Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5965–5968, June 1995
Cell Biology

Cell acidification in apoptosis: Granulocyte colony-stimulating factor delays programed cell death in neutrophils by up-regulating the vacuolar H^+-ATPase

ROBERTA A. GOTTLIEB*, HEIDI A. GIESING*, JESSICA Y. ZHU†‡, ROBERT L. ENGLER†‡, AND BERNARD M. BABIOR*

*Division of Biochemistry, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037; †Research Service, Department of Veterans Affairs Medical Center, San Diego, CA 92089; and ‡Cardiology Division, Department of Medicine, University of California, San Diego, La Jolla, CA 92089

Communicated by Konrad Bloch, Harvard University, Cambridge, MA, March 10, 1995

ABSTRACT **Neutrophils in tissue culture spontaneously undergo programed cell death (apoptosis), a process characterized by well-defined morphological alterations affecting the cell nucleus. We found that these morphological changes were preceded by intracellular acidification and that acidification and the apoptotic changes in nuclear morphology were both delayed by granulocyte colony-stimulating factor (G-CSF). Among the agents that defend neutrophils against intracellular acidification is a vacuolar H^+-ATPase that pumps protons out of the cytosol. When this proton pump was inhibited by bafilomycin A_1, G-CSF no longer protected the neutrophils against apoptosis. We conclude that G-CSF delays apoptosis in neutrophils by up-regulating the cells' vacuolar H^+-ATPase and that intracellular acidification is an early event in the apoptosis program.**

The properties of apoptotic cells have been studied extensively, but little is known about how apoptosis is initiated. There is, however, some evidence suggesting that intracellular acidification may play a role in this process. For example, intracellular acidification increases the susceptibility of cells to killing by heat and chemotherapeutic agents (1–3) and occurs in HL-60 cells undergoing apoptosis in response to etoposide (4). Cell shrinkage, an event characteristic of apoptosis, is also typically seen in cells undergoing intracellular acidification (5). A role for acidification in the DNA breakdown that occurs during apoptosis is suggested by the otherwise perplexing presence in many tissues of an endonuclease that is active only at pH values below 6.6–6.8 (6, 7). In neutrophils, this endonuclease is the only DNase that can be detected (ref. 8; R.A.G., H.A.G., and B.M.B., unpublished data).

In this study, we have examined the relationship between intracellular acidification and apoptosis in cultured human neutrophils. Our results suggest that acidification may be causally related to the destruction of the genome that occurs in these cells when they undergo apoptosis.

MATERIALS AND METHODS

Isolation of Neutrophils and Cell Culture. Purified neutrophils (90–95% pure) were obtained from volunteer donors by 6% dextran sedimentation followed by purification over a discontinuous Percoll (9) or Ficoll/Hypaque (10) gradient, then suspended at 5×10^6 cells per ml in calcium-, magnesium-, and bicarbonate-free Hanks' balanced salt solution supplemented with 20 mM Hepes (pH 7.4) plus 0.25% autologous plasma (HBSS), and cultured for 24 hr at 37°C (11). Where indicated, cultures contained granulocyte colony-stimulating factor (G-CSF; 10^4 units/ml) and/or bafilomycin A_1 {50–200 nM final concentration, added in dimethyl sulfoxide (DMSO) [final DMSO concentration 0.5% (vol/vol)]}. Controls for the bafilomycin experiments received 0.5% DMSO (vol/vol) alone.

Morphologic Scoring of Apoptosis. Neutrophils were cultured as indicated, then applied to a precoated microscope slide (Superfrost Plus, Fisher), and fixed with 2% formaldehyde in phosphate-buffered saline (PBS), followed by a methanol rinse. Cells were then stained with acridine orange and ethidium bromide and examined by fluorescence microscopy (12). A minimum of 300 cells per slide was scored by an observer blinded to the culture conditions.

