120, 507 (1964).
23. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
24. P. Valent, J. Besemer, M. Muhm, K. Lechner, P. Bettelheim, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 5542 (1989).
25. We thank G. Kreil, V. Small, and M. Feldmann for review of the manuscript, P. Höcker for providing human platelets, G. Achatz for computer-aided sequence comparison, and K. Stone for protein sequence analysis. Supported by grant 3/7556 of the Forschungsförderungsfonds für die Gewerbliche Wirtschaft, Vienna, and by grants P7891 and S6002-BIO of the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich.

21 March 1991; accepted 30 May 1991

Protein Kinase Activity Closely Associated with a Reconstituted Calcium-Activated Potassium Channel

SUNGKWON CHUNG, PETER H. REINHART, BRUCE L. MARTIN, DAVID BRAUTIGAN, IRWIN B. LEVITAN*

Modulation of the activity of potassium and other ion channels is an essential feature of nervous system function. The open probability of a large conductance Ca^{2+}-activated K^+ channel from rat brain, incorporated into planar lipid bilayers, is increased by the addition of adenosine triphosphate (ATP) to the cytoplasmic side of the channel. This modulation takes place without the addition of protein kinase, requires Mg^{2+}, and is mimicked by an ATP analog that serves as a substrate for protein kinases but not by a nonhydrolyzable ATP analog. Addition of protein phosphatase 1 reverses the modulation by MgATP. Thus, there may be an endogenous protein kinase activity firmly associated with this K^+ channel. Some ion channels may exist in a complex that contains regulatory protein kinases and phosphatases.

MODULATION OF NEURONAL ACTIVITY underlies many changes in behavior. Such modulation often results from changes in the activity of membrane ion channels. Ca^{2+}-activated K^+ channels, one subclass of the diverse and widespread family of K^+ channels, are intriguing in this regard because they provide a link between second messenger systems and the membrane potential. Some members of this channel subclass are targets for protein phosphorylation, a well-investigated modulatory mechanism for ion channels (*1*). Adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) (*2*) and protein kinase C (PKC) (*3*) both change the activity of Ca^{2+}-activated K^+ channels in a number of tissues.

We have described the properties of several different types of Ca^{2+}-activated K^+ channels from rat brain incorporated into planar lipid bilayers (*4*). Addition of the purified catalytic subunits of PKA and protein phosphatase 2A (PP-2A) can modulate the gating of two different types of large-conductance Ca^{2+}-activated K^+ channels from brain (*5*). These two channel types have the same unitary conductance (240 pS in symmetrical 150 mM K^+) but differ in their gating kinetics, Ca^{2+} sensitivity, pharmacology, and response to phosphorylation by PKA. We have now investigated the modulation by MgATP of the slower gating (*4–6*) of these large-conductance Ca^{2+}-activated K^+ channels.

We recorded single-channel activity for such a channel under conditions in which the open probability was low (Fig. 1A). When 0.5 mM ATP was added to the cytoplasmic side of the channel, the open probability increased substantially (Fig. 1B). With some channels, as little as 50 μM ATP was enough to produce similar increases in open probability. In 25 separate experiments, 16 channels exhibited a large increase in open probability after the addition of ATP, whereas in 9 experiments ATP was without effect. From among the 16 ATP-modulated channels, we chose for quantitative analysis 6 representative channels in which the starting open probabilities were similar. ATP increased the maximal open probability of these channels from 0.058 ± 0.013 (mean ± SEM) to 0.449 ± 0.161. ATP added to the extracellular side of the bilayer did not alter channel activity.

We next examined the time dependence of the open probability changes induced by ATP (Fig. 2A). In 16 experiments the lag time between the addition of ATP and the increase in open probability varied from channel to channel and ranged from less than 20 s to as long as 3 min. The open probability of the channel after the addition of ATP was not constant but oscillated between lower and higher open probability values (Fig. 2, A and B). In the absence of ATP, we never observed these rapid, large changes in channel open probability (Fig. 2C).

