Independent Modes of Natural Killing Distinguished in Mice Lacking *Lag3*

Toru Miyazaki, Andrée Dierich, Christophe Benoist, Diane Mathis

The LAG3 protein has several features in common with CD4, suggesting that it may be important in controlling T cell reactivity. However, mice with a *Lag3* null mutation have now been shown to exhibit a defect in the natural killer cell, rather than the T cell, compartment. Killing of certain tumor targets by natural killer cells from these mice was inhibited or even abolished, whereas lysis of cells displaying major histocompatibility complex class I disparities remained intact. It appears that LAG3 is a receptor or coreceptor that defines different modes of natural killing.

The LAG3 gene, initially discovered in activated natural killer (NK) cells, was later shown to be expressed just as prominently in stimulated T cells (*1*). The gene encodes a transmembrane protein of the immunoglobulin (Ig) superfamily, consisting of four extracellular Ig-like domains, the most external with an unusual looplike insertion. Both *Lag3* and its product (LAG3) exhibit similarities to the CD4 gene and protein: the genes map close together in the mouse and human genomes and the proteins share stretches of sequence homology (*1*, *2*). Evidence also suggests that LAG3, like CD4, interacts with major-histocompatibility complex (MHC) class II molecules (*3*). These similarities have provoked speculation that LAG3 plays a role in controlling T cell responses (*1*, *3*, *4*).

To investigate the function of LAG3, we produced a null mutation in the mouse genome by homologous recombination. A mouse *Lag3* complementary DNA (cDNA) clone was isolated by low-stringency screening of a spleen library with a human *Lag3* probe (*1*), sequenced (*2*), and used to probe a genomic DNA library from embryonic stem cells for *Lag3* gene fragments (Fig. 1A). We then replaced exons 1 to 3 with the neomycin resistance gene (*neo*^{r}) (Fig. 1A). A fragment spanning this region was transferred by electroporation into the D3 line of embryonic stem cells (129 genetic background). Fifteen of 82 neomycin-resistant clones harbored the desired product of homologous recombination, and one of them (clone KE6) was injected into blastocysts to generate chimeras. Chimeric males were crossed with C57BL/6 (B6) females, and the resulting progeny (*Lag3*$^{+/-}$) were intercrossed to generate homozygous mutant mice (*Lag3*$^{-/-}$). Polymerase chain reaction (PCR) analysis of RNA from splenocyte blasts induced by concanavalin A (Con A) confirmed that the mutation abolished *Lag3* expression (Fig. 1B).

At first, the phenotype of the homozygous mutants appeared normal. No developmental or growth abnormalities were observed, and diverse aspects of immune function—in particular, those related to the operation of MHC class II genes—also appeared normal (*2*, *5*). Briefly, normal parameters included (i) positive and negative selection of T cells in the thymus; (ii) numbers and distribution of peripheral CD4$^+$ and CD8$^+$ T cells, their activated and memory subpopulations, and their responses to mitogens and to antigen priming; (iii) generation and function of cytotoxic cells directed against several viruses; (iv) B cell maturation and distribution; and (v) antibody production after primary and secondary immunization.

However, one set of experiments revealed a marked difference between mice that did or did not express *Lag3*: NK activity induced by poly(I)–poly(C) against the YAC-1 tumor line was consistently reduced in the homozygous mutants compared with that in heterozygous (Table 1) or homozygous wild-type littermates. This reduction was partial but reproducible, observed in all of the >25 littermate pairs tested.

It was important to show that this phenotype was not attributable to the mutation itself rather than to linked genes, a concern because *Lag3*, together with the CD4 gene, maps at the boundary of the NK gene com-

Table 1. Effect of *Lag3* disruption on NK cell activity against YAC-1 targets. Lysis of ^{51}Cr-labeled YAC-1 target cells by enriched NK cells (*26*) was determined in duplicate at the indicated effector:target (E:T) ratios in a standard cytotoxicity assay (*6*) at 37°C for 5 hours. Results are expressed as percent-specific cytotoxicity. In experiments 1 and 2, *Lag3*$^{-/-}$ mice were compared with phenotypically wild-type, heterozygous littermates. In experiment 3, the controls were mice heterozygous (phenotypically wild type) for both the *Lag3* mutation and a CD4 null mutation (*8*). Spontaneous ^{51}Cr release by the target cells (18, 22, and 8% in experiments 1 to 3, respectively) has been subtracted from the values shown.