Determination of Intracellular pH. Neutrophils were cultured at 37°C in HBSS. At the indicated time points, cells were loaded with 10 μM carboxy-SNARF-1-AM (Molecular Probes) for 30 min at 37°C, washed in PBS, and resuspended in the same buffer. Cells were analyzed in a Coulter Elite flow cytometer with excitation at 488 nm and emission analysis at 575 and 620 nm (13). Intracellular pH was estimated from the ratio of emission intensities at the two wavelengths, standardizing by comparison with the fluorescence intensity ratios of cells whose intracellular pH values were fixed by incubation with nigericin (10 μM) in high-potassium buffers as described (14). Ten thousand events were collected and data were plotted as forward scatter (size) versus fluorescence ratio (pH).

***In Situ* Nick End Labeling.** Cells were labeled by a modification of the method of Wijsman *et al.* (15). Briefly, neutrophils were fixed for 5 min with 2% formaldehyde in PBS followed by methanol, air-dried on glass slides, and then incubated with a reaction mix containing 0.5 μM biotin-14-dATP, 5 μM dTTP, 5 μM dCTP, and 5 μM dGTP, in 50 mM Tris (pH 7.5) containing 5 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, and 10 units of Klenow fragment per ml, for 30 min at room temperature. The slides were then rinsed with PBS, incubated for 5 min with 0.28% periodic acid, rinsed again, incubated with PBS containing 2% bovine serum albumin for 5 min, and finally rinsed again in PBS. The cells then were incubated with streptavidin-horseradish peroxidase (1:1000) (Kirkegaard & Perry Laboratories) for 30 min at 37°C and then rinsed twice with Tris-buffered saline. Color was developed with Enhance Black (Kirkegaard & Perry Laboratories) according to the manufacturer's instructions. Eosin Y was used as a counterstain.

RESULTS

To examine the possibility that neutrophils acidify their cytoplasm when they undergo apoptosis, we cultured neutrophils for 24 hr, then loaded them with the pH-sensitive dye carboxy-SNARF-1, and determined their size and intracellular pH by flow cytometry scanning (13). Fresh neutrophils appeared as a group of cells that were relatively homogeneous in size and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: G-CSF, granulocyte colony-stimulating factor; DMSO, dimethyl sulfoxide.

showed a narrow distribution of cytosolic pH values centered around pH 7.2 (Fig. 1 *Top*). The cultured neutrophils, however, appeared as a population of acidified cells whose intracellular pH values averaged 6.2 and whose sizes were decreased compared with the fresh cells (Fig. 1 *Middle*). Of the total cultured cells, 32% showed the morphological features of apoptosis, indicating that intracellular acidification preceded the development of the nuclear characteristics of apoptosis.

