TGGGTCCCAGTGA; primer 28, 5′-TGACATCGCC-AAACTGC; primer 29b, 5′-TCCTCTCAGGGTCCA-GGTTA; primer 29, 5′-TCCTGGCAGTGCCTTCA; primer 22, 5′-ACCATCGACCAGCACATC; and primer DS8, 5′-TTGACCATTCACCACATTGGTGTGC.

24. To obtain the genomic structure of the human *SUR* homolog, we screened a normal human lymphocyte genomic bacteriophage library (provided by M. B. Humphrey, Baylor College of Medicine, Houston, TX) (*22*) with a human partial *SUR* cDNA probe, which encompassed 2470 bp at the 3′ end of the cDNA, including the polyadenylation site. Inserts in the bacteriophage clone λG4 were subcloned into pBluescript II (Stratagene). Exon-intron boundaries were defined by comparison of the nucleotide sequences of the human *SUR* gene and the cDNA; these sequences were obtained by means of the dideoxy chain termination method (Sequenase; U.S. Biochemical, Cleveland, OH).
25. Messenger RNA was directly isolated (Oligotex; Qiagen, Studio City, CA) from a freshly frozen pancreatic tissue sample. Reverse transcription (RT) into cDNA was accomplished with the use of random primers (Invitrogen, San Diego, CA) and Superscript II (Gibco BRL). For cloning of the NBF-2 region, an initial PCR amplification with primers 22 (located 5′ of 17) and 29 (*23*) was followed by a second amplification of a portion of the reaction with primers 17 and 29, as described (*4*). The amplified product was cloned into pCR II vector (Invitrogen) and sequenced as above. For detection of the mutation in genomic fragments, 100 ng of genomic DNA was amplified with the use of primers 28 and 29b as above but in the presence of PCR buffer N (Invitrogen), and was then either directly sequenced [S. Khorana, R. F. Gagel, G. J. Cote, *Nucleic Acids Res.* **22**, 3425 (1994)] or cut with 5 U of Msp I (Gibco BRL) at 37°C for 2 hours and run on a 10% polyacrylamide gel. Products on the polyacrylamide gel were visualized by silver staining.
26. Genomic DNA from affected and normal individuals was PCR-amplified with the use of primers 17 and 35al (*23*). Details of the cloning of these PCR products into pRSVhMT2A (*17*) will be provided on request. Constructs were transfected into the human glioblastoma cell line SNB 19 with the use of Lipofectamine (Gibco BRL). RT-PCR analysis was done with primers DS8 and 16, as described (*17*). The plasmids and their cDNA products were sequenced with primer 34al (*23*). Genomic DNA fragments were PCR-amplified with primers 34al and 16 and digested with Nci I, as described above.
27. We thank A. Shenker for helpful discussions and review of the manuscript; M. J. Haddad and G. Cheriyan (both of Saudi ARAMCO) for the surgical pancreatic specimen and for clinical care of their patients; Saudi ARAMCO for samples from Saudi Arabian patients and their families; E. Huang, Y. Yang, G. Gonzalez, and H. Herrera-Sosa for technical assistance; M. B. Humphrey for helpful discussions; and the participating family members. Supported in part by an M. D. Anderson Cancer Center Clinical Research Grant (P.M.T.), NIH grants DK38146 (R.F.G.) and DK44311 (J.B.), and a Juvenile Diabetes Foundation Career Development Award (L.A.B.). P.M.M. was employed by Saudi ARAMCO during the time of this study.

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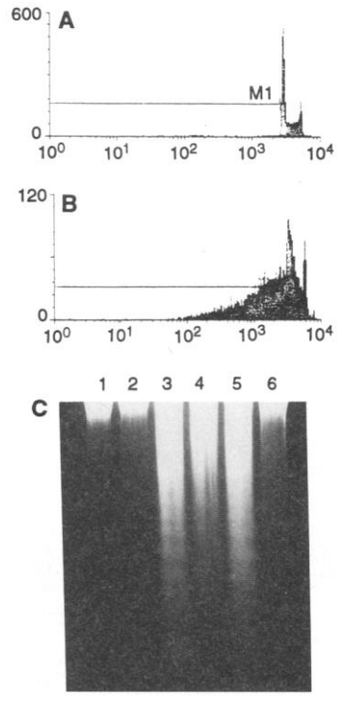

