Published in final edited form as:

Placenta. 2009 May ; 30(5): 464–467. doi:10.1016/j.placenta.2009.03.003.

Cystatin B Associates with Signal Transducer and Activator of Transcription 1 in Monocyte-derived and Placental Macrophages

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Abstract

Cystatin (CSTB, also known as stefin B), a cysteine protease inhibitor, recently was found to be down-regulated in the proteome of uninfected and HIV-1-infected placental macrophages (PM) and associated with restricted HIV-1 replication in PM but not in monocyte-derived macrophages (MDM). We investigated CSTB interactions with signal transducer and activator of transcription 1 (STAT-1) by immunoprecipitation studies because this molecule is known to activate HIV-1 replication. Since both CSTB and STAT-1 are related to HIV-1 replication, we hypothesized that these proteins could be interacting. We applied immunoprecipitation assays to determine STAT-1–CSTB interaction in uninfected and HIV-1-infected PM as compared with MDM. We found that CSTB associates with STAT-1 in PM and MDM. Further analyses indicated that the levels of STAT-1 tyrosine phosphorylation were higher in PM than MDM. High levels of tyrosine phosphorylation previously have been associated with HIV-1 inhibitory activity. This is the first report to demonstrate that cystatin B interact with STAT-1 and that the levels of STAT-1 tyrosine phosphorylation (but not serine phosphorylation) between uninfected and HIV-infected PM and MDM are differentially regulated.

1. Introduction

Cystatin B (CSTB, or stefin B), an inhibitor of cysteine proteases, was recently found to be down-regulated in the proteome of uninfected and HIV-1-infected placenta macrophages (PM) (1). In that study, we reported for the first time that decreased HIV-1 replication was linked to CSTB siRNA treatment, but the mechanism of this inhibition is unknown. CSTB has been shown to induce the production of nitric oxide (NO) [2,3]. One mechanism for that production is activation of the signal transducer and activator of transcription 1 (STAT-1), which induces iNOS [4,5, and 6]. The reported connection of both CSTB and STAT-1 with NO prompted us to question whether the two could be interacting and affecting HIV-1 replication. Previous reports suggested a role for STAT-1 in HIV-1 viral pathogenesis. The pattern of STAT-1 phosphorylation (in tyrosine or serine) could modulate virus replication. The CD8 T-lymphocyte antiviral factor (CAF) activates STAT-1 tyrosine phosphorylation and inhibits activation of HIV-1 long terminal repeat sequences (LTR), resulting in decreased HIV-1

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transcription [7]. Other studies demonstrate that HIV-1 infection induces STAT-1 serine phosphorylation, and this induction is important for viral pathogenesis [8].

In this study, we performed experiments to determine if STAT-1 interacts with CSTB. Our results indicated that STAT-1 co-immunoprecipitated with CSTB. This finding led us to investigate the pattern of activation of STAT-1 in PM and monocyte-derived macrophages (MDM) and to determine whether the levels or types of STAT-1 phosphorylation could explain the differences in HIV-1 replication levels between the highly permissive MDM and the much less permissive PM [9-10]. The association of low levels of CSTB and STAT-1 phosphorylation patterns in PM could provide new insights into the mechanisms used by the placenta to inhibit viral replication.

2. Materials and Methods

2.1 Antibodies

Mouse anti-CSTB antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-phosphotyrosine STAT-1 antibody (Y701) and STAT-1 were purchased from BD Biosciences (San Jose, CA). Rabbit anti-phosphoserine STAT-1 antibody (Ser727) was obtained from Cell Signaling Technology (Danvers, MA). Mouse anti-beta-actin antibody was obtained from Upstate (Charlottesville, VA).

2.2 Cell cultures and whole cell lysates

PM were isolated from term placentas of HIV-1-, HIV-2-, and hepatitis B-seronegative women as previously described [1,9-10]. Peripheral blood mononuclear cells (PBMC) were obtained from leukopheresis of HIV-1-, HIV-2-, and hepatitis B-seronegative healthy women as previously described [11].

After 7 days in culture, PM and MDM were infected *in vitro* with 25 ng of HIV-1_{BaL} per 2×10^5 cells, as previously described [1,9-10]. Four PM and MDM from different donors were maintained for 12 days in culture and monitored for cytopathic effects. Cells were lysed on ice for 15 minutes with lysis buffer (5 mM Tris-HCl buffer, pH 8.0, 0.1% Triton X-100, and protease inhibitor [Sigma]). Cell lysates were cleared by centrifugation and stored at -80°C . Protein concentration was measured by BioRad D_C Protein Assay (BioRad Laboratories, Hercules, CA).