Phenotype	Specific cytotoxicity (%) at E:T ratio				
	200	100	50	25	12
	Experiment 1				
Lag3					
+/−	33	24	16	10	6
+/−	28	17	13	8	7
+/−	41	29	18	13	10
−/−	14	7	4	2	1
−/−	9	6	2	2	1
−/−	18	10	6	3	2
	Experiment 2				
Lag3					
+/−	74	53	38	32	26
+/−	69	47	34	27	20
−/−	43	27	18	14	4
−/−	44	28	18	12	6
	Experiment 3				
Lag3, CD4					
+/−, −/+	32	20	13	9	4
+/−, −/+	28	19	11	7	5
−/−, +/+	17	10	5	2	0
−/−, +/+	11	7	3	1	0

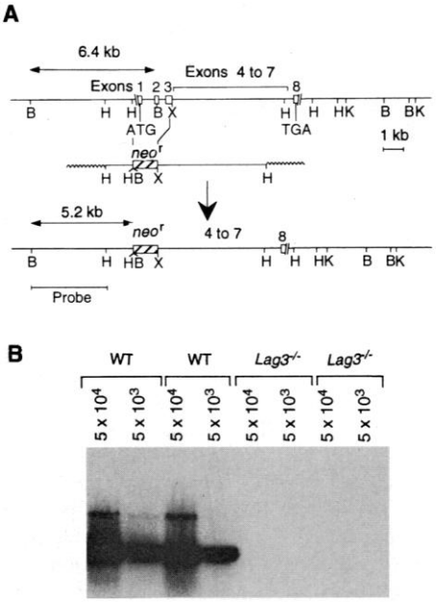

Fig. 1. Disruption of the *Lag3* gene. (**A**) Partial restriction enzyme map of *Lag3* is presented on the top line. Exon positions were determined by hybridization with short oligonucleotide primers. The 5′ and 3′ ends of the *Lag3* mRNA have not been identified, and it is possible that additional exons encode untranslated sequences at either end. Exons 4 to 6 are located in a 5.8-kb Xho I–Hind III fragment but have not been mapped accurately within this fragment. The targeting vector, made by replacing a 2.0-kb Hind III–Xho I fragment that includes exons 1 to 3 with the MC1-*neo* cassette, is shown on the middle line. Wavy lines denote plasmid sequences (pBluescript). The mutant gene resulting from homologous recombination is shown on the bottom. The position of the probe used for Southern (DNA) blot analysis is indicated, as are the 6.4- and 5.2-kb Bam HI fragments that hybridize to this probe in wild-type and mutant DNA, respectively. Restriction enzymes: B, Bam HI; H, Hind III; K, Kpn I; X, Xho I. (**B**) Estimation of *Lag3* expression in the indicated number of Con A blasts from wild-type (WT) or *Lag3*$^{-/-}$ mice by semiquantitative PCR (*24*).

Institut de Génétique et de Biologie Moléculaire et Cellulaire (INSERM, CNRS, ULP), 1 rue Laurent Fries, 67404 Illkirch, C.U. de Strasbourg, France.

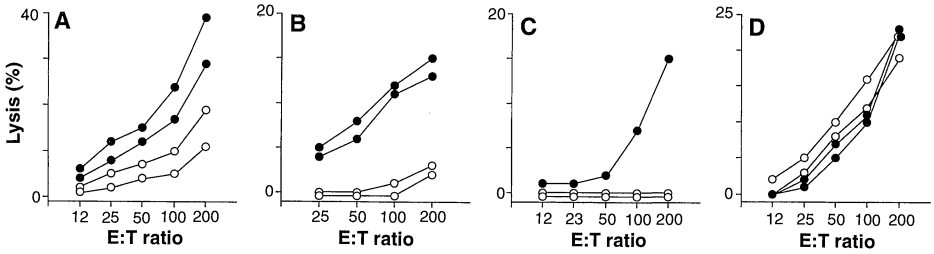

Fig. 2. Mice lacking *Lag3* have decreased NK activity against YAC-1 (**A**), IC-21 (**B**), and J774 (**C**) tumor cells but not against MHC-deficient blasts (**D**). Lysis of target cells was determined as in Table 1. Both IC-21 and J774 cells were obtained from the American Type Culture Collection. MHC-deficient targets were Con A blasts (48-hour culture) from β_2M-deficient mice (*25*). Each curve represents lysis by enriched NK cells (*26*) from individual mice, either *Lag3*$^{-/-}$ animals (○) or heterozygous littermates (●). Spontaneous ^{51}Cr release by the target cells (11, 15, 14, and 12% for YAC-1, IC-21, J774, and Con A blasts, respectively) has been subtracted from the values shown. E:T, effector to target ratio.