Fig. 1. Apoptosis in Jurkat-Tat cells under low serum conditions. Exponentially growing Jurkat (**A**) and Jurkat-Tat-34 (**B**) cells were shifted from 10% to 0.1% FCS, harvested after 48 hours, and subjected to flow cytometric analysis. Fractions of apoptotic cells in control Jurkat cells and Jurkat-Tat-34 were 9.0 ± 0.7% and 73.7 ± 11.4%, respectively. (**C**) Cellular DNA was extracted and subjected to electrophoresis on a 2% agarose gel to detect nucleosome laddering (*26*). Lane 1, HeLa-Tat; lane 2, HeLa; lane 3, Jurkat-Tat-42; lane 4, Jurkat-Tat-44; lane 5, Jurkat-Tat-34; and lane 6, Jurkat.

Induction of Apoptosis in Uninfected Lymphocytes by HIV-1 Tat Protein

Chiang J. Li,* David J. Friedman, Chuanlin Wang, Valeri Metelev, Arthur B. Pardee

Infection by human immunodeficiency virus–type 1 (HIV-1) is typified by the progressive depletion of CD4 T lymphocytes and deterioration of immune function in most patients. A central unresolved issue in acquired immunodeficiency syndrome (AIDS) pathogenesis is the mechanism underlying this T cell depletion. HIV-1 Tat protein was shown to induce cell death by apoptosis in a T cell line and in cultured peripheral blood mononuclear cells from uninfected donors. This Tat-induced apoptosis was inhibitable by growth factors and was associated with enhanced activation of cyclin-dependent kinases.

Termination of the long latency of AIDS in patients infected with HIV-1 is marked by depletion of CD4 T cells. The mechanism by which HIV-1 kills immune cells remains unresolved (*1*), although a growing body of evidence points to a role for programmed cell death, or apoptosis (*1*, *2*). Increased apoptosis of lymphocytes has been detected in primate models of pathogenic lentiviral infections but not in HIV-1–infected chimpanzees that do not develop disease (*2*), suggesting that apoptosis is important in AIDS pathogenesis. Enhanced apoptosis has also been observed in lymph nodes of HIV-1–infected patients (*1*) and in lymphocytes isolated from AIDS patients (*3*).

The basis of HIV-1–enhanced apoptosis is not understood (*1*–*3*). Apoptotic signals generated by the virus must also be transmitted to uninfected cells, because massive T cell destruction can occur in HIV-1–infected individuals when only 1 in 1000 to 1 in 10,000 lymphocytes are productively infected with the virus (*4*). The mean production rate of HIV-1 virions in infected individuals was reported to be 1.1×10^8 to 6.8×10^8 per day, which was much less than the mean destruction rate of lymphocytes (1.8×10^9 to 2×10^9 per day) (*5*). Also, after administration of an antiviral drug to infected patients, lymphocyte depletion increased at a time when virion titer was still lower than prior to treatment (*5*). Thus, destruction of the infected cells by cytotoxic T lymphocytes appears to be insufficient to account for the observed death of T cells.

The viral envelope protein, gp120, has been suggested to activate T cells and prime them for apoptosis (*6*), although several studies do not support this model (*7*). We hypothesized that the HIV-1 transactivator protein, Tat, participates in the induction of lymphocyte apoptosis during infection. Several properties of Tat are compatible with this idea: (i) it is secreted by infected cells and has biological activity on uninfected cells (*8*); (ii) it is likely to accumulate locally in lymphoid tissue where HIV-1 replication is active even during clinical latency (*5*); (iii) it appears to affect cellular gene expression and function (*9*); and (iv) its role in HIV-1 pathogenesis extends beyond its transcriptional function (*10*).