Magnesium was required for the modulation by ATP. When buffer containing 1 mM Mg^{2+} was replaced with Mg^{2+}-free solution containing 20 μM EDTA, 1 mM ATP was no longer able to increase channel activity (n = 5). To examine whether ATP hydrolysis was necessary for the modulation, we tested analogs of ATP. In eight independent experiments, we observed no increase in the open probability in the presence of up to 1 mM of the nonhydrolyzable ATP analog, AMPPNP (adenylylimidodiphosphate). In contrast to AMPPNP, ATPγS [adenosine-5′-*O*-(3-thiotriphosphate)], a hydrolyzable ATP analog that can substitute for ATP in most kinase reactions (*7*), increased the activity of the Ca^{2+}-activated K^+ channel in 12 of 14 experiments (Fig. 3). Quantitative analysis of four representative experiments, with similar initial open probabilities, revealed a greater than tenfold increase in channel open probability in response to ATPγS (from 0.060 ± 0.009 to 0.667 ± 0.083). ATPγS generally took longer to modulate the channel than ATP, consistent with reports that it is used at slower rates in phosphotransferase reactions (*7*). Furthermore, ATPγS induced a more stable, high open probability state (Fig. 3) than did ATP (Fig. 2, A and B).

These results with ATP analogs demonstrated that ATP hydrolysis is required for channel modulation. This is different from the ATP-sensitive channels found in a variety of tissues, in which ATP functions as a ligand to alter channel properties by binding

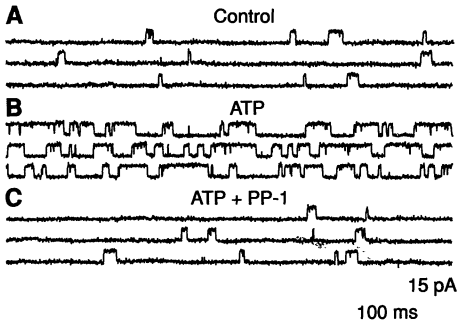

Fig. 1. Activation of a large-conductance Ca^{2+}-activated K^+ channel in lipid bilayers by MgATP and reversal by PP-1. Voltage across the bilayer was clamped at +10 mV, and the reversal potential for K^+ was set to −28 mV. For all traces, channel openings are shown as upward deflections from the closed-level current. (**A**) Representative control openings of the channel before the addition of ATP in three continuous traces. (**B**) Traces 3 min after the addition of 0.5 mM ATP (Calbiochem) to the cytoplasmic side. (**C**) The effect of MgATP is reversed by the addition of 30 nM PP-1 in the continued presence of MgATP.

S. Chung, P. H. Reinhart, I. B. Levitan, Graduate Department of Biochemistry and Center for Complex Systems, Brandeis University, Waltham, MA 02254.
B. L. Martin and D. Brautigan, Section of Biochemistry, Division of Biology and Medicine, Brown University, Providence, RI 02912.

*To whom correspondence should be addressed.

reversibly to an allosteric site, without ATP hydrolysis (*8*). To test the possibility that phosphorylation is involved in the effects of ATP on the Ca^{2+}-activated K^+ channel, we added the purified catalytic subunit of protein phosphatase 1 (PP-1), an enzyme specific for phosphoserine and phosphothreonine residues, to the cytoplasmic side of the channel after channel activation by ATP. PP-1 (10 to 40 nM) reversed the ATP enhancement of activity (Fig. 1C) (n = 4), further supporting the notion that the ATP modulation is the result of phosphorylation. Treating channels with PP-1 without first adding ATP did not induce any change in the open probability (n = 4).

We determined the time dependence of the change in open probability brought about by PP-1 (Fig. 2B). PP-1 induced a decrease in open probability within 1 to 3 min and eventually the open probability returned to the control value. The channel activity remained at the control value for at least 1 hour, which is as long as we can routinely maintain the bilayer. This action of PP-1 was not due to hydrolysis of ATP because ATP is not a substrate for the enzyme (*9*). In contrast to the effect of PP-1, PP-2A (up to 60 nM) failed to reverse the ATP modulation (n = 4). This lack of reversal by PP-2A indicates a degree of substrate specificity described only rarely for these two phosphatases (*5, 10, 11*).

