Rapid Communication

The γc-cytokine regulated transcription factor, STAT5, increases HIV-1 production in primary CD4 T cells

Nithianandan Selliah a, Mingce Zhang a, Dennis DeSimone a, Hellen Kim a, Michael Brunner a, Richard F. Ittenbach b, Hallgeir Rui c, Randy Q. Cron a,d, Terri H. Finkel a,d,*

a Division of Rheumatology, The Children's Hospital of Philadelphia, PA 19104, USA
b Division of Biostatistics, The Children's Hospital of Philadelphia, PA 19104, USA
c Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC 20057, USA
d Department of Pediatrics, The University of Pennsylvania, Philadelphia, PA 19104, USA

Received 8 August 2005; returned to author for revision 6 September 2005; accepted 21 September 2005
Available online 14 November 2005

Abstract

Although HIV-1 (HIV) replicates poorly in non-dividing CD4 lymphocytes, resting T cells contribute to the latent reservoir. The γc-related cytokines reverse this block to HIV infection; however, the molecular mechanisms controlling this process are not understood. We asked whether the γc-cytokine regulated transcription factor, signal transducer and activator of transcription 5 (STAT5), activates HIV transcription. We identified three regions in the long terminal repeat (LTR) as close matches to the STAT5 consensus-binding site and show that STAT5 binds the LTR during HIV infection. Expression of Janus kinase 3 (JAK3) or STAT5 in primary human CD4 T cells activated LTR transcription, while transactivation-incompetent dominant-negative STAT5 inhibited JAK3-induced LTR activity and infection of activated HIV-producing CD4 T-cells. In addition, overexpression of STAT5 increased virus production in unstimulated primary T cells – both the number of p24+ cells and their level of p24 production – suggesting that STAT5 promotes a permissive state for HIV infection. These data may have implications for regulation of latency and therapeutic strategies for control of HIV disease.

© 2005 Elsevier Inc. All rights reserved.

Keywords: HIV; Signal transduction; STAT5; Cytokines; Gene regulation; LTR

Introduction

HIV entry into activated CD4 T cells leads to a productive infection. In contrast, the virus enters but remains latent in naïve or resting CD4 T cells, in the absence of T cell stimulation (reviewed in Blankson et al., 2002; Stevenson, 2003). Although these cells appear resistant to productive infection in vitro, active HIV gene expression has been demonstrated in resting CD4 lymphocytes within peripheral blood and lymphoid tissues of HIV-positive individuals (Blak et al., 2000; Ostrowski et al., 1999; Zhang et al., 1999). Factors that promote infection of resting CD4 T cells might contribute to the regulation of HIV latency and viral spread (Blankson et al., 2002). Recent data suggest that the restriction to viral infection can be reversed by genetic manipulation of the host cell transcription factors, NFAT2 (by constitutive expression) (Kinoshita et al., 1998) or NF-κB (by inhibition of the cellular factor, Murr-1) (Ganesh et al., 2003). In addition, the γc-cytokines increase susceptibility of resting CD4 T cells to HIV infection (Ducrey-Rundquist et al., 2002; Unutmaz et al., 1999), although their mechanism of action has not been defined.

The cytokines that support viral infection, IL-2, IL-4, IL-7 or IL-15 (Unutmaz et al., 1999), bind to the γc-related cytokine receptors. Ligand binding results in tyrosine phosphorylation and activation of the Janus family kinase, JAK3. The latent cytoplasmic transcription factor, STAT5, is recruited to the cytokine receptor and phosphorylated by JAK3. Phosphorylated STAT5 then enters the nucleus to regulate gene transcription (Darnell, 1997). STAT5 is a critical component of the IL-2 receptor-mediated proliferative
signal and regulates expression of mitogenic and survival genes (Lord et al., 2000). STAT5 has not previously been implicated in regulation of the HIV LTR (Pereira et al., 2000).

Here, we show that overexpression of STAT5 promotes a permissive state for HIV infection. We demonstrate that STAT5 binds and activates the viral LTR in stimulated and unstimulated primary CD4 T cells, and that functional expression of STAT5 is correlated with increased virus production. These data provide a mechanism by which cytokines induce susceptibility of resting CD4 T cells to HIV infection.