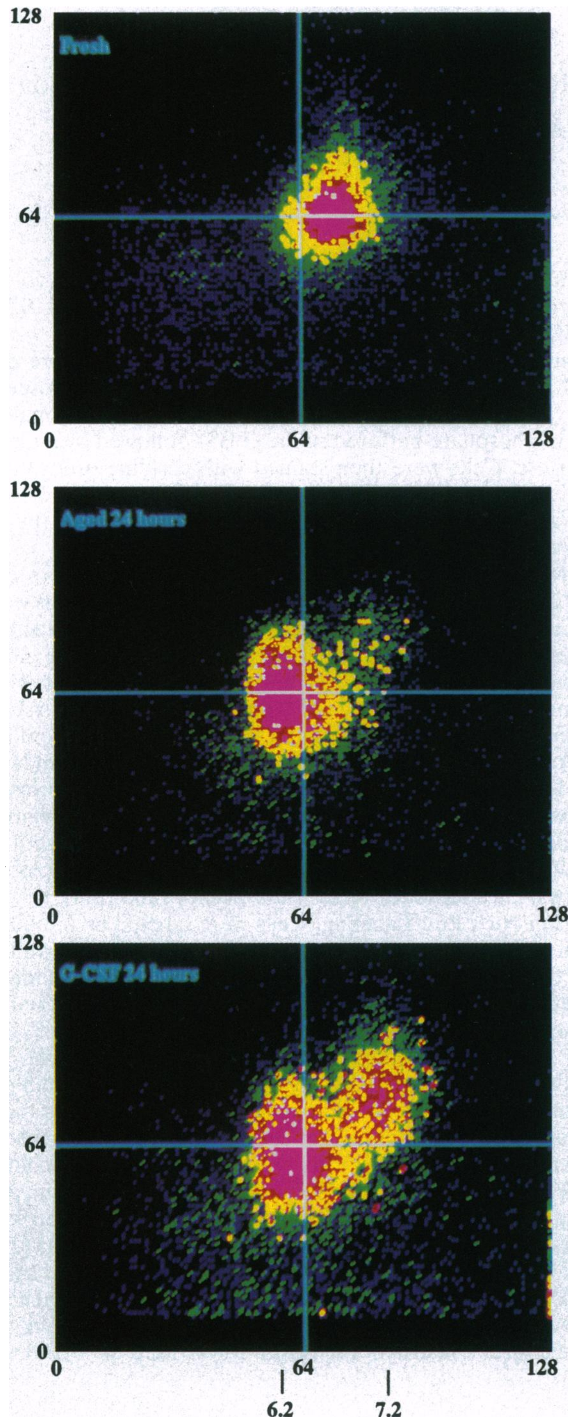

FIG. 1. Flow cytometric analysis of neutrophils for size and intracellular pH. Purified neutrophils were examined immediately ("fresh") or cultured for 24 hr in the presence or absence of G-CSF, then loaded with carboxy-SNARF-1, and examined by flow cytometry. Relative cell size was evaluated by forward scatter (*y* axis), and intracellular pH was evaluated by the ratio of emissions at 575 and 620 nm (*x* axis). (*Top*) Fresh cells. (*Middle*) Cells cultured without G-CSF. (*Bottom*) Cells cultured in the presence of G-CSF (10^4 units/ml). Emission ratios corresponding to pH 6.2 and pH 7.2 are indicated at the bottom. Results are representative of experiments with three different preparations of neutrophils. The units on the two axes are arbitrary.

Apoptosis in neutrophils is known to be delayed by treating the cells with G-CSF (11, 16, 17). To assess the effect of G-CSF on cell size and intracellular pH, neutrophils from the same preparation were cultured 24 hr in the presence of this cytokine. When these neutrophils were cultured for 24 hr and then examined by flow cytometry, two groups of cells were seen (Fig. 1 *Bottom*). One group (24% of the total) consisted of cells whose size and pH distributions were similar to those of the fresh neutrophils, while the other group consisted of smaller cells of lower pH that resembled neutrophils cultured in the absence of the cytokine. The number of morphologically apoptotic cells was reduced to 20% of the total cell population. Thus the protection against apoptosis conferred on cultured neutrophils by G-CSF was accompanied by a reduction in the rate at which the cells acidify.

In the foregoing experiments, neutrophils were cultured for 20–24 hr. Acidification actually began much earlier, however. Fig. 2 shows that within 3 hr after initiating the cultures (the earliest time point examined), the cells had distributed themselves into two discrete populations: a major population with an unaltered intracellular pH and a minor population with an acidified cytoplasm. The same two groups of cells were seen at 5, 8, 12, and 16 hr (data not shown), the acidified group growing steadily larger with time at the expense of the group with unaltered pH. The finding that the cells separated themselves into two well-defined groups, rather than remaining as a single population but with intracellular pH values that spread progressively over time into the acid range, suggested that in a particular cell, the transition to an acidic pH occurred relatively rapidly.