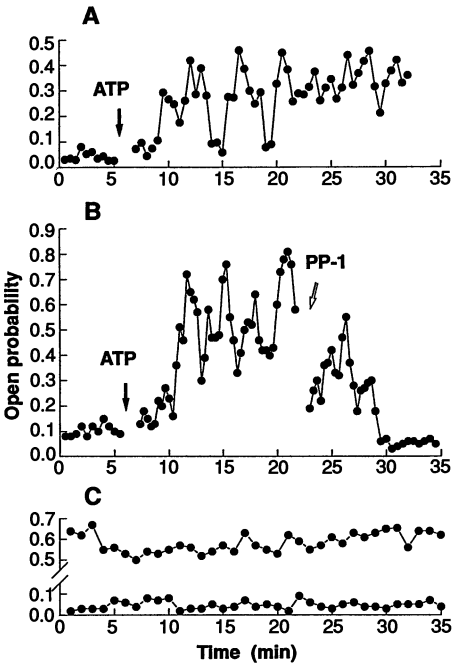

Fig. 2. The time course of the activity of the Ca^{2+}-activated K^+ in the absence or presence of ATP and PP-1. Representative experiments for each experimental condition are shown. The threshold for detecting opening and closing transitions was set to 50% of the open current for each individual event. Channel open probability was calculated continuously, and each point represents the value for a 30-s sweep. (**A**) Activation of the channel by ATP. We adjusted the initial open probability to approximately 0.05 by adding either $CaCl_2$ or EGTA. The filled arrow marks the time at which 0.25 mM ATP was added. (**B**) The reversal by PP-1 of ATP activation. The open arrow marks the time at which 30 nM PP-1 was added. (**C**) Control channel activity. Open probabilities of other channels were adjusted to approximately 0.05 or 0.60 and monitored continuously without addition of ATP or PP-1.

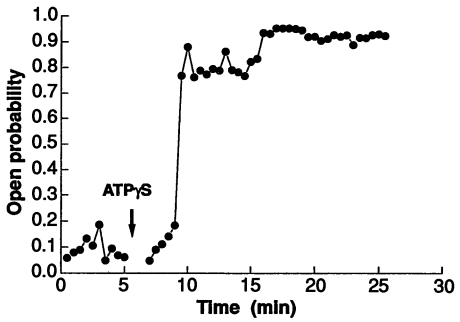

Fig. 3. Time course of the modulation of the Ca^{2+}-activated K^+ channel by ATPγS (Boehringer Mannheim). The initial open probability was adjusted and calculated as in Fig. 2. The filled arrow marks the time at which 0.25 mM ATPγS was added. The results are representative of the 12 (of 14) experiments in which ATPγS modulated channel activity.

Because we did not add exogenous protein kinase in these experiments, an endogenous kinase activity must be responsible for the increase in open probability. ATP can modulate the channel even after the bilayer chamber has been perfused with excess buffer to remove any soluble proteins, as well as to remove the membrane vesicles that have been added to the bilayer chamber (n = 4). The results imply the participation of a protein kinase activity that inserts with the channel into the lipid bilayer. Furthermore, it can be calculated, from measurements of protein kinase activity in our vesicle preparation and from knowledge of protein diffusion rates in lipid bilayers, that random interactions between the channel protein and protein kinase molecules moving independently in the bilayer are too infrequent to explain the time course of modulation by ATP (*12*). A more likely explanation is that the endogenous protein kinase is either part of the channel protein or is so intimately associated with the channel that it diffuses together with it in the bilayer. The oscillations in open probability seen in the presence of ATP (Fig. 2, A and B) may indicate that some PP-1–like activity is also associated with the reconstituted K^+ channel. Another type of K^+ channel can be phosphorylated by ATP in the absence of exogenous protein kinase (*13*).

The endogenous protein kinase activity associated with ATP modulation of this Ca^{2+}-activated K^+ channel remains to be characterized. Because the activity of this same channel can be decreased by PKA, and the actions of PKA are reversed by PP-2A but not by PP-1 (*5*), the endogenous protein kinase is not PKA. Thus, the channel can be modulated in opposite directions by two distinct protein kinases and two distinct protein phosphatases. Our findings suggest that there is a regulatory complex tightly associated with some kinds of ion channels.

