Table 3. Contribution of the amino- and carboxy-terminal domains, and the first transmembrane domain (TMD) to CXCR4 oligomerization as analyzed by bioluminescent resonance energy transfer (BRET) analysis

Sample	BRET level[†]	*n*	*P*-value	Relative BRET efficiency (%)[‡]
Amino- and carboxy-terminal domain				
CXCR2/4-GFP + CXCR4-Rluc	1.163 ± 0.106	6	n.s.	71.9
Lyn My-R4cyt-GFP + CXCR4-Rluc	0.758 ± 0.057	4	<0.001	4.8
CXCR4 d-31-GFP + CXCR4-Rluc	1.111 ± 0.083	8	n.s.	98.9
3TMD homotypic interaction				
3TMD125-GFP + 3TMD125-Rluc	0.806 ± 0.071	5	<0.001	30.8
3TMD367-GFP + 3TMD367-Rluc	0.917 ± 0.061	6	<0.01	64.0
3TMDs heterotypic interaction				
3TMD125-GFP + 3TMD367-Rluc	0.806 ± 0.010	3	<0.001	46.3
3TMD367-GFP + 3TMD125-Rluc	0.885 ± 0.012	3	<0.05	64.7
3TMD–5TMD interaction				
5TMDΔ23-GFP + 3TMD125-Rluc	0.776 ± 0.020	4	<0.001	48.3
5TMDΔ23-GFP + 3TMD367-Rluc	0.877 ± 0.030	4	<0.01	73.3
5TMDΔ34-GFP + 3TMD125-Rluc	0.914 ± 0.041	4	<0.05	79.8
5TMDΔ34-GFP + 3TMD367-Rluc	1.009 ± 0.061	4	n.s.	103.0
5TMDΔ45-GFP + 3TMD125-Rluc	0.931 ± 0.024	4	<0.05	84.5
5TMDΔ45-GFP + 3TMD367-Rluc	0.937 ± 0.010	4	n.s.	86.5
5TMDΔ67-GFP + 3TMD125-Rluc	0.826 ± 0.031	4	<0.01	59.5
5TMDΔ67-GFP + 3TMD367-Rluc	0.892 ± 0.011	4	<0.05	75.8
3TMD125-GFP + 5TMDΔ23-Rluc	0.841 ± 0.088	4	<0.01	57.3
3TMD125-GFP + 5TMDΔ34-Rluc	0.819 ± 0.044	4	<0.01	52.8
3TMD125-GFP + 5TMDΔ45-Rluc	0.846 ± 0.049	4	<0.01	58.3
3TMD125-GFP + 5TMDΔ67-Rluc	0.824 ± 0.061	4	<0.01	53.0
3TMD367-GFP + 5TMDΔ23-Rluc	0.990 ± 0.041	5	n.s.	91.8
3TMD367-GFP + 5TMDΔ34-Rluc	0.989 ± 0.028	4	n.s.	93.8
3TMD367-GFP + 5TMDΔ45-Rluc	0.958 ± 0.042	4	n.s.	85.5
3TMD367-GFP + 5TMDΔ67-Rluc	0.984 ± 0.053	4	n.s.	91.8

[†]The average and standard deviation for the indicated number of independent experiments.
The statistical significance of each BRET efficiency was tested compared to the BRET signal of CXCR4-GFP and CXCR4-Rluc (Student's *t*-test). n.s., not significant.
[‡]The relative BRET efficiency was calculated for each experiment and the average is shown.

Table 4. Detection of higher-order multimerization of CXCR4 by bioluminescent resonance energy transfer (BRET)–bimolecular fluorescence complementation (BiFC) analysis

Sample	BiFC–BRET signal[†]	*n*	*P*-value
Specificity control			
mKGN 1000 ng + mKGC 1000 ng + Rluc	0.615 ± 0.070	3	
CXCR4 mKGN 500 ng + CXCR4 mKGC 500 ng + Rluc	0.624 ± 0.049	6	
CXCR3 mKGN 1000 ng + CXCR3 mKGC 1000 ng + CXCR3 Rluc	0.569 ± 0.021	3	
CXCR4–CXCR3 interaction			
CXCR3 mKGN 1000 ng + CXCR3 mKGC 1000 ng + CXCR4 Rluc	0.610 ± 0.065	3	n.s.
CXCR4 mKGN 31.3 ng + CXCR4 mKGC 31.3 ng + CXCR3 Rluc	0.593 ± 0.021	4	n.s.
CXCR4 mKGN 62.5 ng + CXCR4 mKGC 62.5 ng + CXCR3 Rluc	0.582 ± 0.011	4	n.s.
CXCR4 mKGN 125 ng + CXCR4 mKGC 125 ng + CXCR3 Rluc	0.586 ± 0.015	4	n.s.
CXCR4 mKGN 250 ng + CXCR4 mKGC 250 ng + CXCR3 Rluc	0.568 ± 0.011	4	n.s.
CXCR4 mKGN 500 ng + CXCR4 mKGC 500 ng + CXCR3 Rluc	0.573 ± 0.014	4	n.s.
CXCR4–CXCR4 interaction			
CXCR4 mKGN 31.3 ng + CXCR4 mKGC 31.3 ng + CXCR4 Rluc	0.634 ± 0.023	6	n.s.
CXCR4 mKGN 62.5 ng + CXCR4 mKGC 62.5 ng + CXCR4 Rluc	0.644 ± 0.035	7	n.s.
CXCR4 mKGN 125 ng + CXCR4 mKGC 125 ng + CXCR4 Rluc	0.676 ± 0.016	7	<0.05
CXCR4 mKGN 250 ng + CXCR4 mKGC 250 ng + CXCR4 Rluc	0.707 ± 0.030	7	<0.01
CXCR4 mKGN 500 ng + CXCR4 mKGC 500 ng + CXCR4 Rluc	0.717 ± 0.054	10	<0.01