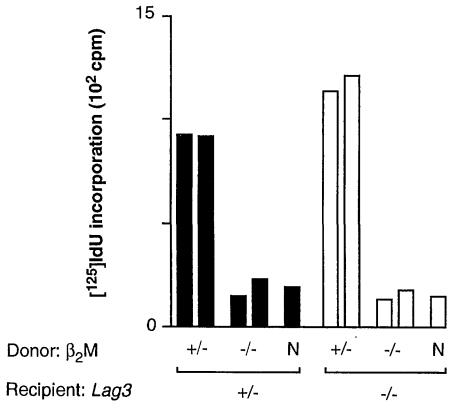

Fig. 3. Mice lacking *Lag3* are fully competent to reject bone marrow grafts from β_2M$^{-/-}$ mice. Incorporation of 5-[^{125}I]iodo-2′-deoxyuridine ([^{125}I]IdU) into recipient spleen cells was measured after transfer to test for rejection of bone marrow grafts by NK cells (*27, 28*). Bone marrow donors were β_2M-deficient mice (−/−) or normal heterozygous littermates (+/−), as indicated; N indicates no graft. *Lag3*$^{-/-}$ homozygotes or heterozygous littermates were used as irradiated recipients.

plex, which contains several genes important in NK cell function, including the Ly49, CD69, and NK-RP1 gene families (*6*). Allelic polymorphism between the B6 and 129 strains at these loci is evidenced by differential staining of cells with monoclonal antibodies and by differential responsiveness to bone marrow allografts (*7*). Thus, the 129 allele of a linked recessive gene might cosegregate with the *Lag3* mutation and confer low NK activity in the homozygous mutants. To exclude this possibility, we derived positive controls that were double heterozygotes: for the *Lag3* mutation on one copy of chromosome 6 and for a CD4 null mutation (*8*) on the other. These mice were phenotypically wild type, but both copies of the NK gene complex originated from the 129 strain. Reduced killing by NK cells from *Lag3*$^{-/-}$ mice compared with that from cells from *Lag3*$^{+/-}$ littermates was still apparent (Table 1).

To evaluate the generality of the NK defect in *Lag3*$^{-/-}$ mice, we measured lysis of other NK targets that differ in various respects. YAC-1 is a T lymphoma line, whereas IC-21 and J774 are macrophage-derived tumor lines; YAC-1 and IC-21 are sensitive to both Ly49$^+$ and Ly49$^-$ NK cells, whereas J774 is sensitive to only Ly49$^-$ cells (*9*). A marked reduction in NK activity was also observed with the two macrophage tumor targets (Fig. 2, A to C).

Different results were obtained with MHC class I–deficient targets. Class I molecules are a major determinant of susceptibility to natural killing: Inhibition of NK activity is induced by the recognition of supratypes of MHC molecules by a family of receptors on NK cells; derepression and lysis occur in the absence of self-MHC molecules (*10*). Thus, Con A blasts from mice devoid of β_2-microglobulin (β_2M) are readily lysed by NK cells from normal mice (*11*). In contrast to their reduced activity against tumor targets, NK cells from *Lag3*$^{-/-}$ mice effectively lysed MHC class I–deficient targets (Fig. 2D). We also assessed the rejection of bone marrow grafts from β_2M$^{-/-}$ mice, another assay of NK reactivity to self-class I deficiency (*12*). Mice displaying class I molecules, whether or not they expressed *Lag3*, rejected bone marrow cells from β_2M$^{-/-}$ animals, as indicated by the representative splenic 5-[^{125}I]iodo-2′-deoxyuridine incorporation assay shown in Fig. 3. Thus, according to both in vitro and in vivo assays, recognition of MHC disparity by the NK system is not affected by the absence of LAG3, in contrast to recognition of certain tumor targets.