REFERENCES AND NOTES

1. I. Levitan, *Annu. Rev. Neurosci.* **11**, 119 (1988).
2. J. DePeyer, A. Cachelin, I. Levitan, H. Reuter, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4207 (1982); D. Ewald, A. Williams, I. Levitan, *Nature* **315**, 503 (1985); J. Lechleiter, D. Dartt, P. Brehm, *Neuron* **1**, 227 (1988); J. Sadoshima, N. Akaike, H. Kanaide, M. Nakamura, *Am. J. Physiol.* **255**, H754 (1988); H. Kume, H. Tokuno, T. Tomita, *Nature* **341**, 152 (1989); S. Sikdar, R. Mcintosh, W. Mason, *Brain Res.* **496**, 113 (1989); M. Gray, J. Greenwell, A. Garton, B. Argent, *J. Membr. Biol.* **115**, 202 (1990).
3. J. Baraban, S. Snyder, B. Alger, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2538 (1985); D. Doerner, T. Pitler, B. Alger, *J. Neurosci.* **8**, 4069 (1988); L. Kaczmarek, *Adv. Second Mess. Phosphorylation Res.* **22**, 113 (1988); M. Shearman, K. Sekiguchi, Y. Nishizuka, *Pharmacol. Rev.* **41**, 211 (1989).
4. P. Reinhart, S. Chung, I. Levitan, *Neuron* **2**, 1031 (1989).
5. P. Reinhart, S. Chung, B. Martin, D. Brautigan, I. Levitan, *J. Neurosci.* **11**, 1627 (1991).
6. Rat brain plasma membrane vesicles were prepared as described (*4*). Briefly, brains were excised and immediately homogenized in an ice-cold isotonic sucrose buffer [0.25 M sucrose, 2.5 mM KCl, 0.1 mM EGTA, 0.1 mM dithiothreitol, and 20 mM Hepes (pH 7.2)]. The homogenate was centrifuged at 800*g* for 3 min, the pellet washed once, and the supernatants centrifuged at 30,000*g* for 10 min. We resuspended the pellet in a hypotonic lysis buffer [0.1 mM dithiothreitol, 2.5 mM KCl, and 5 mM tris-HCl (pH 8.2)] and allowed it to stand on ice for 20 min. The membranes were then rehomogenized, pelleted, and layered under a discontinuous step-gradient of Percoll (Pharmacia) (25, 18, 10, and 0%; v/v). The gradient was centrifuged at 45,000*g* for 2 min, and the membrane fraction banding at the 0 to 10% interface was collected. The Percoll was removed by centrifugation, and the membranes were frozen at −70°C. We obtained single-channel records by incorporating these plasma membrane vesicles into planar lipid bilayers (*4*). Membranes were added to the cytoplasmic solution consisting of 150 mM KCl, 1.05 mM $CaCl_2$, 1 mM EGTA, 1 mM $MgCl_2$, 7 mM KOH, and 10 mM Hepes (pH 7.4). We adjusted the free Ca^{2+} concentration by adding more $CaCl_2$ or more EGTA. The extracellular solution consisted of 0.1 mM EGTA, 7 mM KOH, and 10 mM Hepes (pH 7.4). Ca^{2+}-activated K^+ channels inserted predominantly in one direction, with the Ca^{2+}-sensing side (that is, the cytoplasmic side) facing the solution to which vesicles were added. Of the two large conductance Ca^{2+}-activated K^+ channels in this preparation, only the slower-gating type 2 channel (*4, 5*) was studied in the present experiments. The activity of the faster-gating type 1 channel is not affected by ATP in the absence of exogenous protein kinase. Occasionally, a vesicle preparation contained channels that could not be modulated by ATP or any kinase or phosphatase tested. We do not know the basis for this variability. After the incorporation of a channel, further fusion was suppressed by adding 50 mM KCl to the extracellular side, thereby reducing the transmembrane osmotic gradient. Voltages were expressed in cellular convention, that is, the voltage of the cytoplasmic side with respect to the extracellular side. The data were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices) and digitized at 4 kHz. Catalytic subunits of PP-1 and PP-2A were

