Expression of the c-myc Proto-oncogene Is Essential for HIV-1 Infection in Activated T Cells
By Yu Sun* and Edward A. Clark*‡

From the *Regional Primate Research Center and the ‡Department of Microbiology, University of Washington, Seattle, Washington 98195

Summary
We previously found that activation of primary CD4+ T cells via both the T cell antigen receptor (TCR) and CD28 is required for HIV-1 DNA to be translocated from the cytoplasm to the nucleus. Here we report that expression of c-Myc protein in CD4+ T cells is induced only after such costimulation. In addition, cyclosporin A not only inhibits nuclear import of HIV-1 DNA but also inhibits expression of c-Myc protein. Because of these correlations, we tested whether c-Myc is necessary for nuclear import of HIV-1 DNA. Specific c-myc antisense, but not sense or non-sense, phosphorothioate oligodeoxynucleotides selectively induced the accumulation of two NH2-terminally truncated c-Myc proteins and abolished HIV-1 genome entry into host nuclei. Consequently, both virus replication and HIV-1-induced apoptotic cell death were inhibited. Specific c-myc antisense oligonucleotide inhibited HIV-1 infection under conditions that did not affect cell cycle entry or proliferation. Thus, c-Myc appears to regulate HIV-1 DNA nuclear import via a mechanism distinct from those controlling entry into the cell cycle.

Key words: c-Myc • HIV-1 DNA • T cell • nuclear import • apoptosis

The life cycle of human immunodeficiency virus type 1 (HIV-1) in infected cells can be divided into pre- and postintegrated stages. After HIV-1 binds to the surface of T cells through interaction of the envelope protein gp120 with CD4 and a seven-span transmembrane chemokine receptor, the virus fuses with the host cell, enters the cytoplasm, and disassociates from the cell membrane (1–4). Viral reverse transcription is initiated, then linear double-stranded viral DNA is synthesized, followed by formation of virus preintegration complexes (PIC) (5). Double-stranded viral DNA within these complexes migrates to the host nucleus and integrates into the host cell genome, or forms circular molecules without the capacity to integrate (6, 7). After a latency period, proviruses can be induced by a variety of stimuli to replicate and this in turn can lead to depletion of CD4+ T cells by a process of programmed cell death (8, 9).

Full-length HIV-1 DNA synthesis and translocation to the nucleus are dependent upon activation of T cells (10–16). Two T cell activation signals are required for the synthesis and nuclear translocation of simian immunodeficiency virus (SIV) or HIV-1 DNA (14, 17): one signal through the TCR, which normally regulates the G0 to G1 transition, induces full-length viral DNA synthesis; the second signal, through CD28 or the IL-2 receptor complex (IL-2R), which regulates the G1 to S transition, controls viral DNA entry into the nucleus. Furthermore, cyclosporin A (CSA), a T cell activation inhibitor, and mimosine, a late G1 phase inhibitor, abrogate nuclear import of SIV or HIV-1 genomes (14, 17, 18). However, the cellular factors involved in regulation of this process are not well understood.

One candidate molecule regulated by lymphocyte activation is c-Myc, a transcription factor that has been implicated in regulation of cell activation, differentiation, cell cycle progress, transformation, and apoptosis (19–25). The c-myc proto-oncogene is an immediate-early gene rapidly induced during the G0 to G1 transition in activated T cells (20–22). An IL-2R-dependent signaling pathway is required for induction of c-myc expression (26–30) and CSA suppresses c-myc gene transcription (31). These observations suggested that c-Myc might play a key role in the regulation of HIV-1 DNA nuclear import.