Most lymphocytes in vivo are quiescent. We therefore investigated the effect

C. J. Li, D. J. Friedman, C. Wang, A. B. Pardee, Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA.
V. Metelev, Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545, USA.
*To whom correspondence should be addressed.

of Tat expression on apoptosis of serum-deprived T cells. Jurkat–T lymphocytes were compared with three Jurkat-derived cell lines that were stably transfected with the HIV-1 *tat* gene driven by the promoter of human BK virus (Jurkat-Tat-34, Jurkat-Tat-42, and Jurkat-Tat-44) (*11*). The expression of Tat was confirmed by immunoblotting and by transactivation assay (*12*). Apoptosis was quantified by flow cytometry after staining of cells with propidium iodide (*13*). After being transferred to a medium containing a low concentration (0.1%) of fetal calf serum (FCS) for 48 hours, the Jurkat-Tat-34 cells, but not the Jurkat cells, showed signs of extensive apoptosis (Fig. 1, A and B). Similar results were obtained with the other Jurkat-Tat-42 and Jurkat-Tat-44 cells (*14*). After 48 hours, DNA in the Tat-expressing cells showed the characteristic fragmentation pattern of apoptosis (Fig. 1C). Cell death was confirmed by trypan blue entry (*14*). Expression of Tat did not affect levels of c-myc (*15*) or the suppressor of apoptosis, Bcl-2 (*16*), arguing against their involvement in Tat-mediated apoptosis (*17*).

At first sight, it is surprising that most Jurkat-Tat cells survive when they express a gene that induces apoptosis. Because deprivation of growth factors greatly increases apoptosis of lymphocytes cultured from HIV-1–infected patients (*18*), we tested whether Tat-induced apoptosis, like that induced by c-myc (*16*), is diminished by growth factors. Far fewer exponentially growing Jurkat-Tat cells, ~24% of Jurkat-Tat-42 cells (Fig. 2, A and B), and ~14% of Jurkat-Tat-34 cells underwent apoptosis as compared to the serum-deprived cells. In these apoptotic cells, Tat induced the typical morphological changes that define apoptosis: chromatin condensation and the formation of apoptotic bodies (Fig. 2C). Expression of Tat by the Epstein-Barr virus promoter in Jurkat cells showed similar apoptotic properties (*19*). In contrast, Tat did not induce apoptosis of an epithelial cell line. A HeLa-Tat cell line (*11*) that constitutively expresses Tat at a level comparable to that in Jurkat-Tat cells did not undergo apoptosis when cultured in optimal (10%) or suboptimal (0.1%) concentrations of FCS (*14*), suggesting that Tat is not an apoptotic signal for every cell type. Other workers have reported that Tat expression protects against apoptosis in epithelial cells and a different Jurkat cell line grown in serum-free conditions (*20*). The reason for this discrepancy is not clear.

Addition of recombinant Tat protein to human peripheral blood mononuclear cells (PBMCs) from seronegative donors (*12*)

