The CD8+ cell non-cytotoxic antiviral response affects RNA polymerase II-mediated human immunodeficiency virus transcription in infected CD4+ cells

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A CD8+ cell non-cytotoxic antiviral response (CNAR), mediated by a CD8+ cell antiviral factor (CAF), is associated with a long-term healthy state in human immunodeficiency virus (HIV) infection. CNAR/CAF reduces viral transcription without a known effect on specific viral sequences in the HIV genome. In studies to define the mechanism involved in the block in viral transcription, we now report that transcription from the HIV-LTR reporter is reduced in infected CD4+ cells upon treatment with CAF. In agreement with this observation, the amount of RNA polymerase II (RNAPII) on the HIV promoter and other viral regions was strongly diminished in HIV-infected CD4+ cells co-cultivated with CNAR-expressing CD8+ cells. These results demonstrate further that CNAR/CAF has a specific role in regulating HIV transcription and a step during the preinitiation complex assembly appears to be sensitive to CNAR/CAF.

INTRODUCTION

Long-term survival from human immunodeficiency virus (HIV) infection is associated with a CD8+ cell non-cytotoxic antiviral response (CNAR) (Walker et al., 1986). This innate cellular immune activity involves inhibition of viral transcription and is active against all HIV-1, HIV-2 and simian immunodeficiency virus isolates tested (Chen et al., 1993; Levy et al., 1996; Mackewicz et al., 1995; Tomaras et al., 2000; Walker et al., 1986). CNAR is mediated by the production of a soluble CD8+ cell antiviral factor (CAF) (Brinchmann et al., 1990; Levy, 2003; Walker & Levy, 1989). Healthy HIV-infected individuals, many of whom have lived for more than 10 years to over 35 years without treatment, show the presence of CNAR and the production of CAF by their CD8+ cells (Mackewicz et al., 1991; Barker et al., 1998; Gómez et al., 1994). Thus, the characterization of CNAR/CAF and its specific mechanism of action is a major objective of our research.

Previous work has indicated that several regions of the HIV-LTR promoter are not involved in the inhibition of transcription by CNAR/CAF (Bonneau et al., 2008; Shridhar et al., 2014). Deletion of these promoter sequences did not lead to the production of viruses that are resistant to this CD8+ cell antiviral response. These findings have suggested that intracellular transcriptional factors may be targeted by CNAR/CAF and this regulatory action is responsible for the block in HIV transcription.

In approaches to define the processes involved in the block in viral transcription, we have found that HIV-infected cells exposed to CAF or CD8+ cells expressing CNAR have a reduced amount of RNA polymerase II (RNAPII) on the HIV promoter and other viral genetic regions. A role of positive transcriptional elongation factor b (P-TEFb) is suggested, but a direct effect on P-TEFb complexes was not observed. The results suggest that a step during the preinitiation complex assembly appears to be sensitive to CNAR/CAF.

RESULTS

The effect of CAF on P-TEFb-mediated HIV-LTR transcription

To determine the mechanism by which CNAR/CAF affects transcription, a transcriptional reporter system was used (Fig. 1). It consists of a plasmid reporter PG6LTR
When we co-expressed the Gal-CycT1 chimera with pG6LTRCAT in the Jurkat cells, the levels of CAT activity increased 17-fold over basal levels (Fig. 1b, compare bar 2 to bar 1). To assess the effect on the transcriptional activity of the CAT reporter, we incubated transfected cells with CAF-containing culture fluids from CD8+ cells from three CNAR-positive subjects. Strikingly, incubation with CAF fluid decreased reporter activity substantially (Fig. 1b, compare bars 3, 4 and 5 to bar 2). The CD8+ cell fluids from CNAR-negative uninfected subjects did not affect CAT transcription levels (Fig. 1b, compare bars 6 and 7 to bar 2). This experiment indicated that CAF from CNAR-positive subjects can regulate the transcription of an HIV promoter-based reporter system.

**Effect of CAF on P-TEFb**

It is well-established that P-TEFb is associated in two forms in cells: either in the catalytically inactive form in complex with other proteins including Hexim1 (so-called the large complex of P-TEFb) or in a catalytically active free form (small complex) (Peterlin & Price, 2006). Because the above-described HIV-LTR based reporter system is dependent on Cdk9 activity, we incubated CD4+ cells with either CAF positive or negative fluids for 48 h and then separated the protein complexes from the CD4+ lysates by glycerol gradient centrifugation. Western blot analyses of the glycerol gradient fractions with Hexim1 and Cyclin T1 antibodies showed that the amounts of P-TEFb in the large and small complexes remained the same upon culture of the infected CD4+ cells with either CAF-positive or negative fluid (data not shown). Moreover, the total amounts of the Cyclin T1, Cdk9 and Hexim1 proteins were the same in the CD4+ cells treated with both the CAF-positive and negative culture fluids (Fig. S1, available in the online Supplementary Material).