Here we present evidence that expression of c-Myc occurs as a consequence of T cell costimulation. In addition, blocking c-Myc by CSA correlates with this drug’s inhibitory effect on translocation of HIV-1 genome to the nucleus. Furthermore, specific c-myc antisense, but not corresponding sense, non-sense, or scrambled phosphorothioate oligodeoxynucleotides (PS-ODNs), selectively abolished HIV-1 DNA entry into host nuclei and induced 46- and 50-kD truncated c-Myc proteins whose NH2-terminal

1Abbreviations used in this paper: c-MycS, c-Myc short; CSA, cyclosporin A; PS-ODN, phosphorothioate oligodeoxynucleotides; PIC, pre-integration complexes; TUNEL, TdT-mediated dUTP nick-end labeling.
transactivation domains are deleted. As a result, both replication and the cytotoxic effects of HIV-1 were inhibited. Specific c-myc antisense PS-ODN s inhibited HIV-1 infection without affecting cell cycle entry or proliferation, suggesting that c-Myc regulates HIV-1 DNA nuclear import via a mechanism distinct from those controlling entry into the cell.

Materials and Methods

Reagents. PS-ODNs used in this study were synthesized by Oligo Etc. Sequences used were as previously described (23): c-myc antisense, AACGTTGAGGGGCAT, located in exon 2 of initiation site of translation; sense c-myc, ATGCCCTCAACGGT; non-sense, AGTGGCGGAGACTCT; and scrambled, AACGTTGAGGGGCAT containing a GGGG motif (32). The oligonucleotides were dissolved in 30 mM Hepes (pH 7.0). Purified mAbs to human CD8 (G10-1, IgG2a), CD16 (FC-2, IgG2b), CD20 (1F5, IgG2a), and HLA-DR (HB10a, IgG2a) were produced in our lab and used to purify human primary CD4⁺ T cells as previously described (14). Goat anti-mouse IgG conjugated to magnetic microbeads was purchased from Miltenyi Biotec. Reagents. PS-ODNs used in this study were synthesized by Oligo Etc. Sequences used were as previously described (23): c-myc antisense, AACGTTGAGGGGCAT, located in exon 2 of initiation site of translation; sense c-myc, ATGCCCTCAACGGT; non-sense, AGTGGCGGAGACTCT; and scrambled, AACGTTGAGGGGCAT containing a GGGG motif (32). The oligonucleotides were dissolved in 30 mM Hepes (pH 7.0). Purified mAbs to human CD8 (G10-1, IgG2a), CD16 (FC-2, IgG2b), CD20 (1F5, IgG2a), and HLA-DR (HB10a, IgG2a) were produced in our lab and used to purify human primary CD4⁺ T cells as previously described (14). Goat anti-mouse IgG conjugated to magnetic microbeads was purchased from Miltenyi Biotec.

Results and Discussion

In our previous study we found that HIV-1 nuclear import required a CSA-sensitive pathway, and that both TCR and CD28 ligation are essential for this process (14). Similarly, the expression of c-Myc in primary CD4⁺ T cells required costimulation with CD3 and CD28 mAbs (Fig. 1A); neither CD3 nor CD28 ligation alone induced c-Myc expression. Time course experiments showed that c-Myc expression increased by 4 h, peaked at 24 h after costimulation, and was sustained for 48 h. Moreover, CSA inhibited c-Myc expression (Fig. 1A, bottom). Because of this correlation, we tested whether c-Myc might be a key regulator of HIV-1 DNA nuclear import in primary T cells. Since no c-Myc–specific inhibitor is yet available, we used a c-myc antisense PS-ODN to inhibit c-Myc function. By competitively inhibiting HIV-1 reverse transcriptase binding to the virus genome-cellular primer complex, PS-
ODNs have an inhibitory effect on the initiation of HIV-1 reverse transcription in a sequence-independent manner (35, 36). However, sequence-independent PS-ODNs do not exhibit any anti-HIV activity once initiation of virus reverse transcription has begun (35–38). To avoid nonspecific anti–HIV-1 activity of sequence-independent PS-ODNs, we first infected activated CD4⁺ T cells with HIV-1 for 24 h and then administrated graded doses of c-myc antisense, sense, or non-sense PS-ODNs to the infected cells. As shown in Fig. 1B, initiation of reverse transcription (LTR/LTR product) and full-length viral DNA synthesis (LTR/gag product) were not affected by the c-myc antisense PS-ODN.