2.3 Western blot analysis

Twenty micrograms each of uninfected and HIV-1-infected whole cell lysates were diluted with Laemmli sample buffer (BioRad Laboratories, Hercules, CA), and each sample was applied to each well of a 15-well 4%-20% Tris-HCl Ready Gel (BioRad) and transferred to 0.45 μm nitrocellulose membranes (BioRad). After blocking with 3% BSA in TBS, membranes were incubated with mouse anti-CSTB (1:500), mouse STAT-1 (1:500), phospho-STAT-1 (Y701) (1:500), and phospho STAT-1 (S727) (1:500); this step was followed by incubation with the corresponding secondary antibody conjugated with horseradish peroxidase (HRP). Mouse anti-beta-actin antibody (1:10000) was used as the loading control for normalization. The U87 whole cell lysate, whole HeLa cells, and HeLa cells plus IFN lysates were used as positive controls for cystatin B, STAT-1, and phospho-STAT-1 antibodies, respectively (Santa Cruz Biotechnology). Signal was detected using a Visualizer™ Chemiluminescent Substrate HRP detection system (Upstate). The density of protein bands was determined using the Versa Doc System with Quantity One Software (BioRad) and normalized against β -actin. The pattern of STAT-1 phosphorylation was expressed as a ratio between β -actin-normalized phosphorylation levels and β -actin normalized levels of STAT-1. Two separate blots containing three different donors per group were assayed.

2.4 Immunoprecipitation assays

Monoclonal antibodies against CSTB and STAT-1 (Santa Cruz Biotechnology) were used for protein-specific immunoprecipitation from cell lysates using an Exacta homologous IP/WB kit (Santa Cruz Biotechnology), according to the manufacturer's instructions. In brief, whole cell lysates were pre-cleared by incubating with immunoprecipitation beads for 1 hour, and then samples were incubated with antibody-conjugated beads overnight. Following several washes, the immunoprecipitated proteins were resolved and probed as described in the Western blot section. Pre-cleared lysates were incubated with un-conjugated beads as controls for nonspecific binding. Duplicate experiments of four independent donors were performed.

2.5 Statistical analyses

An un-paired two-tailed Student's t-test was used to compare the densitometry values of the blots obtained from Quantity One Software (BioRad). Results are shown as mean $\pm$ SEM.

3. Results

3.1 Immunoprecipitation

We immunoprecipitated CSTB and tested its association with STAT-1 (Figure 1, panel A). When we immunoprecipitated CSTB from uninfected and HIV-1-infected PM and from uninfected and HIV-1-infected MDM and tested the immunoprecipitates by Western blotting for CSTB and STAT-1, we found a band of 83 kDa corresponding to STAT-1 and the CSTB 11 kDa band (Figure 1A). When we immunoprecipitated STAT-1 from uninfected PM and MDM and from HIV-1-infected PM and MDM, and tested the immunoprecipitates for STAT-1 and CSTB, we found an immunoreactive band for STAT-1 in both PM and MDM but a CSTB band was found only in MDM (Figure 1, panel B). The absence of a CSTB band in uninfected and HIV-infected PM could be due to the fact that CSTB levels are lower in PM than in MDM, as demonstrated previously [1]. On the basis of the CSTB association with STAT-1, we hypothesized that PM and MDM could also have different activation levels of this signal transducer.

To determine if PM and MDM have differences in STAT-1 levels, we measured both total protein and their phosphorylated forms by Western blots (Figure 1, panel C). We compared the level of STAT-1PY701 to total STAT-1 and found that both uninfected ($p=0.02$) and HIV-infected ($p=0.03$) PM have higher levels of STAT-1PY701 than do uninfected and infected MDM (Figure 1, panels C1 and C5). We did not detect any difference between PM and MDM in the level of STAT-1P Ser727 (Figure 1, panels C2 and C6).