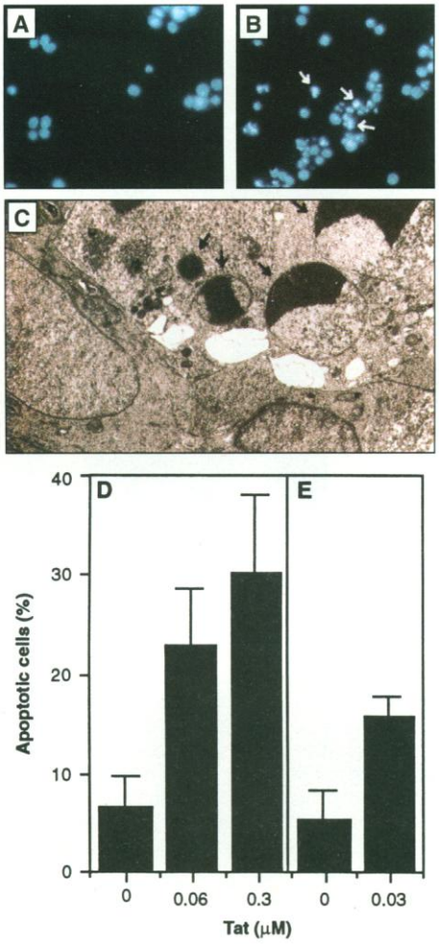

Fig. 2. HIV-1 Tat as an apoptotic signal for exponentially growing Jurkat cells and for PBMCs. (**A**) Jurkat and (**B**) Jurkat-Tat-42 cells were cultured at 1.5 × 10^5 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, and G418 (400 μg/ml). The cells were harvested for apoptotic analysis when they reached a density of about 6 × 10^5 cells/ml on day 3, and after methanol fixation they were stained with Hoechst 33528 (*23*). Arrowheads mark apoptotic nuclei. (**C**) Electron microscopic analysis (magnification, ×9161) of Jurkat-Tat-42; growth conditions were as in (A). Arrowheads mark apoptotic cells displaying chromatin condensation. (**D** and **E**) Apoptosis in human PBMCs activated by exogenous Tat protein. The PBMCs were prepared by Ficoll-Hypaque gradient centrifugation of blood from seronegative donors (*12*) and were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS plus L-glutamine. Blood samples from 10 different donors were examined. Recombinant Tat protein was added after 2 hours of culture. In (D) the Tat protein was >90% pure; in (E) it was >95% pure. After 72 hours, the PBMCs were stained with propidium iodide and the proportion of apoptotic cells determined by flow cytometric analysis (*13*).

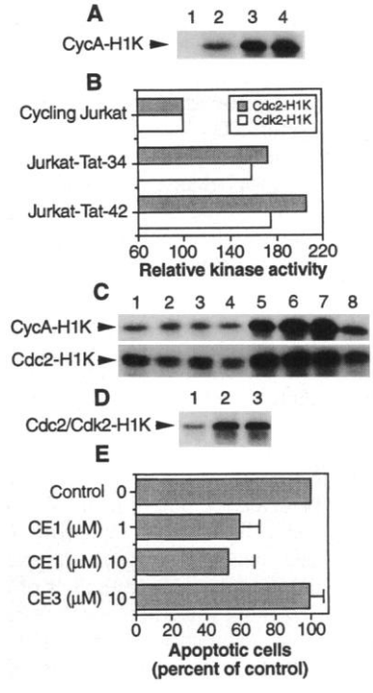

Fig. 3. Activation of Cdks by HIV-1 Tat. (**A**) Activity of CycA-H1K in exponentially growing Jurkat and Jurkat-Tat cells. Lane 1, immunoprecipitation by control serum of nuclear extract from Jurkat-Tat-34 cells. Other lanes contained immunoprecipitates produced by anti–Cyc A. Lane 2, Jurkat; lane 3, Jurkat-Tat-34; lane 4, Jurkat-Tat-42. Cells were harvested during the exponential phase of growth and nuclear extracts were prepared as in (*27*). Ten micrograms of protein extract were incubated with 2 μl of preimmune serum or anti–Cyc A for 2 hours or overnight. The immunoprecipitation and H1 kinase assay were performed as in (*27*). (**B**) Activities of Cdc2-H1K and Cdk2-H1K in proliferating Jurkat-Tat cells. Reactions were with antibodies against the COOH-terminal domains of Cdc2 (LDNQIKKM) (2 μl) or Cdk2 (DVTKPVPHLRL) (2 μl) (*28*). Kinase activity in exponentially growing Jurkat cells was normalized to 100 for comparisons with Jurkat-Tat cells. (**C**) Effect of shift to low serum on Cdc2-H1K and CycA-H1K activity in Tat-expressing cells. Lanes 1 to 4, Jurkat cells; lanes 5 to 8, Jurkat-Tat-34 cells. Cells in the exponential phase of growth were shifted from 10% to 0.1% FCS and harvested at 0 hours (lanes 1 and 5), 12 hours (lanes 2 and 6), 24 hours (lanes 3 and 7), or 48 hours (lanes 4 and 8). The activity of Cdc2-H1K at 12 and 24 hours after serum deprivation increased by 18.2% (lane 6) and 64.8% (lane 7), respectively. CycA-H1K activity increased by 48% by 24 hours (lane 7). The low kinase activity in lane 8 may be due to massive cell lysis after 48 hours in low serum. (**D**) Activation by Tat of total Cdk activity in human PBMCs (*29*). Lane 1, untreated PBMCs; lanes 2 and 3, PBMCs cultured with Tat (0.25 μM) (RepliGen) for 24 hours. (**E**) Inhibition of Tat-induced apoptosis by an antisense oligodeoxynucleotide phosphorothioate (CE1) against the highly conserved region of the human cyclin E, A, and B genes. CE1 is TAACCAATCCAGAAGAATTGCTCGCAT. As a control, we used a scrambled oligodeoxynucleotide phosphorothioate (CE3) of sequence GTAAGTCATTCCACATTTCCCTTCTCC. Jurkat-Tat-34 or Jurkat-Tat-44 cells were plated at 0.5 × 10^5 cells/ml in RPMI 1640 with 10% FCS and G418 (400 μg/ml) in the presence or absence of CE1 or CE3. Cell death was quantified at 72 hours by trypan blue exclusion or flow cytometry (*13*).

also induced apoptosis (Fig. 2, D and E). Tat induced apoptosis in T cells, but not in monocytes (*21*), and CD4 T cells did not appear to be substantially more sensitive than CD8 cells (*21*). Cells that stained negative for both CD4 and CD8 were moderately sensitive (*21*).