However, nuclear import of HIV-1 DNA (LTR circles) was blocked by c-myc antisense PS-ODN even at doses as low as 1 μM. Doses below 0.2 μM were less efficient at inhibiting LTR circle formation (data not shown). Neither c-myc sense nor non-sense PS-ODN had any effect on viral DNA nucleus translocation up to 8 μM (Fig. 1B). Consequently, HIV-1–infected cells treated with c-myc antisense PS-ODN did not produce p24 gag protein or undergo apoptosis (Fig. 1C). Under conditions in which HIV-1 had already entered the nucleus (e.g., at 48 h), c-myc antisense PS-ODN did block viral p24 expression (data not shown). Lack of an effect by c-myc antisense PS-ODN on full-length viral DNA synthesis was not simply because the oligonucleotides were added too late to the cultures (after 24 h infection), as full-length viral DNA was not detectable until at least 40 h after HIV infection in activated CD4⁺ T cells (reference 14 and data not shown). Thus, c-myc antisense PS-ODN apparently selectively acts on the stage of HIV-1 DNA nuclear import.

We next studied whether c-myc antisense PS-ODN specifically inhibited full-length c-Myc protein expression. Using mAb 9E10 specific to the COOH-terminal end of c-Myc (39), we consistently observed that in the presence of c-myc antisense, sense, or non-sense PS-ODNs, the two major forms of c-Myc proteins, p64 and p67, remained relatively unchanged (Fig. 2). However, c-myc antisense PS-ODN selectively induced the accumulation of 46- and 50-kD proteins, whose expression levels were higher than...
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that of the full-length c-Myc. Neither the c-myc sense nor non-sense PS-ODN induced accumulation of these two proteins (Fig. 2, top). These data are consistent with previous studies showing that expression of c-Myc short (c-MycS) proteins in some tumor cell lines arises from two translational initiation sites downstream of the full-length c-Myc start codon (40–45). These downstream-initiated c-MycS proteins lack most of the NH2-terminal transactivation domain; they are produced through a leaky scanning mechanism, since optimization of the traditional initiation codon for full-length c-Myc results in less synthesis of the c-MycS proteins (45). Because the c-myc antisense oligonucleotide we used corresponds to the initiation site of full-length c-Myc mRNA, and the two smaller proteins we detected are about the same size as c-MycS isoforms, it seemed likely that the 46- and 50-kD proteins are produced through the same mechanism leading to deletion of the NH2-terminal region. This possibility was substantiated by the fact that antibodies specific to either NH2-terminal phosphorylated Thr58/Ser62 or the whole NH2-terminal region of c-Myc, respectively (bottom). The migration of mol wt markers is indicated on the left. Similar results were obtained in three additional experiments.

Figure 2. c-myc antisense PS-ODN induces accumulation of 46- and 50-kD NH2-terminally truncated c-Myc proteins in anti-CD3 and anti-CD28 activated CD4+ T cells. Human PBL CD4+ T cells were stimulated with CD3 and CD28 mAbs for 24 h followed by incubation with PS-ODNs for another 24 h at indicated concentrations. c-Myc proteins were detected by Western blotting with 9E10 mAb (top), or with an antibody specific to phosphorylated Thr58/Ser62 of c-Myc (middle), or with an antibody specific to the NH2-terminal region of c-Myc, respectively (bottom). The migration of mol wt markers is indicated on the left. Similar results were obtained in three additional experiments.