Among the mechanisms used by cells to rid themselves of excess H^+ is the vacuolar H^+-ATPase, a proton pump that secretes H^+ from the cytoplasm into intracellular endosomes or the extracellular space (18). In resting neutrophils, the vacuolar H^+-ATPase has very low activity, but its activity is greatly increased when the cells are stimulated by phorbol esters (19). This ATPase may also be up-regulated by G-CSF, because we found that the intracellular pH of the unacidified neutrophil population increased by 0.064 ± 0.017 pH unit (mean ± SE), a relatively small but statistically significant amount ($n = 7$; $P < 0.01$), after exposure to G-CSF for 2–20 hr. To determine if the resistance of G-CSF-treated neutrophils to apoptosis was related to the up-regulation of this proton pump and the corresponding increase in the cells' ability to defend themselves against intracellular acidosis, we examined the effect of bafilomycin, a powerful and specific inhibitor of vacuolar H^+-ATPases (20), on the rate of apoptosis in neutrophils cultured with and without G-CSF. The K_1 for bafilomycin is 0.2 nM; we treated cells with 50 mM bafilomycin, a concentration expected to inhibit >98% of the vacuolar H^+-ATPase activity (20). The results are shown in Fig. 3. Bafilomycin had only a very small effect on the rate of apoptosis in cells incubated without G-CSF, consistent with the low activity of the vacuolar H^+-ATPase in unstimulated cells. The protection conferred by G-CSF, however, was almost completely abrogated by bafilomycin, suggesting that the effect of G-CSF was almost entirely due to the increase in proton export caused by a G-CSF-mediated up-regulation of the cells' vacuolar H^+-ATPase.

An alternative explanation for the results in Fig. 3 is a hitherto unsuspected effect of bafilomycin on signal transduction in G-CSF-treated neutrophils. This possibility was examined by looking for the expected up-regulation of CD11b on the surfaces of the cytokine-treated cells (21). The results (Table 1) showed that surface CD11b levels on G-CSF-treated cells increased as expected when compared with untreated neutrophils and that bafilomycin, this time used at a concen-