purified by a modification of the procedure in (14).
7. D. Gratecos and E. Fischer, *Biochem. Biophys. Res. Commun.* **58**, 960 (1974).
8. F. Ashcroft, *Annu. Rev. Neurosci.* **11**, 97 (1988).
9. M. Meisler and T. Langan, *J. Biol. Chem.* **244**, 4961 (1969); K. Hsiao, A. Sandberg, H.-C. Li, *ibid.* **253**, 6901 (1978).
10. T. Ingebritsen and P. Cohen, *Science* **221**, 331 (1983).
11. A. Fernandez, D. Brautigan, M. Mumby, N. Lamb, *J. Cell Biol.* **111**, 103 (1990).
12. If channel insertion into the bilayer results from random all-or-none fusion of vesicles (packets of channels), then channel density and vesicle fusion rates can be obtained by counting the total number of Ca^{2+}-activated K^+ channels that incorporate into the bilayer [R. Ashley, *J. Membr. Biol.* **111**, 179 (1989)]. Our results indicate that on average a single vesicle contains a single Ca^{2+}-activated K^+ channel. To calculate the maximal number of protein kinase molecules in a vesicle, we assayed the PKC activity in our vesicle preparation. This kinase was chosen because it is the most abundant protein kinase in rat brain. The specific activity of PKC in our washed membrane vesicle preparation was less than 0.3 nmol of phosphate milligram protein^{-1} min^{-1}. Because the turnover number of this enzyme in rat brain is 180 to 500 s^{-1} [K. Huang *et al.*, *J. Biol. Chem.* **263**, 14839 (1988)], there are at most 0.2 to 1.0×10^9 molecules of PKC per microgram of vesicle protein. Because the total number of vesicles in 1 μg of protein is 10^9 to 10^{10} {assuming an average vesicle diameter of 100 nm and a protein to lipid ratio of 1:1 [V. P. Whittaker *et al.*, *Biochem. J.* **90**, 293 (1964)]}, the average number of protein kinase molecules per vesicle is 0.02 to 1.0. Thus, one Ca^{2+}-activated K^+ channel and at most one PKC molecule insert into the 100-μm-diameter bilayer with a single vesicle fusion. If the kinase and channel are inserted into the bilayer adjacent to each other and are free to diffuse randomly, then we can calculate the average distance (r) separating them after t (time) = 10^3 s [we always take at least a 20-min (10^3 s) control record before adding the ATP] by the equation, $r = 2(Dt)^{1/2}$. For proteins in lipid membranes the diffusion constant (D) is 3×10^{-9} cm^2 sec^{-1} [R. Cone, *Nature* **247**, 438 (1974); D. Tank *et al.*, *J. Cell Biol.* **92**, 207 (1982)]; r is approximately 20 μm. If we now put a constraint on the distance the kinase and channel can separate from one another by assuming that there is a reflecting boundary at $b = 2r$ (that is, every molecule that diffuses as far as $2r$, or 40 μm, diffuses back inward), assume a capture radius a of 1 nm of the channel for the kinase, and further assume that every hit between kinase and channel leads to a successful phosphorylation and consequent channel modulation, then we can solve the equation $\tau(r) = (b^2/2D)\ln(r/a)$ [H. Berg and E. Purcell, *Biophys. J.* **20**, 193 (1977)] for the average interaction time $\tau(r)$ to yield 8×10^4 s or approximately 1 day. As there is no such reflecting boundary in the bilayer, the interaction time is probably much longer. That is, it would take at least 1 day on average for an interaction between independently moving channel and kinase molecules to produce channel modulation. However, activation of the channel occurs routinely within 20 to 180 s after adding ATP. Only if kinase molecules and channels are distributed in different vesicle populations with different fusion rates or if the endogenous kinase is very much more abundant than PKC can our results be explained by random interactions between independent kinase and channel molecules in the bilayer.
13. H. Rehm *et al.*, *Biochemistry* **28**, 6455 (1989).
14. D. Brautigan, C. Shriner, P. Grippuso, *J. Biol. Chem.* **260**, 4295 (1985).
15. Supported by NIH grant NS17910 (to I.B.L.) and NIH grant DK31374 (to D.B.). We thank C. Miller for bringing the Berg and Purcell paper (*12*) to our attention, and C. Miller, J. Lisman, D. Oprian, T. Egan, and P. Katz for their comments on the manuscript.