Figure 3. c-myc antisense PS-ODN has no effect on cell cycle progression or proliferation of T cells under conditions of inhibition of HIV-1 infection. Human PBL CD4+ T cells were stimulated with CD3 plus CD28 mAbs for 24 h and then incubated with PS-ODNs or the cell cycle inhibitor mimosine. (A) At day 4, cells were stained with propidium iodide and DNA content was detected by flow cytometry. The percentages of cells in each cell cycle phase were determined with the use of MCycle plus software (Phoenix Flow Systems). (B) Cells were pulsed with 0.5 μCi of [3H]thymidine for 16 h before harvesting at day 3 and incorporated [3H]thymidine was monitored by beta counter. One of three representative experiments is shown.
PS-ODN most likely is mediated by these NH$_2$-terminally truncated c-Myc proteins. Finally, we tested whether c-myc antisense PS-ODN could inhibit the entry of cell cycle and proliferation induced in primary CD4$^+$ T cells after TCR and CD28 ligation. Treating CD4$^+$ T cells with 6 μM of c-myc antisense oligonucleotide, which efficiently blocked HIV-1 LTR circle formation, could not inhibit cell cycle progression (Fig. 3 A). Similarly, c-myc antisense, sense, and non-sense PS-ODN had no effects on CD4$^+$ T cell proliferation induced by CD3 plus CD28 mAbs (Fig. 3 B). These data are consistent with previous findings that NH$_2$-terminally truncated c-Myc proteins do not interfere with cell growth (45, 49).

The study presented here reveals a novel function of c-Myc for regulation of HIV-1 nuclear import. Blocking of HIV-1 DNA nuclear import by c-myc antisense PS-ODN appeared to be mediated through the presence of 46- and 50-kD NH$_2$-terminally truncated c-Myc proteins, which do not affect cell cycle progression or cell proliferation (Fig. 3, A and B). Our data imply that the mechanism by which c-Myc controls HIV-1 DNA nuclear import is distinct from those controlling cell cycle progression. However, precisely where and how c-Myc is required for HIV-1 DNA nuclear import in proliferating CD4$^+$ T cells remains to be discovered. NH$_2$-terminal-defective c-Myc proteins are able to heterodimerize with Max, translocate to nucleus, repress gene expression, stimulate cellular proliferation, and induce cell apoptosis (49). However, c-Myc proteins are not able to activate gene transcription (49). It is probable that c-Myc regulates HIV-1 DNA nuclear import through its transactivation activity by regulation downstream gene expression. The ability of HIV-1 to infect nondividing cells, such as monocytes, terminally differentiated macrophages, mucosal dendritic cells, or γ-irradiated cells, is believed to be a unique feature since oncoretroviruses only can establish infection when the cells undergo mitosis (50–57). The ability of HIV-1 to infect nondividing cells is presumably related to the fact that its PIC can be recognized by the cell nuclear import machinery (58–61) and actively transported through nucleoporins (62). Moreover, a cellular serine/threonine protein kinase, mitogen-activated protein kinase (MAPK), can associate with HIV-1 PIC to facilitate nuclear targeting of viral DNA (63–66). It is unclear whether HIV-1 DNA nuclear import in proliferating CD4$^+$ T cells is regulated through the identical pathway seen in nondividing cells. A reasonable possibility is that c-Myc affects the expression of genes encoding cellular proteins involved in nuclear transport. Further elucidation of the role of c-Myc in regulation of expression of cellular nuclear importing molecules might help us to understand how c-Myc regulates HIV-1 DNA nuclear import.

We thank Ms. M. Domenowske for preparation of figures; Dr. Aaron Marshall and Ms. Kate Elias for editorial assistance; Drs. James Mullins and Michael Kalze for critical review of the manuscript; Drs. Andrew Craxton, Raymond T. Doty, and Aaron Marshall and Mr. Aimin Jing for helpful discussion; and members of the Clark laboratory for technical assistance.

This work was supported by National Institutes of Health grant R R 00166.

Address correspondence to Edward A. Clark, Regional Primate Research Center, Box 357330, University of Washington, Seattle, WA 98195. Phone: 206-543-8706; Fax: 206-685-0305; E-mail: eclark@bart.rprc.washington.edu

Received for publication 21 O dober 1998 and in revised form 12 February 1999.

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