Fig. 1. STAT5 binds the HIV LTR. (A) Sequence of the HIV 3' LTR. Putative STAT5 binding sites are indicated in bold and underlined. Boxed sites are dual NFκB/NFAT sites. Forward and reverse arrows indicate primers for real-time PCR, which include STAT5 binding sites S2–S3. (B) STAT5 binds the HIV LTR in CD4 T cells in vitro. Nuclear extracts from PHA plus IL-2-stimulated primary human CD4 T cells were incubated with radiolabeled oligonucleotides corresponding to the HIV LTR site S2 (lanes 1–5), without (lane 1) or with unlabeled self-competitor probe (lane 2) or with antibodies specific for STAT1 (lane 3), STAT3 (lanes 4) or STAT5 (lane 5). A representative experiment of 5 performed is shown. Binding of the LTR S2 site is inhibited by unlabeled self-oligonucleotide and by antibody to STAT5 but not by antibodies to STAT1 or STAT3. (C–F) STAT5 binds the HIV LTR in infected CD4 T cells in vivo. (C–D) Protein cross-linked chromatin was isolated from HIV-infected WE17/10 T-cells and immunoprecipitated with antibodies to STAT5, NFκB, or an isotype antibody control. NFκB was used as a positive control and demonstrated the feasibility of this assay to detect transcription factor binding to the LTR in vivo. Immunoprecipitated DNA or a DNA-free negative control was amplified by conventional (C) or real-time (D) PCR using primers and a probe specific to the proximal HIV LTR, including sites S2–S3 (see A). A representative experiment of 3 performed is shown. Note the qualitative and quantitative (8- to 16-fold) increases in PCR amplification of LTR STAT5 sites S2–S3 after immunoprecipitation of bound STAT5 or NFκB, compared to a mouse IgG isotype control. (E–F) Primary human CD4 T cells were activated with PHA plus IL-2, infected with HIV NL4-3, and then stimulated for 2 h with PHA (10 μg/ml; E) or with PMA plus ionomycin (F). ChIP was performed on cross-linked and sonicated DNA using anti-STAT5 (red, green, yellow triplicate lines) or isotype control (violet, purple, blue lines) antibodies. Immunoprecipitated DNA was amplified with primers specific for the proximal HIV LTR. Two representative experiments of four performed are shown. Note the 4- to 8-fold differences (i.e., 2–3 amplification cycles) between STAT5 and control.
HIV-Infected WE17/10 T-cells

Antibody: STAT5 NFκB IgG Buffer

HIV-infected Primary CD4 T-cells

Stimulation:

PHA

PMA + Ionomycin

Fig. 1 (continued).
Results and discussion

STAT5 binds to the HIV LTR in vitro and in vivo

We identified 3 regions in the LTR (sites 1–3, S1–S3; Fig. 1A) as close matches to the STAT5 consensus-binding sequence and demonstrated STAT5 binding to the LTR in vitro by EMSA (Fig. 1B). Nuclear extracts from PHA-stimulated primary human CD4 T cells were incubated with radiolabeled oligonucleotides corresponding to the HIV LTR S2 site. Two gel-retarded complexes were identified by the mobility shift assay (Fig. 1B, lane 1). These complexes were specific in that they were competed away by 100-fold excess of unlabeled (cold) self-probe (Fig. 1B, lane 2). The upper complex migrated identically to STAT5 consensus-binding sequences from the Bcl-xL promoter or the prolactin response element of the β-casein promoter (data not shown). To characterize the STAT proteins bound to this probe, nuclear extracts were first incubated with specific anti-STAT antibodies prior to incubation with the oligonucleotide probe. Antibody directed to STAT5 consistently blocked formation of both bands (Fig. 1B, lane 5), whereas antibody to STAT1 or STAT3 had no effect (Fig. 1B, lanes 3 and 4). Similar results were obtained using the LTR S3, but not the LTR S1, site (data not shown). These in vitro binding studies suggest that STAT5 from primary human T cell extracts binds to the HIV LTR.