19 April 1991; accepted 12 June 1991

Selective Inhibition of Leukemia Cell Proliferation by BCR-ABL Antisense Oligodeoxynucleotides

CEZARY SZCZYLIK,* TOMASZ SKORSKI,† NICHOLAS C. NICOLAIDES, LIVIA MANZELLA, LUCIA MALAGUARNERA, DONATELLA VENTURELLI, ALAN M. GEWIRTZ, BRUNO CALABRETTA‡

To determine the role of the BCR-ABL gene in the proliferation of blast cells of patients with chronic myelogenous leukemia, leukemia blast cells were exposed to synthetic 18-mer oligodeoxynucleotides complementary to two identified BCR-ABL junctions. Leukemia colony formation was suppressed, whereas granulocyte-macrophage colony formation from normal marrow progenitors was unaffected. When equal proportions of normal marrow progenitors and blast cells were mixed, exposed to the oligodeoxynucleotides, and assayed for residual colony formation, the majority of residual cells were normal. These findings demonstrate the requirement for a functional BCR-ABL gene in maintaining the leukemic phenotype and the feasibility of gene-targeted selective killing of neoplastic cells.

THE PHILADELPHIA CHROMOSOME (Ph[1]) translocation is the most common genetic abnormality in human leukemias (*1*). At the molecular level, the most notable feature is the translocation of the proto-oncogene ABL from chromosome 9 to the breakpoint cluster region (BCR) on chromosome 22, resulting in the formation of BCR-ABL hybrid genes (*2*). The ABL proto-oncogene normally encodes a protein with tyrosine kinase activity (*3*); this activity is augmented in cells carrying BCR-ABL hybrid genes (*4*). The BCR-ABL transcripts are found in the vast majority of chronic myelogenous leukemia (CML) patients and in Ph[1] acute lymphocytic leukemia patients (*5*).

CML invariably progresses from the chronic phase into the blast crisis. In chronic phase CML, the increase in mature and immature myeloid elements in bone marrow and peripheral blood is the most characteristic feature (*6*). Kinetic studies indicate that these abnormal cells do not proliferate or mature faster than their normal counterparts. Instead, the basic defect underlying the abnormal degree of granulopoiesis in CML appears to reside in the expansion of the myeloid progenitor cell pool in bone marrow and peripheral blood (*6*). Nevertheless, the generation of terminally differentiated cells indicates that the process of hematopoiesis retains some normal features. In contrast, during blast transformation, the leukemic cells exhibit a marked degree of differentiation arrest with a "blast" phenotype (7). The role of the BCR-ABL transcript in the pathogenesis of the abnormal hematopoiesis of CML has been investigated by introducing BCR-ABL constructs in mice and demonstrating the occurrence of a CML-like syndrome (*8*). To determine the functional relevance of the BCR-ABL protein for the proliferation of leukemia cells (CML-BC), we selectively inhibited BCR-ABL protein synthesis by an antisense strategy (*9*).

Clonogenic assays of leukemia cells freshly obtained from individuals with myeloid CML-BC often revealed the formation of numerous colonies. In most cases, a BCR-ABL transcript was amplified by the reverse transcriptase–polymerase chain reaction (RT-PCR) technique with a 5′ primer corresponding to 22 bases of BCR exon 2 and a 3′ primer complementary to 22 bases of ABL exon 2. Because blast colonies derived from cells of one patient were particularly numerous, they were pooled for RNA extraction; a region of 257 nucleotides corresponding to the BCR-ABL transcript was amplified by RT-PCR and cloned by blunt-end ligation into the Bluescript SK vector (Stratagene) that had been linearized by Sma I digestion. Sequence analysis of ten

C. Szczylik, T. Skorski, N. C. Nicolaides, L. Manzella, L. Malaguarnera, D. Venturelli, Department of Pathology and Fels Institute for Cancer Research and Molecular Biology, Temple University Medical School, Philadelphia, PA 19140.
A. M. Gewirtz, Department of Pathology and Medicine, University of Pennsylvania, Philadelphia, PA 19103.
B. Calabretta, Department of Pathology and Fels Institute for Cancer Research and Molecular Biology, Temple University Medical School, Philadelphia, PA 19140 and Instituto Regina Elena, Roma 00165, Italy.

Present address for all authors except A. M. Gewirtz, Department of Microbiology, and Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107.
*On leave of absence from Postgraduate Medical Center CSK WAM, Warszawa, Poland.
†On leave of absence from Medical Center of Postgraduate Education, Warszawa, Poland.
‡To whom correspondence should be addressed at the Department of Microbiology, Thomas Jefferson University.