These observations suggest that LAG3 might participate in recognition by or signal transduction in NK cells. However, it is possible that LAG3 plays a more indirect role, influencing NK ontogeny or selection (we could not ascertain directly the number of NK cells in *Lag3* mutant mice because the 129 alleles of NK complex genes linked to the mutation are not detected by available antibodies to NK1.1 or Ly49A). Additional evidence indicates that LAG3 directly participates in target cell recognition. First, with mice lacking both LAG3 and β_2M, we showed that *Lag3* is not required for the generation of NK cell–susceptible MHC class I–deficient targets (*13*). Second, we made use of an antiserum generated against a peptide derived from LAG3; this reagent was specific for LAG3, as shown by its ability to stain activated T cells from wild-type but not *Lag3*$^{-/-}$ mice (*14*). This antiserum stained essentially all NK1.1$^+$ splenocytes of B6 mice that had been treated with poly(I)–poly(C) (Fig. 4A); in contrast, NK cells from uninduced spleens were largely negative. The antiserum inhibited the ability of NK cells from normal mice to lyse YAC-1 cells (Fig. 4B), but as expected, it did not affect the residual activity in *Lag3*$^{-/-}$ animals; similar results were obtained with IC-21 and J774 targets (*13*). In contrast, the antiserum had no effect on the killing of β_2M-deficient blasts. The inhibition by the antiserum of YAC-1 lysis by NK cells from normal mice was only partial, even at saturating doses, reminiscent of the partial defect in *Lag3*$^{-/-}$ mice. The inhibitory activity of the antiserum was observed when it was added during the cytotoxicity assay or when it was incubated with the effector NK cells before the assay (Fig. 4C), indicating that LAG3 operates on the NK effectors themselves.

These data indicate that LAG3 plays a role in the lysis of a subset of target cells by NK cells. Such a function is consistent with its expression pattern and with its genomic localization, adjacent to the NK gene complex on mouse chromosome 6 (*2, 6*); indeed, it might now be justified to extend the borders of the complex to include *Lag3*. The precise molecular role of LAG3 remains unclear. It could be an activatory recognition receptor. Like several of the other NK cell recognition molecules (for example, p58 and 2B4) (*15*), LAG3 consists of multiple Ig-like domains; however, its cytoplasmic tail contains no obvious signal transduction motif. Instead, the key to understanding LAG3 function may lie in its similarities to CD4. Thus, LAG3 may function as an NK cell coreceptor. The lysis of IC-21 cells, which is dependent on LAG3, also appears to involve NK-RP1: The ability to kill IC-21 cells is abolished by blocking or loss of NK-RP1 (*16*). These observations suggest that NK-RP1 and LAG3 might function together to effect IC-21 cell lysis, consistent with a coreceptor function.

The more general implications of our data concern the target structures recognized by NK cells. In recent years, the "missing self" model (*17*) has become an accepted paradigm, explaining the NK cell sensitivity of many tumor lines and the