To determine whether STAT5 binding to LTR occurs in vivo, we used the ChIP assay (Schubert et al., 2002). We immunoprecipitated sonicated chromatin from an HIV-infected IL-2 dependent CD4 T cell line, WE17/10 (Figs. 1C–D) or from HIV-infected primary human CD4 T cells (Figs. 1E–F) and analyzed immunoprecipitates by conventional or real-time PCR. As detected by ethidium bromide staining, relative PCR amplification was greater after DNA immunoprecipitation by antibody to STAT5 than by control antibody (Fig. 1C), demonstrating LTR binding by STAT5 in vivo. Although we cannot rule out an indirect interaction of STAT5 and LTR, our in vitro data suggest that STAT5 binds directly to the LTR sites assayed here by ChIP (Fig. 1B). We quantified binding of STAT5 by amplifying the immunoprecipitated DNA with primers and a probe designed to detect LTR STAT5 sites S2–S3, using real-time PCR. The STAT5 and NFκB curves showed fewer amplification cycles required to reach threshold levels compared to the mouse isotype control (i.e., 2–3 fewer cycles for primary T cells, 3–4 fewer cycles for the T cell line), demonstrating reproducible levels (4- to 16-fold above background) of STAT5 and NFκB binding to the HIV LTR in vivo. In contrast, we found no evidence of transcription factor binding to the coding region of HIV, using primers and a probe specific to the gag gene (data not shown). Therefore, similar to the in vitro binding studies (Fig. 1B), STAT5 is capable of binding the HIV LTR in vivo in infected primary CD4 T cells.

STAT5 regulates viral transcription

Does STAT5 binding to the HIV LTR alter its function? We transfected unstimulated primary human CD4 T cells or Hela cells expressing the IL-2 receptor (Hela-IL2R) with an LTR-driven reporter construct, with or without co-transfection of JAK3 or STAT5 expression vectors. Expression of STAT5 increased LTR activity in Hela-IL2R cells by more than 200-fold (Fig. 2A), while 2- to 3-fold increases in transcription were observed in primary T cells (Fig. 2B). These increases are similar quantitatively to prior studies of transcriptional activation in primary CD4 T cells (Cron et al., 1999, 2000; Sweetser et al., 1998), possibly due to tighter transcriptional regulation compared to activated tumor cells. Since resting T cells have a limited amount of JAK3, ectopic JAK3 expression also increased LTR activity, likely via auto-phosphorylation and activation of endogenous STAT5 (Fig. 2B). In contrast, expression of transactivation-incompetent DNSTAT5 significantly inhibited JAK3-induced LTR activity (Fig. 2C) and virus expression in PHA-stimulated HIV-infected primary CD4 T cells (Fig. 2D). Importantly, these differences did not appear to be due to increased cell death induced by the DNSTAT5 vector, as measured by trypan blue or scatter change (by flow cytometry) (data not shown). In addition, to correct for possible differences in cell viability induced by the DNSTAT5 expression vector, the calculation of luciferase activity was based upon equal numbers of live cells in each sample. Finally, virus expression in the PHA-stimulated HIV-infected primary CD4 T cells after transfection with DNSTAT5 was analyzed in the live GFP+ cell population, to correct for the possibility of differential cell death and to analyze virus production solely in the transfected cell population. These data suggest that STAT5 regulates transcriptional activation of the HIV LTR in primary CD4 T cells.
STAT5 increases virus expression in primary CD4 T cells

To determine the effect of STAT5 on HIV infection in the absence of T cell stimulation, we expressed STAT5 in unstimulated primary CD4 T cells and measured the late viral gene product, p24, in total cells and in GFP+ (transfected) vs. GFP− (non-transfected) populations. Measurement of cell-associated and supernatant p24 antigen has demonstrated good agreement between the concentration of p24 antigen and virion equivalents measured by RT-PCR (O’Doherty et al., 2000). In STAT5-transfected cells, a maximum increase in HIV production was achieved between 48 and 72 h after electroporation (data not shown). Fig. 3A is representative of 7 replicate experiments, showing a 2- to 3-fold increase in %p24+ cells, in the presence of STAT5. In addition, experiments measuring p24 in culture supernatants by ELISA showed an increase from...
14 ± 12 pg/ml immediately after virus inoculation to 211 ± 25 pg/ml by day 3 post-infection, in STAT5-transfected unstimulated CD4 T cells (n = 3, P = 0.002). These increases, though small, correlate with the effect of STAT5 on LTR activity (Fig. 2B) and are in agreement with the reported effects of \( \gamma \)-cytokines on virus expression in resting primary CD4 T cells (Scripture-Adams et al., 2002; Unutmaz et al., 1999). Importantly, we observed a significant increase both in \%p24+ cells and in levels of p24 expression in STAT5 transfected (GFP+), compared to non-transfected (GFP−) controls (Figs. 3B–C). As above, these differences did not appear to be due to enhanced viability induced by the STAT5 vector; the viability of STAT5-transfected cells was not significantly different from control vector-transfected cells after 3 days of transfection (average percent of viable GFP+ cells = 27.79 ± 3.73 vs. 25.89 ± 3.83, respectively). In addition, in order to analyze virus production solely in the transfected cell population and to correct for the possibility of differential cell death, we gated selectively on the live, transfected (GFP+) cells and assessed p24 positivity, as shown in Figs. 3B and C. These data confirm that STAT5 expression is associated with a higher percentage of p24-positive cells, expressing higher levels of p24 per cell, compared to cells expressing the control vector. Thus, STAT5 increases HIV production in primary CD4 T cells.