**Chromatin immunoprecipitation (ChIP) studies**

To determine further the effect of CNAR/CAF on HIV transcription, we performed chromatin immunoprecipitation (ChIP) assays measuring the presence of RNAPII on the HIV genome in the infected CD4+ cells co-cultured with CD8+ cells from subjects that showed CNAR activity (Fig. 2). This experiment allowed us to measure the amount of RNAPII on the HIV provirus under the presence or absence of CNAR activity. We designed primers for the ChIP for the HIV promoter (LTR) and for two coding regions of HIV (gag and tat genes) (Fig. 2a). CD4+ cells were activated with phytohaemagglutinin (PHA) and IL-2 and infected with a single round HIV (pNL4-3 ΔEnv GFP virus). The CD4+ cells were then incubated with CD8+ cells from a subject with CNAR or CD8+ cells from a normal uninfected subject that served as a control. After 2 days, the CD8+ cells were removed and the ChIP assay was performed with lysates from the HIV-infected CD4+ cells.
These ChIP studies with RNAPII antibody showed about a tenfold enrichment of RNAPII on the LTR promoter as well as on the tat and gag genes in comparison to the control ChIP with IgG empty beads (Fig. 2b, compare gray and black bars in IgG and RNAPII ChIP). Notably, incubation of the infected CD4+ cells with CNAR-positive CD8+ cells from HIV-infected subjects with CNAR. The results from the current study come from two independent experiments and quantitative PCR (qPCR) was performed in triplicate for each experiment. CNAR denotes results with CD8+ cells from CNAR-positive subjects; CTRL denotes results with CD8+ cells from normal uninfected subjects. The line bars represent mean and SD for two independent studies.

**DISCUSSION**

Previous studies have indicated that the CNAR suppresses HIV replication by blocking viral transcription (Chen et al., 1993; Mackewicz et al., 1995; Tomaras et al., 2000).
studies further demonstrate that CNAR/CAF activity is associated with transcriptional inhibition at the HIV-LTR. First, incubation of CAF-containing fluid from CNAR-positive subjects inhibited transcriptional activity of the integrated HIV-LTR transcriptional reporter while the fluid from the CNAR-negative subjects did not (Fig. 1). This anti-HIV activity of CD8+ cell fluids had no apparent effect on the formation or the amount of P-TEFb complexes (Fig. S1). Second, ChIP analyses of HIV-infected CD4+ cells revealed markedly diminished amounts of RNAPII on the HIV genome (both promoter and coding regions) upon co-culture with CD8+ cells from CNAR-positive subjects (Fig. 2). It is possible that the effects of CNAR/CAF on the virus genome result from a block in transcription of certain cellular proteins needed for HIV transcription. However, the levels of Cdk9 and Cyclin T1 were not substantially affected (Fig. S1). Moreover, previous work in our laboratory and others have shown that CNAR does not affect the level of maintenance genes expressed, such as glyceraldehyde-3-phosphate dehydrogenase and the IL-2 receptor (Mackewicz et al., 2000) nor does this CD8+ cell anti-HIV response suppress reporter protein production in cells transfected with CMV (cytomegalovirus) or SV40 constructs (Shridhar et al., 2014). Therefore, the current results further suggest a previously unrecognized role of CNAR/CAF in the regulation of HIV-specific transcription.

Importantly, HIV transcription is dependent on the kinase activity of P-TEFb (Peterlin & Price, 2006). Moreover, although P-TEFb regulates transcription of many human genes (Chao et al., 2000; Rahl et al., 2010) the HIV-LTR seems to be much more sensitive to P-TEFb activity for optimal HIV transcription (Ott et al., 2011; Peterlin & Price, 2006). Notably, in addition to the well-established role of P-TEFb in transcriptional elongation of HIV and human genes, P-TEFb can regulate the HIV-specific transcription complex assembly at the preinitiation step (Brady & Kashanchi, 2005; Raha et al., 2005). Importantly, in addition to the Hexim1-containing LC, P-TEFb is also found in several other complexes including super elongation and Brd complexes (Peterlin & Price, 2006). These newly discovered P-TEFb complexes differ in their function and kinase activity (including their ability to activate HIV transcription).

We can then speculate that one of those complexes might be important for HIV-specific transcription complex assembly at the preinitiation step and is sensitive to CNAR/CAF. However, given that CNAR/CAF leads to diminished levels of RNAPII on the HIV-LTR promoter and other viral genetic regions (Fig. 2), virtually any step during the preinitiation complex assembly could be sensitive to inhibition by this CD8+ cell non-cytotoxic anti-HIV activity.

**METHODS**

**CAF positive/CAF negative fluid.** The HIV-infected subjects whose CD8+ cells produce CAF came from a cohort of healthy long-term survivors (LTS) who have been part of the Levy laboratory studies for many years (Barker et al., 1998; Levy, 1993). These individuals have been infected for more than 10 years, remain healthy with a CD4+ cell count above 500 cells µl⁻¹ and have a low viral load (< 10 000 RNA copies ml⁻¹). The fluids used were selected at random from several LTS out of at least 20 who have been followed for many years. Healthy uninfected subjects, whose CD8+ cells did not show CNAR or CAF production, were blood donors to the Blood Centers of the Pacific, San Francisco.