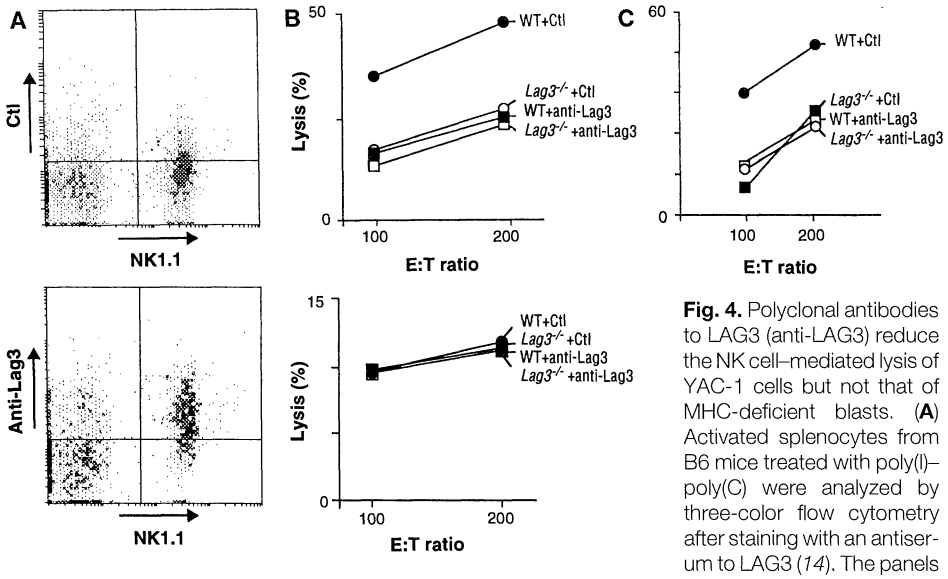

Fig. 4. Polyclonal antibodies to LAG3 (anti-LAG3) reduce the NK cell–mediated lysis of YAC-1 cells but not that of MHC-deficient blasts. (**A**) Activated splenocytes from B6 mice treated with poly(I)–poly(C) were analyzed by three-color flow cytometry after staining with an antiserum to LAG3 (*14*). The panels show the staining of gated $CD3^-IgM^-$ cells by a combination of NK1.1 and either antiserum to LAG3 (bottom) or a control antiserum from a mouse similarly immunized with keyhole limpet hemocyanin (Ctl) (top). (**B**) Killing of YAC-1 (top) or $\beta_2M^{-/-}$ Con A blasts (bottom) by enriched NK cells (*26*) from LAG3-deficient (open symbols) or wild-type (WT) (filled symbols) littermates was assayed in the presence of 5% antiserum to LAG3 (squares) or control antiserum (circles). (**C**) Effector cells were incubated with 10% antiserum to LAG3 or control antiserum and washed before testing in the cytotoxicity assay. Spontaneous ^{51}Cr release by the target cells (16 and 5% for YAC-1 and β2-$M^{-/-}$ blasts, respectively) has been subtracted from the values shown.

phenomenon of hybrid resistance, as well as providing a molecular basis for the relation between inhibitory receptors and MHC class I alleles (*9*, *18*, *19*). However, not all NK cell activity appears to involve recognition of missing class I molecules: (i) NK cell clones have been detected that are apparently oblivious to MHC disparities and levels of expression (*19*, *20*). (ii) NK cell lysis of some tumor lines is only partially or not at all affected by the abundance of class I molecules (*21*). (iii) NK cell activity in class I–deficient mice is reduced but not completely abolished (*11*, *22*).

Our data may provide a framework for these observations, suggesting that the NK system operates in two modes. LAG3 was important for the lysis of tumor targets, whereas the targets whose lysis was independent of LAG3 were nontransformed cells taunting natural killers by an absence of MHC class I molecules. This difference is reminiscent of recent studies showing a differential influence of class I molecules on normal and tumor targets (*23*). The explanation for this dichotomy and for the observation that the inhibition of LAG3 function, through mutation or antibody blockade, did not completely inhibit NK activity may lie in what actually constitutes the dominant trigger for NK action, as well as in the balance between activatory and inhibitory signals. With nontransformed targets, derepression of killer activity in the context of altered or absent MHC molecules may be the primary trigger, a positive signal being conferred relatively nonspecifically by a broadly distributed ligand. With tumor cells, a more potent activatory signal, recognized by a structure that includes LAG3, would be the primary trigger. These two modes could operate in an additive manner; for example, we have observed that the low level of NK activity against YAC-1 targets that persists in $\beta_2M^{-/-}$ mice virtually disappears in $\beta_2M^{-/-}$ and $Lag3^{-/-}$ double mutants (*13*). The two modes could also be interconnected: MHC inhibition of tumor cell lysis has been demonstrated, and we have also shown that killing of the class I–deficient RMA-S tumor line, which is substantially more efficient than killing of its class I–positive parent RMA, is partially affected by the *Lag3* null mutation (*13*); the staining pattern with the antiserum to LAG3, which recognizes essentially all NK cells, irrespective of Ly49 subtype expression, also indicates that LAG3 coexists with the inhibitory receptors on the same cells. LAG3-dependent and -independent modes of natural killing may thus be operational in the same cell.

