletsky, A., Harding, M., and Handschumacher, R. (1986) *J. Immunol.* **137,** 1054–1059
20. Shieh, B., Stamnes, M., Seavello, S., Harris, G., and Zuker, C. (1989) *Nature* **338,** 67–70
21. Tropschug, M., Nicholson, D. W., Hartl, F.-U., Köhler, H., Pfanner, N., Wachter, E., and Neupert, W. (1988) *J. Biol. Chem.* **263,** 14433–144440
22. Liu, J., and Walsh, C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4028–4032
23. Hayano, T., Takahashi, N., Kato, S., Maki, N., and Suzuki, M. (1991) *Biochemistry* **30,** 3041–3048
24. Ryffel, B., Woerly, G., Greiner, B., Haendler, B., Mihatsch, M., and Foxwell, B. (1991) *Immunology* **72,** 399–404
25. Zydowsky, L., Ho, S., Baker, C., McIntyre, K., and Walsh, C. (1992) *Protein Sci.* **1,** 961–969
26. Price, E., Zydowsky, L., Jin, M., Baker, C., McKeon, F., and Walsh, C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1903–1907
27. Caroni, P., Rothenfluh, A., McGlynn, E., and Schneider, C. (1991) *J. Biol. Chem.* **266,** 10739–10742
28. Fischer, G., Wittman-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. (1989) *Nature* **337,** 476–478
29. Takahashi, N., Hayano, T., and Suzuki, M. (1989) *Nature* **337,** 473–475
30. Sigal, N., Dumont, F., Durette, P., Siekierka, J., Peterson, L., Rich, D., Dunlap, B., Staruch, M., Melino, M., Koprak, S., Williams, B., Witzel, B., and Pisano, J. (1991) *J. Exp. Med.* **173,** 619–628
31. Liu, J., Farmer, J., Lane, W., Friedman, J., Weissman, I., and Schreiber, S. (1991) *Cell* **66,** 1–9
32. Tropschug, M., Barthelmess, I., and Neupert, W. (1989) *Nature* **342,** 953–955
33. Wray, W., Boulikas, T., Wray, V., and Hancock, R. (1981) *Anal. Biochem.* **118,** 197–203
34. Danielson, P., Forss-Petter, S., Brow, M., Calavetta, L., Douglass, J., Milner, R., and Sutcliffe, J. (1988) *DNA* **7,** 261–267
35. Theriault, Y., Logan, T., Meadows, R., Yu, L., Olejniczak, E., Holzman, T., Simmer, R., and Fesik, S. (1993) *Nature* **361,** 88–91
36. Friedman, J., and Weissman, I. (1991) *Cell* **66,** 799–806
37. Duke, R., Chervenak, R., and Cohen, J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 6361–6365
38. Sulston, J. (1976) *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **275,** 287–298
39. Ucker, D., Obermiller, P., Eckhart, W., Apgar, J., Berger, N., and Meyers, J. (1992) *Mol. Cell. Biol.* **12,** 3060–3069
40. Ke, H., Zydowsky, L., Liu, J., and Walsh, C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 9483–9487