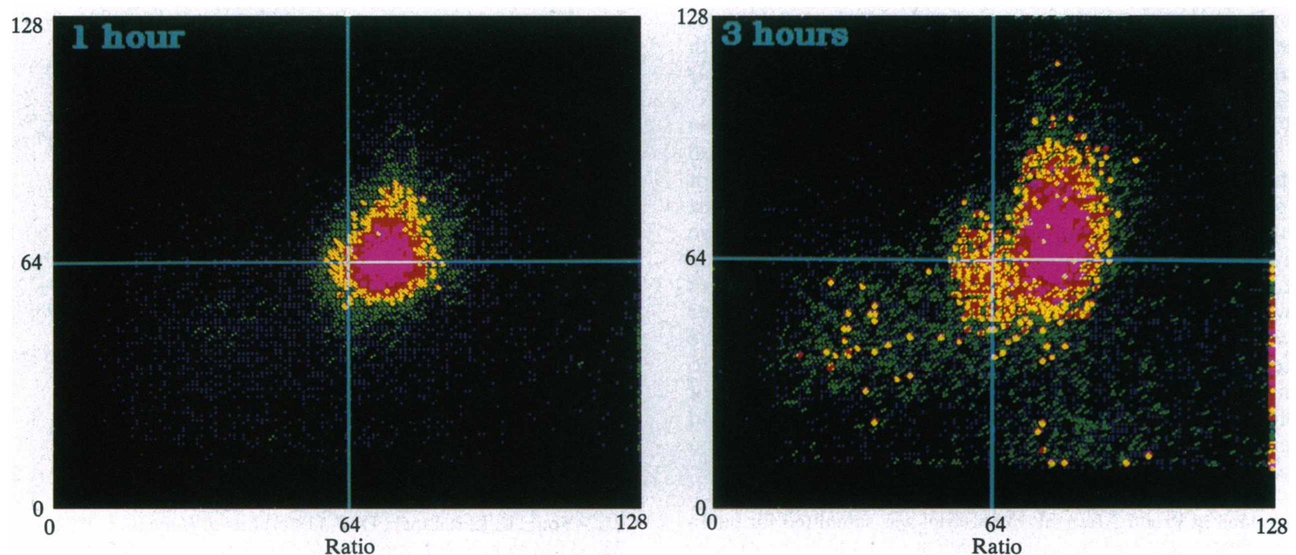

FIG. 2. Flow cytometric analysis of neutrophils for size and pH 3 hr after isolation. Purified neutrophils were cultured 2 hr (see legend to Fig. 1), then loaded with carboxy-SNARF-1, and examined by flow cytometry as above. (*Left*) Fresh cells. (*Right*) Cells cultured for 3 hr. A population of acidified cells not present in the fresh population is apparent in the 3-hr culture.

tration of 200 nM, had no effect on the up-regulation of CD11b expression in either group of cells. Furthermore, all neutrophils in the untreated and bafilomycin-treated groups appeared to respond to G-CSF, because the flow cytometric measurements of surface CD11b levels in both groups of neutrophils showed only a single population of cells, not a dual population as would be expected if some cells responded to G-CSF and others did not.

DISCUSSION

Neutrophils in culture are known to undergo apoptosis at a rate that results in the death of the entire population over the course of 72 hr (16). We have obtained evidence that before the nuclear changes of apoptosis are detected, the intracellular pH of the cultured neutrophils falls from a normal value averaging 7.2 to values averaging 6.2. The change in intracellular pH must occur relatively rapidly, because the cultured cells appear as two discrete groups, not as a single group whose intracellular pH values vary smoothly between the two extremes as would be expected if the fall in intracellular pH were slow.

Neutrophils defend themselves against intracellular acidification in a number of ways. They contain Na^+/H^+ exchanger 1 (NHE-1), the universal Na^+/H^+ antiporter that exports protons by exchanging them for extracellular Na^+ (22). They also contain a voltage-activated channel through which the protons that accumulate through the activity of the respiratory burst oxidase are eliminated (23). Finally, they contain a bafilomycin-sensitive vacuolar H^+-ATPase whose activity is low in resting cells but increases considerably when the cells are stimulated (19). Our finding that the delay in apoptosis induced by G-CSF is abolished by inhibiting the vacuolar H^+-ATPase strongly suggests that the effect of G-CSF is mediated through an increase in proton export via this H^+-ATPase. Additional evidence that proton export is important to the suppression of apoptosis is the study showing that the Na^+/H^+ exchanger was somehow involved in the suppression of apoptosis in hemopoietic stem cells by stem cell factor and

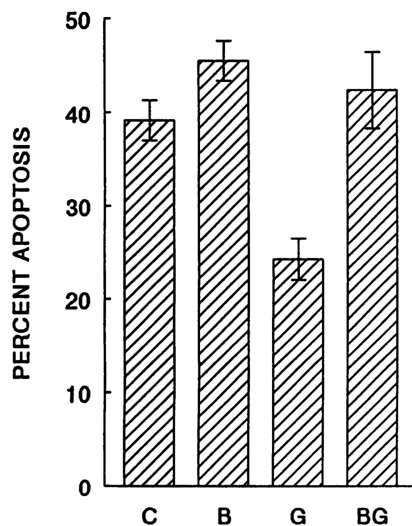

FIG. 3. Effect of G-CSF and bafilomycin on neutrophil apoptosis. Neutrophils were purified and cultured for 20 hr as described in the legend to Fig. 1. Where indicated, the culture medium contained G-CSF at 10^4 units/ml and/or 50 nM bafilomycin. Cultures not containing bafilomycin received DMSO alone [0.05% (vol/vol)]. The cells were then stained with acridine orange and ethidium bromide and examined by fluorescence microscopy. Results represent the mean ± SE of five experiments. C, control; B, bafilomycin alone; G, G-CSF alone; BG, bafilomycin plus G-CSF. Analysis by paired t test gave the following P values: G vs. C, $P < 0.005$; G vs. BG, $P < 0.02$; C vs. BG, not significant.