REFERENCES AND NOTES

1. F. Triebel *et al.*, *J. Exp. Med.* **171**, 1393 (1990).
2. T. Miyazaki *et al.*, in preparation.
3. E. Baixeras *et al.*, *J. Exp. Med.* **176**, 327 (1992); B. Huard, P. Prigent, M. Tournier, D. Bruniquel, F. Triebel, *Eur. J. Immunol.* **25**, 2718 (1995).
4. R. M. Locksley, S. L. Reiner, F. Hatam, D. R. Littman, N. Killeen, *Science* **261**, 1448 (1993).
5. T. Miyazaki, A. Dierich, C. Benoist, D. Mathis, *Int. Immunol.*, in press.
6. W. M. Yokoyama, *Annu. Rev. Immunol.* **11**, 613 (1993); S. F. Ziegler *et al.*, *J. Immunol.* **152**, 1228 (1994); W. M. Yokoyama *et al.*, *ibid.* **147**, 3229 (1991); R. Giorda and M. Trucco, *ibid.*, p. 1701; W. M. Yokoyama, P. J. Kehn, D. I. Cohen, E. M. Shevach, *ibid.* **145**, 2353 (1990); E. R. Stoneman *et al.*, *J. Exp. Med.* **182**, 305 (1995).
7. C. L. Sentman, V. Kumar, G. Koo, M. Bennett, *J. Immunol.* **142**, 1847 (1989).
8. N. Killeen, S. Sawada, D. R. Littman, *EMBO J.* **12**, 1547 (1993).
9. F. M. Karlhofer, R. K. Ribaudo, W. M. Yokoyama, *Nature* **358**, 66 (1992).
10. K. Kärre, *Semin. Immunol. Ser* **5**, 127 (1993); G. Trinchieri, *J. Exp. Med.* **180**, 417 (1994); W. M. Yokoyama, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3081 (1995).
11. N.-S. Liao, M. Bix, M. Zijlstra, R. Jaenisch, D. Raulet, *Science* **253**, 199 (1991); P. Höglund *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10332 (1991).
12. Y. Y. L. Yu, V. Kumar, M. Bennett, *Annu. Rev. Immunol.* **10**, 189 (1992).
13. T. Miyazaki, unpublished data.
14. Antiserum to LAG3 was produced by repeated immunization of $Lag3^{-/-}$ mice with a LAG3-derived 26-residue peptide (Ser-Gly-Gln-Pro-Thr-Pro-Ile-Pro-Ala-Leu-Asp-Leu-His-Gln-Gly-Met-Pro-Ser-Pro-Arg-Gln-Pro-Ala-Pro-Gly-Arg) conjugated to ovalbumin. The antiserum was adsorbed before use with total spleen cells from LAG3-deficient mice, and its specificity was ascertained by its ability to stain Con A blasts from normal, but not from LAG3-deficient, mice.
15. W. M. Yokoyama, *Curr. Opin. Immunol.* **7**, 110 (1995); D. Raulet and W. Held, *Cell* **82**, 697 (1995).
16. K. Bezouska *et al.*, *Nature* **372**, 150 (1994); J. C. Ryan, E. C. Niemi, M. C. Nakamura, W. E. Seaman, *J. Exp. Med.* **181**, 1911 (1995).
17. K. Karre, in *Mechanisms of Cytotoxicity by NK Cells*, R. B. Herberman and D. M. Callewaert, Eds. (Academic Press, Orlando, FL, 1985), pp. 81–96; H. G. Ljunggren and K. Kärre, *Immunol. Today* **11**, 7 (1990).
18. E. Ciccone *et al.*, *J. Exp. Med.* **175**, 709 (1992); E. Ciccone *et al.*, *ibid.* **176**, 963 (1992); M. Colonna, E. G. Brooks, M. Falco, G. B. Ferrara, J. L. Strominger, *Science* **260**, 1121 (1993); M. Cella, A. Longo, G. B. Ferrara, J. L. Strominger, M. Colonna, *J. Exp. Med.* **180**, 1235 (1994); A. Moretta *et al.*, *ibid.*, p. 545.
19. V. Litwin, J. Gumperz, P. Parham, J. H. Phillips, L. L. Lanier, *J. Exp. Med.* **178**, 1321 (1993).
20. F. M. Karlhofer, M. M. Orihuela, W. M. Yokoyama, *ibid.* **181**, 1785 (1995).
21. G. E. Piontek *et al.*, *J. Immunol.* **135**, 4281 (1985); G. Dennert, C. Landon, E. M. Lord, D. W. Bahler, J. G. Frelinger, *ibid.* **140**, 2472 (1988).
22. H. G. Ljunggren, L. Van Kaer, H. L. Ploegh, S. Tonegawa, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6520 (1994).
23. C. L. Sentman *et al.*, *J. Immunol.* **153**, 5482 (1994); A. J. Zajac, D. Muller, K. Pederson, J. A. Frelinger, D. G. Quinn, *Int. Immunol.* **7**, 1545 (1995).
24. Reverse transcription–PCR was performed mostly as described [B. C. Byrne *et al.*, *Nucleic Acids Res.* **16**, 4165 (1988)]. Briefly, cDNA was synthesized for 45 min at 42°C from total RNA, isolated from 5×10^6 Con A blasts, with 200 ng of random hexamers and AMV (avian myeloblastosis virus) reverse transcriptase. PCR was then performed with portions of this cDNA corresponding to 5×10^4 or 5×10^3 cells as template, a 5′-specific primer complementary to a sequence in exon 1 of *Lag3* (5′-GAGGCCCGGGACATAGAGGAGAT), and a 3′-specific primer complementary to a sequence in exon 2 (5′-TAGAAAGTTAGGATCCAGGTTG). The PCR mixture contained deoxynucleoside triphosphates each at a concentration of 200 μM, 2.5 U of Taq polymerase, primers each at a concentration of 1 μM, 50 mM KCl, 10 mM tris-HCl (pH 8.3), 15 mM $MgCl_2$, and 0.1% gelatin in a total volume of 20 μl. PCR products (5 μl) were separated by electrophoresis on a 3% agarose gel, transferred to a nylon membrane, and exposed to a ^{32}P-labeled *Lag3* cDNA fragment.
25. B. H. Koller, P. Marrack, J. W. Kappler, O. Smithies, *Science* **248**, 1227 (1990).
26. To obtain enriched NK cells, we injected mice with 200 μg of poly(I)–poly(C) (Sigma). After 48 hours, spleen cells were isolated and incubated for 30 min on ice with 10% (v/v) culture supernatants of hybridomas