In conclusion, we have for the first time demonstrated a role for STAT5 in regulating HIV gene expression. We show that STAT5 binds to the LTR in vivo during HIV infection. This binding activates LTR transcription in otherwise unstimulated primary CD4 T cells and is correlated with increased virus production. STAT5 overexpression increases the number of p24+ cells, as well as their level of p24 production, suggesting that STAT5 promotes a permissive state for HIV infection. While our data demonstrate that STAT5 increases virus production in unstimulated primary T cells, it will be important to determine whether STAT5 induces permissiveness in fully quiescent (G0) T cells or requires early events of activation or cell cycle entry. Of note, STAT5’s role in HIV infection is
supported by our data showing inhibition of virus expression by DNSTAT5 in activated HIV-infected T cells.

Our data may have relevance to recent studies showing susceptibility of resting CD4 T cells to HIV infection in the presence of the γ-cytokines, IL-2, IL-4, IL-7, and IL-15 (Ducrey-Rundquist et al., 2002; Unutmaz et al., 1999). In vitro, HIV enters resting CD4 lymphocytes but, instead of replicating as in activated T cells, remains latent in the absence of T cell stimulation (Chou et al., 1997; Scales et al., 2001; Zack et al., 1992), reviewed in Blankson et al., 2002; Stevenson, 2003; Zack et al., 1992). Although these cells are highly resistant to productive infection, active HIV gene expression has been demonstrated in resting CD4 lymphocytes in vivo, within peripheral blood and lymphoid tissues of HIV-positive individuals (Blaak et al., 2000; Ostrowski et al., 1999; Zhang et al., 2004). The nature of the induced factors that lead to HIV permissiveness and the molecular mechanisms of virus release from resting CD4 T cells are not understood. Barriers that preclude infection include host restriction factors, inefficiency of reverse transcription, energy levels that are too low for effective nuclear import, blocks to integration and lack of activation-dependent host transcription factors (Chiu et al., 2005; Kinoshita et al., 1998; Pereira et al., 2000; Stevenson, 2004). Here, we show that the γ-c-regulated T cell transcription factor, STAT5, binds to and activates the HIV LTR and virus transcription, suggesting at least one mechanism by which γ-cytokines reverse the block to productive infection in resting T cells. This is consistent with recent data suggesting that HIV can accumulate stable long reverse transcripts, albeit inefficiently, and integrate within resting lymphocytes (Stevenson, 2004; Swiggard et al., 2004, 2005). In addition, data showing that the γ-cytokine, IL-7, can drive infected T cells out of latency (Scripture-Adams et al., 2002) argue that the JAK3/STAT5 pathway may play a role in reactivation of latent provirus. Whether STAT5 also acts to reverse the block to reverse transcription (like NFAT2) or to promote integration is currently under investigation.

In conclusion, we have for the first time demonstrated a role for STAT5 in regulating HIV production. Future therapeutic strategies targeting JAK3 or STAT5 (Changelian et al., 2003), in combination with current anti-retroviral therapy, may allow elimination of both productive and latently infected T cells from HIV-infected individuals.