4. Discussion

This study was designed to determine the interaction of CSTB with STAT-1 in both uninfected and HIV-1-infected MDM and PM. STAT-1 is an important molecule for HIV-1 replication [12]. Our data indicate an association of CSTB with STAT-1 and suggest it could influence HIV-1 viral life cycle. We investigated the activation levels of STAT-1 in PM and MDM to determine if they could account for differences in HIV-1 replication. To our surprise the level of STAT-1PY701 was higher in PM than MDM. The high levels of STAT-1 tyrosine phosphorylation could represent a pathway of HIV-1 restriction in PM, and although levels of STAT-1 tyrosine phosphorylation decrease after HIV-1 infection, they are still higher than in MDM ($p=0.03$). The downregulation of STAT-1PY701 upon HIV-1 infection in PM suggests the virus changes the pattern of activation toward a serine phosphorylation, reported to be augmented by HIV-1 infection [8]. How this shift in phosphorylation patterns benefits the virus is not completely understood, but certainly this question should thoroughly be explored. STAT-1PY701 has been associated with inhibition of HIV-1 replication when induced by CD8-

antiviral factor (CAF) [7]. This factor blocks HIV-1 LTR transcription by activating the interferon activating factor-1 (IRF-1), which in turn is responsible for STAT-1 phosphorylation [7]. We do not know the extent to which IRF contributes to HIV-1 inhibition in PM, but we posit that CSTB could directly or indirectly influence STAT-1 activation patterns and affect the levels of IRF-1. CSTB present in MDM could be inhibiting a kinase responsible for STAT-1 tyrosine phosphorylation, as reported for kininogen, a cystatin-like molecule that inhibits extracellular signal-regulated kinase (ERK-1) [13]. The potential kinase affected by CSTB could be from the Janus kinase family reported to induce tyrosine phosphorylation in STAT members [14].

In conclusion, we found that the low levels of CSTB in placental macrophages are associated with high levels of STAT-1 tyrosine phosphorylation. We propose the following mechanism for restriction of HIV replication in PM: that low levels of CSTB allow the activation of a tyrosine kinase that promotes STAT-1 tyrosine phosphorylation (STAT-1PY701). The STAT-1PY701 induces IRF-1 that decreases replication of HIV-1 by interfering with its LTR-driven replication (Figure 2). This is the first report that associates CSTB with STAT-1 and provides information on the levels of STAT-1 and its phosphorylated forms in MDM and PM. Future studies will determine if the proposed mechanism is operative in PM.

Acknowledgements

We acknowledge the help and dedication of Dr. Melendez laboratory staff. The NIH AIDS Research and Reference Reagent Program provided the HIV-BAL isolate. This work was supported by the following NIH grants: NINDS 1 U54NS430, MBRS-CORE-SO6GMO822, RCMI-CRC P20RR11126, RCMI-G12RR03051 of the proteomics core. Ms. Luciano received an NIH-MBRS-RISE GM61838 award.

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Figure 1. CSTB associates with STAT-1 in MDM and PM whole cell lysates

Immunoprecipitated lysates with monoclonal CSTB antibody were probed for STAT-1 and CSTB and showed STAT-1 is present in the immunoprecipitated CSTB. STAT-1 was recognized by the positive control antibody (STAT-1 CTRL). Pre-cleared lysates were incubated with un-conjugated beads that served as non-specific binding control (non-specific CTRL) with no recognition of either STAT-1 or CSTB. U87 cell lysates used as CSTB positive control gave a positive signal for both CSTB and STAT-1 (panel A).

Immunoprecipitated lysates for STAT-1 were probed for STAT-1 and CSTB. CSTB is present in the immunoprecipitated STAT-1. CSTB band was detected in MDM and MDM-HIV but not in PM and PM-HIV samples (panel B). HeLa cell lysates were used as CSTB positive control.

Patterns of STAT-1 phosphorylation were expressed as a ratio between total levels of STAT-1 (panel C). All blots were b-actin normalized and measured by densitometry (panels D and E). Blots shows PM have higher levels of tyrosine phosphorylation (PY701) than do MDM ($p=0.02$) (panels C and D). Tyrosine phosphorylation levels were also higher in PM than in PM-HIV ($p=0.03$). PM-HIV had higher levels of PY701 than MDM-HIV ($p=0.03$) (panels C and D). Levels of serine phosphorylation were similar between samples (panels C and E). Beta-actin (loading control) shows a similar protein content among all the samples (panel C). ($n=3$, Error bars = $\pm$ SEM).

Figure 2. Possible mechanism underlying HIV-1 restriction in placental macrophages

We posit that cystatin B could inhibit a kinase responsible for STAT-1 tyrosine phosphorylation. In PM, we observed low levels of CSTB that correlate with higher levels of STAT-1 PY. This STAT-1 PY interacts with interferon regulatory factor 1 (IRF-1), and the two translocate to the nucleus and inhibit HIV-1 long terminal repeat (LTR). This inhibition results in decreased production of HIV-1 viral progeny (in purple), characteristic of HIV-1 replication in PM.