RL72 (IgM antibodies to mouse CD4), 31M (IgM antibodies to mouse CD8), and BP-107 (antibodies to mouse MHC class II A^b) [R. Ceredig, J. W. Lowenthal, M. Nabholz, H. R. MacDonald, *Nature* **314**, 98 (1985); F. W. Symington and J. Sprent, *Immunogenetics* **14**, 53 (1981); M. Sarmiento, A. L. Glasebrook, F. W. Fitch, *J. Immunol.* **125**, 2665 (1980)]. Cedarlane Lo-Tox complement was added to 10% concentration, and the cells were incubated for a further 1 hour at 37°C. After two washes in Dulbecco's minimum essential medium containing 5% fetal bovine serum, live cells were counted and resuspended (1×10^7 cells per milliliter) for use in cytotoxicity assays.

27. W. J. Murphy *et al.*, *J. Exp. Med.* **166**, 1499 (1987).
28. Recipient mice were exposed to γ radiation (9.4 Gy) and then injected intravenously with 5×10^6 bone marrow cells from $\beta_2M^{-/+}$ or $\beta_2M^{-/-}$ mice (*12*). After 5 days, the mice were injected intraperitoneally with 3 μCi of 5-[^{125}I]iodo-2′-deoxyuridine. Isotope incorporated by the spleens of the recipients was determined the next day after extensive rinsing of the spleens with phosphate-buffered saline.

29. We thank E. Long and D. Raulet for helpful comments on the manuscript; D. Littman for the CD4 mutant mice; M. Digelmann, J.-M. Kuhry, P. André, and M. Duval for help in creating the knockout mice; C. Schricke and F. Fischer for help with care of the mice; P. Gerber and C. Ebel for assistance; P. Eberling and F. Ruffenach for peptides and oligonucleotides; and C. Waltzinger for help with flow cytometry. Supported by funds from INSERM, CNRS, and the Centre Hospitalier Universitaire Régional, and by grants to D.M. and C.B. from the Association pour la Recherche sur le Cancer and the Human Frontier Science Program. T.M. was supported by a fellowship from the Human Frontier Science Program Organization.

24 October 1995; accepted 18 January 1996

A Mammalian Histone Deacetylase Related to the Yeast Transcriptional Regulator Rpd3p

Jack Taunton, Christian A. Hassig, Stuart L. Schreiber*

Trapoxin is a microbially derived cyclotetrapeptide that inhibits histone deacetylation in vivo and causes mammalian cells to arrest in the cell cycle. A trapoxin affinity matrix was used to isolate two nuclear proteins that copurified with histone deacetylase activity. Both proteins were identified by peptide microsequencing, and a complementary DNA encoding the histone deacetylase catalytic subunit (HD1) was cloned from a human Jurkat T cell library. As the predicted protein is very similar to the yeast transcriptional regulator Rpd3p, these results support a role for histone deacetylase as a key regulator of eukaryotic transcription.

Reversible acetylation of lysine residues clustered near the NH_2-terminus of nucleosomal histones is thought to modulate the accessibility of transcription factors to their respective enhancer and promoter elements. Transcriptionally silent regions of the genome are enriched in underacetylated histone H4 (*1*), and histone hyperacetylation facilitates the ability of transcription factor TFIIIA to bind to chromatin templates (*2*). Although a histone acetyltransferase gene (*HAT1*) has been identified in yeast (*3*), the molecular entities responsible for histone deacetylation are unknown.

A requirement for a functional histone deacetylase in cell cycle progression has been implicated by the discovery that two cytostatic agents, trapoxin and trichostatin (Fig. 1A), inhibit histone deacetylation in cultured mammalian cells and in fractionated cell extracts. In addition to causing G_1 and G_2 phase cell cycle arrest, these natural products alter gene expression and induce certain mammalian cell lines to differentiate (*4*). Whereas sodium butyrate also has these properties, both trapoxin and trichostatin are five orders of magnitude more potent.