Prior to Tat-induced apoptosis, resting PBMCs became bigger and expressed CD25, signs of cell activation (*21*). We therefore hypothesized that Tat might affect a cell cycle regulator. Eukaryotic cell cycle progression is governed by activation and inactivation of cyclin-dependent kinases (Cdks) (*22*). Premature activation of $p34^{Cdc2}$ kinase has been implicated in apoptosis of a transformed T cell line (*23*), and cyclin A–associated Cdks have been shown to be activated during apoptosis in HeLa cells (*23*). We found that cyclin A–associated Cdk activity, measured with histone H1 as the substrate (cyc A–H1K), was four to five times higher in Jurkat-Tat cells than in normally cycling Jurkat cells (Fig. 3A). The activities of both the cyclin A–associated Cdk2 and Cdc2 kinases (*22*) were equally enhanced by Tat expression in Jurkat-Tat cells (Fig. 3B). The Cdk kinase activities in Jurkat-Tat-34 cells were not reduced, but appeared to be augmented further in low serum conditions prior to apoptosis (Fig. 3C).

Exogenous Tat added to PBMCs markedly enhanced total Cdk activity (Fig. 3D). This activation of Cdk was not accompanied by cell cycle progression: >95% of cells remained in G_0/G_1 before and after the 72 hours of treatment with Tat (*21*). Enhanced Cdk activity (Fig. 3C) was seen at 24 hours, prior to the DNA fragmentation and nuclear collapse (*21*) that were detected at 72 hours (Fig. 2, D and E). These results suggest that Tat released from an infected cell may activate Cdk in uninfected bystander cells.

Expression of Tat in cycling Jurkat cells enhanced the activities of Cdks (Fig. 3, A and B). It did not affect their cell cycle progression (*14*), arguing against the idea that the effect of Tat on Cdk activity is secondary to its possible perturbation of the cell cycle.

As a preliminary test of dependence of apoptosis on enhanced Cdk activity, Jurkat-Tat-34 cells were exposed for 72 hours to an antisense oligodeoxynucleotide corresponding to the highly conserved region of human cyclins A, B, and E. Apoptosis was inhibited with the cyclin-specific antisense, but was unaffected by the nonspecific control oligodeoxynucleotide (Fig. 3E). These results suggest that Tat-induced apoptosis is mediated through activation of Cdk activities.

Inappropriate activation of Cdks by Tat is consistent with the observation that uninfected lymphocytes are abnormally activated in HIV-1–infected patients (*1*). Unlike quiescent T cells, activated T cells respond to antigen stimulation by undergoing apoptosis rather than an immune response (*24*). We propose that T cells in HIV-1–infected individuals are stimulated by Tat in lymphoid tissue, which prematurely activates Cdks and prevents the cells from returning to a quiescent state. When, from time to time, the T cells respond to an antigen, they are depleted by apoptosis. As this process is repeated, the depletion of T cells continues progressively. Other factors, such as Fas antigen, may synergize with Tat to induce apoptosis (*25*).

What might be the evolutionary advantage for HIV-1 of encoding a protein that primes its host cells for apoptosis? One possibility is that T cells serve as transient hosts for virus until Tat accumulates to a critical level. Once that level is reached, the host cells will undergo apoptosis. This self-limiting property in host cell killing may be critical for the establishment of a persistent infection.

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28. Abbreviations for the amino acid residues are as follows: D, Asp; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; T, Thr; and V, Val.
29. Human PBMCs were prepared as in (*22*). PBMCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. Cells were treated with recombinant Tat (0.25 μM) for 24 hours and harvested for immunoprecipitation and H1 kinase analysis. For immunoprecipitations, we used 1 μl of anti-Cdc2 and 1 μl of anti-Cdk2.

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