Table 1. Effect of G-CSF and bafilomycin on neutrophil CD11b levels

	Fluorescence, arbitrary units		
	No G-CSF	G-CSF	Ratio*
No bafilomycin	68.5 ± 4.7	94.0 ± 9.2	1.37 ± 0.15
Bafilomycin	71.1 ± 5.1	94.4 ± 7.4	1.33 ± 0.15

Neutrophils were purified and cultured under the conditions indicated. G-CSF was used at 10^4 units/ml, and bafilomycin was used at 200 nM. Incubations not containing bafilomycin received a similar volume of DMSO as described in the legend to Fig. 3. Cells were incubated for 30 min at 37°C and then placed on ice with and treated with phycoerythrin-conjugated anti-CD11b antibody (Dako) at 1:10 according to the manufacturer's recommendation. They were then washed in PBS/2% bovine serum albumin, fixed with formaldehyde, and analyzed by flow cytometry (13). Results shown are the mean ± SD of three experiments.

*G-CSF/no G-CSF.

granulocyte/macrophage colony-stimulating factor (24). It appears likely that the anti-apoptotic effect of other growth factors on other systems may be mediated through a similar mechanism.

What is the relationship between intracellular acidification and the apoptosis program? We have shown that cultured neutrophils acidify before displaying the nuclear changes of apoptosis, some cells acidifying as early as 3 hr after the start of culture, and that apoptosis in cultured neutrophils can be delayed by the up-regulation of the H^+-ATPase, an enzyme that exports protons from the cytosol. Other studies have shown that many agents that delay apoptosis, including other growth factors, phorbol esters, extracellular matrix, and the protooncogene Ras, are able to activate ion transporters to promote intracellular alkalinization (25–34). On the basis of the foregoing results, representing findings from our own and other laboratories, we propose intracellular acidification as a possible effector of the apoptosis program.

Note Added in Proof. After this manuscript was submitted for publication, papers appeared by Li and Eastman (35) and by Perez-Sala, Collado-Escobar, and Mollinedo (36) that also report correlations between intracellular acidification and apoptosis.

We are grateful to Ms. Judy Nordberg of the Veterans Affairs Medical Center cell sorter facility for expert flow cytometric analysis. This research was supported in part by U.S. Public Health Service Grants AI-24227, AI-30742, AI-28479, and RR-00833 and by the Department of Veterans Affairs. R.A.G. is supported by a Minority Scientist Award from the American Heart Association.