Trapoxin is an "irreversible" inhibitor of histone deacetylase activity, and its molecular structure offers clues as to how it could form a covalent bond with a nucleophilic active site residue. First, trapoxin contains an electrophilic epoxyketone that is essential for biological activity (*5*). Second, the aliphatic epoxyketone side chain is approximately isosteric with *N*-acetyl lysine (Fig. 1A). Trapoxin likely acts as a substrate mimic, with its expoxyketone poised to alkylate an active site nucleophile. We therefore regarded trapoxin as a tool that could reveal the molecular identity of histone deacetylase, so that its role in transcriptional regulation and cell cycle progression could be elucidated.

Tritium-labeled trapoxin was prepared by total synthesis and used to identify trapoxin binding proteins in crude extracts from bovine thymus. We used a charcoal precipitation assay to detect a specific trapoxin binding activity primarily in the nuclear fraction of the extracts (*6*). The binding activity was saturable with nanomolar concentrations of [^{3}H]trapoxin and was not detected in the presence of unlabeled trapoxin. Trichostatin also competed for binding with [^{3}H]trapoxin, which suggests that both of these compounds exert their cellular effects by targeting the same molecule.

If trapoxin and trichostatin induce cell cycle arrest by directly inhibiting histone deacetylase, then their binding and enzymatic activities should copurify. To investigate this possibility, we fractionated nuclear thymus proteins by ammonium sulfate precipitation and MonoQ anion-exchange chromatography (*7*). Two peaks of histone deacetylase activity eluted from the MonoQ column between 250 and 350 mM NH_4Cl (Fig. 1B). Trapoxin binding activity, as revealed by the charcoal precipitation assay (40 nM [^{3}H]trapoxin), precisely coeluted

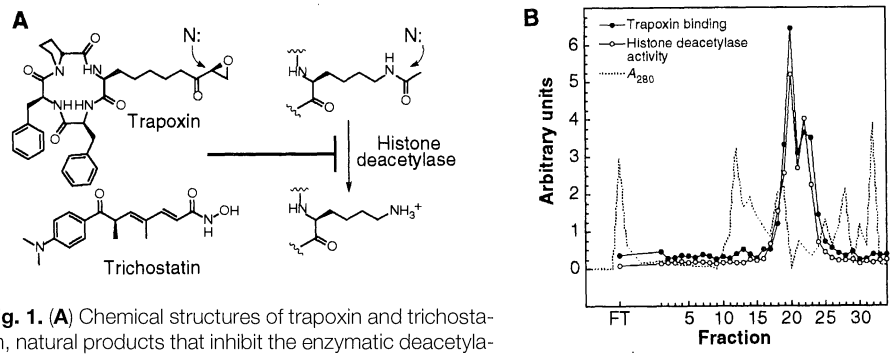

Fig. 1. (**A**) Chemical structures of trapoxin and trichostatin, natural products that inhibit the enzymatic deacetylation of lysine residues near the NH_2-terminus of histones. The epoxyketone side chain of trapoxin is approximately isosteric with *N*-acetyl lysine and likely alkylates an active site nucleophile. (**B**) Copurification of trapoxin binding and histone deacetylase activities. Nuclear proteins from bovine thymus were precipitated with ammonium sulfate and fractionated on a MonoQ column (*7*). Trapoxin binding was assayed by charcoal precipitation with [^{3}H]trapoxin (*6*). For the histone deacetylase assay, a peptide corresponding to bovine histone H4 (residues 1 to 24) was synthesized. The peptide was chemically acetylated with sodium [^{3}H]acetate (5.3 Ci/mmol, New England Nuclear)–BOP reagent (Aldrich) and purified by reversed-phase HPLC. Two microliters of [^{3}H]peptide (~40,000 dpm) was used per 200-μl assay. After incubation at 37°C for 1 hour, the reaction was quenched with 1 M HCl and 0.16 M acetic acid (50 μl). Released [^{3}H]acetic acid was extracted with 600 μl of ethyl acetate and quantified by scintillation counting. Pretreatment of crude or partially purified enzyme with trapoxin or trichostatin (20 nM) for 30 min at 4°C abolished deacetylase activity. A_{280}, absorbance at 280 nm; FT, flow-through.

Howard Hughes Medical Institute, Departments of Chemistry and Chemical Biology, Molecular and Cellular Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA.

*To whom correspondence should be addressed.