Using immunomagnetic (IM) beads (Life Technologies), CD8+ cells were obtained from PBMC from these HIV-infected subjects and stimulated for 3 days with anti-CD3 beads. Fluids were collected and assayed for anti-HIV activity by standard procedures (Mackewicz et al., 1994). Briefly, CD4+ T-cells obtained by IM beads from the PBMC from normal donors were stimulated with phytohaemagglutinin (PHA) (3 µg ml⁻¹) for 3 days, washed and inoculated with the X4-tropic HIV isolate, HIV1EF2. This virus is chemokine-resistant and sensitive to CAF. After 1 h, the CD4+ cells were washed and CD8+ cell fluids were added to the culture. Fluids from the CD4+ cell cultures were changed on day 2 and day 4 post-infection and assayed for virus replication by the reverse transcriptase (RT) assay (Hoffman et al., 1985). Reduction of HIV replication by more than 50% was considered positive. Negative fluids obtained from CD8+ cells from uninfected controls showed less than 15% inhibition of HIV replication.

**Transfection transfection and CAT assay.** A chloramphenicol acetyltransferase (CAT) assay was performed as described previously (Taubet al., 2002). Briefly, Jurkat T-cells were transfected with 0.5 µg of the pCMV-EGFP reporter plasmid and 2 µg of the Gal-CycT1 or Gal-empty vector plasmid using Mirus transIT transfection reagent. After 8 h, the medium was changed and cells were incubated with CAF or control CD8+ cell fluids for 48 h. Subsequently, the CD4+ cells were harvested and the CAT activity in the cell lysate was measured by a scintillation counter. The protein content of the whole-cell lysate for normalization was determined using a BCA protein determination kit (Pierce). In these cell culture studies, no cell toxicity or reduction in cell growth was observed.

**Plasmid constructs.** Full-length pNL4-3-ΔE-EGFP was obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (catalogue number 11100; provided by Drs Haili Zhang, Yan Zhou and Robert Siliciano). The viral envelope pMD.G was obtained as a generous gift from the D. Trono laboratory (Lausanne).

**Transfection for virus recovery.** Culture of 293T cells was performed in Dulbecco’s modified Eagle’s medium (1 x) (Life Technologies) supplemented with 10% FBS, 2 mM l-glutamine and 1 mM sodium pyruvate. Cells were plated at 3.5 x 10⁴ per 100 ml of medium in a 100 mM plate 1 day prior to transfection. Three hours before transfection, the cell culture medium was replaced with fresh growth medium. Transfections were performed using a Profection Mammalian Transfection System-Calcium Phosphate kit (Promega Corporation). Briefly, 15 µg pNL 4-3-ΔE-EGFP and 5 µg pMD.G were used for each transfection in a 100 mm plate. Reagents were combined and incubated according to the protocol. At 16 h post-transfection, the medium was discarded, and fresh medium added to the culture. The culture fluid was then collected 48 and 72 h post-transfection. These fluids were spun at 1300 g for 15 min, and 500 µl samples taken from each collected time point and centrifuged at 12 000 g for 2 h. Supernatants were aspirated and samples were quantified for the amount of the vesicular stomatitis virus (VSV)-HIV-GFP virus by the RT assay (Hoffman et al., 1985).

**ChIP assay with HIV infected CD4+ cells and Western blot analysis.** Approximately 6 x 10⁵ PHA-stimulated CD4+ cells were infected with a single round of VSV-HIV-GFP virus derived as described above. After 1 h, 2.4 x 10⁴ CD4+ cells were washed and

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Evidence for a soluble inhibitor.

Reverse (5987–6011). Glycerol gradient centrifugation was performed forward (1700–1722), reverse (1852–1875); Tat: forward (5862–5886), reverse (5987–6011). Glycerol gradient centrifugation was performed as described previously (Blazek et al., 2011; Taube et al., 2002). Immunoprecipitations were performed with 5 μg of RNAPII antibody (sc-899; Santa Cruz Biotechnology). Quantitative PCRs (qPCRs) were conducted with the Quantitect kit (QIAGEN). Approximately 2 × 10^6 of the infected CD4+ cells were used for the ChIP procedure that was performed as described (Blazek et al., 2011). CD8+ cells from normal uninfected subjects served as controls. For these studies, equal numbers of cells were used and they grew at the same rate. Cell toxicity was assessed by trypan blue dye exclusion and no difference in the experimental versus control cultures was observed. Antibodies used for Western blot analyses were Cdk9 (Santa Cruz Biotechnology; sc484), CyclinT1 (Santa Cruz Biotechnology; sc8126) and Hexim1 (Everest Biotech; EB06964).

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