Materials and methods

Isolation of CD4 T cells

CD4 T cells were isolated by negative selection from heparinized venous blood of healthy adult human donors, as described (Hamilton et al., 2003; Selliah and Finkel, 2001). Isolated cells were 90–95% CD3+ CD4+.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were isolated from phytohemagglutinin (PHA)-stimulated human CD4 T cells. EMSA was performed as described (Cron et al., 1999), using LTR oligonucleotides (IDT, Coralville, IA) containing potential STAT5 binding sites (Fig. 1A). Antibodies incubated with extract, 20 min prior to adding probe, were mouse monoclonal anti-STAT1 (Zymed Laboratories, South San Francisco, CA), rabbit polyclonal anti-STAT3 (Upstate Biotechnology, Waltham, MA), or rabbit polyclonal anti-pan STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described (Schubert et al., 2002). WE17/10 T cells (NIH ARRRP, Bethesda, MD) were infected with HIV NL4-3 [multiplicity of infection (MOI) 0.05] and cultured with IL-2 (30 U/ml, ARRRP) for 3 days. Cells were fixed, lysed and sonicated, and soluble chromatin collected as supernatant after centrifugation. Lysates were pre-cleared with salmon sperm DNA–protein A-agarose beads (Upstate Biotechnology) and immunoprecipitated with beads plus anti-STAT5, anti-NFκB (p65; Santa Cruz Biotechnology) or mouse IgG (Pharmingen, San Diego, CA). Immunoprecipitated DNA was eluted and reverse cross-linked, then extracted, precipitated, washed, and resuspended in Tris/EDTA buffer. HIV-1 LTR sequence primers (F, 5'-TTGACAGCCGCCTAG-CATT-3'; R, 5'-CAGGCCTCCCTGGAAAGTC-3'; IDT) were used to amplify immunoprecipitated DNA as template. Samples were done in triplicate with reagents contained in TaqMan Universal Master Mix (PerkinElmer Life Sciences, Boston, Massachusetts) according to the manufacturer’s instructions. Fluorescence signals were detected during each of 40 cycles (denaturing for 15 s at 95 °C, annealing/extension for 1 min at 60 °C) by binding of TaqMan probe (5'-36-FAM/ CATCCGTGGCCCGAGACTG/3' BHQ-1-3') to double-stranded DNA products. Real-time quantitative PCR was done in an ABI Prism 7000 Sequence Detection System (PerkinElmer Life Sciences). Graphs were generated with software included with this system.

Transfections, infections, and luciferase assays

CD4 T cells were transiently transfected with enhanced green fluorescent protein (EGFP) vector (0.5 μg, pEGFP-F, Clontech, Palo Alto, CA) ±STAT5 expression vector (1.5 μg, pEGF-STAT5), using AMAXA technology (Cron, 2003) (AMAXA® Biosystems, Cologne, Germany). This protocol results in 50–80% transfection efficiency of primary human CD4 T cells (Cron, 2003), without observed effects on resting T cell phenotype (Ganesh et al., 2003). Twenty-four hours post-transfection, cells were infected with HIV NL4-3 with DEAE-dextran (MOI 0.05) and analyzed 3 days later by flow cytometry, as described (Rapaport et al., 1998), using labeled KC57 to detect a late viral gene product, p24 antigen. This IgG1 antibody recognizes p55, p39, p33, and p24 kDa proteins of the core antigens of HIV. Prior studies have demonstrated flow cytometric detection of infected lymphocytes in culture and from HIV-infected patients by intracellular staining with anti-p24 antibody (Cory et al., 1987; Costigliola et al., 1992;
Jason and Inge, 1999; Mascola et al., 2002; McSharry et al., 1990; Steele-Mortimer et al., 1990; Vanham et al., 2000). Supernatants collected from infected cultures were assayed for p24 antigen by enzyme-linked immunosorbent assay (ELISA; Beckman Coulter, Inc., Fullerton, CA). For luciferase assays, CD4 T cells were co-transfected as described (Cron et al., 2000) with LTR-firefly luciferase reporter gene (1 μg), JAK3, STAT5 or dominant-negative STAT5 (DNSTAT5) (Ahonen et al., 2000) expression vectors (1.5 μg), and Renilla luciferase-expressing control plasmid (pRL-null, 0.5 μg). Cells were then incubated for 6 h, and IL-2 (100 U/ml) was added for the final 2 h prior to lysis. Cell viability was determined, and samples were normalized to equalize numbers of viable cells prior to lysis.

Acknowledgments

The authors are indebted to Stefania Gallucci, Rick Bushman, Drew Weissman and Una O’Doherty for critical review of the manuscript and to John O’Shea for the kind gift of human STAT5 and JAK3 expression vectors.

Supported by the National Institutes of Health (NIH) P30 AI45008, NIH RO1 AI35513, the Joseph L. Hohlrand Chair, the Bender Foundation (T.H.F.), NIH T32 AR07442 (N.S.), the Mary L. Smith Charitable Trust, the Elizabeth Glaser Pediatric AIDS Foundation (R.Q.C.), the Penn Cancer Center, the NIH AIDS Research and Reference Reagent Program (ARRRP), and the University of Pennsylvania Center for AIDS Research.

The laboratories of R.Q.C. and T.H.F. contributed equally to this study.

References