1. Song, C. W., Lyons, J. C., Griffin, R. J., Makepeace, C. M. & Cragoe, E. J., Jr. (1993) *Cancer Res.* **53,** 1599–1601.
2. Maidorn, R. P., Cragoe, E. J., Jr., & Tannock, I. F. (1993) *Br. J. Cancer* **6,** 297–303.
3. Newell, K., Wood, P., Stratford, I. & Tannock, I. (1992) *Br. J. Cancer* **66,** 311–317.
4. Barry, M. A., Reynolds, J. E. & Eastman, A. (1993) *Cancer Res.* **53,** 2349–2357.
5. Grinstein, S., Woodside, M., Sardet, C., Pouyssegur, J. & Rotin, D. (1992) *J. Biol. Chem.* **267,** 23823–23828.
6. Bernardi, G. & Griffe, M. (1964) *Biochemistry* **3,** 1419–1426.
7. Barry, M. A. & Eastman, A. (1993) *Arch. Biochem. Biophys.* **300,** 440–450.
8. Lamers, M. C., DeGroot, E. R. & Roos, D. (1981) *Eur. J. Immunol.* **11,** 757–764.
9. Haslett, C., Guthrie, L. A., Kopaniak, M. M., Johnston, R. J. & Henson, P. M. (1985) *Am. J. Pathol.* **119,** 101–110.
10. Markert, M., Andrews, P. C. & Babior, B. M. (1984) *Methods Enzymol.* **105,** 358–365.
11. Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M. & Haslett, C. (1989) *J. Clin. Invest.* **83,** 865–875.
12. Duke, R. C. & Cohen, J. J. (1992) *Curr. Protoc. Immunol.* **3,** 17.
13. Bassnett, S., Reinisch, L. & Beebe, D. C. (1990) *Am. J. Physiol.* **258,** C171–C178.
14. Musgrove, E. A. & Hedley, D. W. (1990) *Methods Cell Biol.* **33,** 59–69.
15. Wijsman, J. H., Jonker, R. R., Keijzer, R., Van de Velde, C. J. H., Cornelisse, C. J. & Van Dierendonck, J. H. (1993) *J. Histochem. Cytochem.* **41,** 7–12.
16. Colotta, F., Re, F., Polentarutti, N., Sozzani, S. & Mantovani, A. (1992) *Blood* **80,** 2012–2020.
17. Lee, A., Whyte, M. K. B. & Haslett, C. (1993) *J. Leukocyte Biol.* **54,** 283–288.
18. Forgac, M. (1989) *Physiol. Rev.* **69,** 765–796.
19. Nanda, A., Gukovskaya, A., Tseng, J. & Grinstein, S. (1992) *J. Biol. Chem.* **267,** 22740–22746.
20. Crider, B. P., Xie, Y-S. & Stone, D. K. (1994) *J. Biol. Chem.* **269,** 17379–17381.
21. Yong, K. L. & Linch, D. C. (1993) *Eur. J. Haematol.* **49,** 251–259.
22. Grinstein, S. & Furuya, W. (1986) *Am. J. Physiol.* **259,** C55–C65.
23. Nanda, A. & Grinstein, S. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10816–10820.
24. Caceres-Cortes, J., Rajotte, D., Dumouchel, J., Haddad, P. & Hoang, T. (1994) *J. Biol. Chem.* **269,** 12084–12091.
25. Motyka, B., Griebel, P. J. & Reynolds, J. D. (1993) *Eur. J. Immunol.* **23,** 1314–1321.
26. Walker, P. R., Kwast-Welfeld, J., Gourdeau, H., Leblanc, J., Neugebauer, W. & Sikorska, M. (1993) *Exp. Cell Res.* **207,** 142–151.
27. Hagag, N., Lacal, J. C., Graber, M., Aaronson, S. & Viola, M. V. (1987) *Mol. Cell. Biol.* **7,** 1984–1988.
28. Owen, N. E., Knapik, J., Strebel, F., Tarpley, W. G. & Gorman, R. R. (1989) *Am. J. Physiol.* **256,** C756–C763.
29. Satoh, T., Nakafuku, M. & Kaziro, Y. (1992) *J. Biol. Chem.* **267,** 24149–24152.
30. Duronio, V., Welham, M. J., Abraham, S., Dryden, P. & Schrader, J. W. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1587–1591.
31. Schwartz, M. A., Cragoe, E. J., Jr., & Lechene, C. P. (1990) *J. Biol. Chem.* **265,** 1327–1332.
32. Lindberg, F. P., Gresham, H. D., Schwartz, E. & Brown, E. J. J. (1993) *Cell Biol.* **123,** 485–496.
33. Frisch, S. M. & Francis, H. (1994) *J. Cell Biol.* **124,** 619–626.
34. Bates, R. C., Buret, A., Van Helden, D. F., Horton, M. A. & Burns, G. F. (1994) *J. Cell Biol.* **125,** 403–415.
35. Li, J. & Eastman, A. (1995) *J. Biol. Chem.* **270,** 3203–3211.
36. Perez-Sala, D., Collado-Escobar, D. & Mollinedo, F. (1995) *J. Biol. Chem.* **270,** 6235–6242.