[†]The average and standard deviation for the indicated number of independent experiments are shown.
The statistical significance of each BiFC–BRET signal was tested compared to the signal of CXCR4 mKGN + CXCR4 mKGC + Rluc (Student's *t*-test). n.s., not significant. The gray scale code represents the BRET efficiency: >100%, black; >80% dark gray; >60%, gray; >40%, light gray; >20%, white gray; <20%, white.

doi: 10.1111/j.1349-7006.2008.00997.x

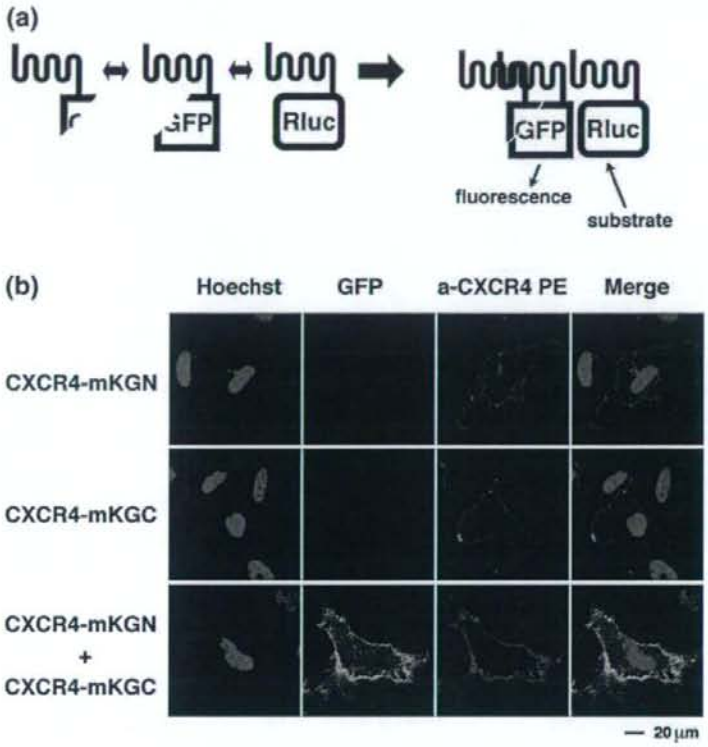

Fig. 5. Bioluminescent resonance energy transfer (BRET)–bimolecular fluorescence complementation (BiFC) analysis to demonstrate the presence of a higher-order molecular clustering of CXCR4. (a) Schematic drawing of the BiFC–BRET assay system. The bioluminescence energy from renilla luciferase (Rluc) was absorbed by green fluorescent protein (GFP; mKG) reconstituted by amino- and carboxy-terminal fragments to emit green fluorescence. (b) Detection of a BiFC signal by coexpressing CXCR4-mKGN and CXCR-mKGC. Cell surface expression of CXCR4-mKGN and CXCR4-mKGC was detected by an immunofluorescence assay using anti-CXCR4 R-phycoerythrin (PE) (a-CXCR4 R-phycoerythrin). The green fluorescence was detected only when two constructs were cointroduced into cells (bottom). Green, red, and blue represent GFP, PE, and the Hoechst 33258-stained nucleus, respectively. Magnification, ×630; scale bar = 20 μm.

(Table 4). These data indicate that the BiFC–BRET signal is specific for CXCR4, and that CXCR4 forms a molecular complex consisting of more than two molecules *in vivo*. The BiFC–BRET signals were lower than the BRET signals, presumably because of the decreased efficiencies of green fluorescence activation by mKGN and mKGC fragments relative to full-length GFP. Taken together, our *in vivo* biophysical analysis favors a multimerization model for CXCR4.

Discussion

In the present work, we demonstrated that CXCR4 forms a complex consisting of more than two molecules (a multimer) in the absence of CXCL12/SDF-1α by using a combination of BRET and BiFC techniques. The BiFC–BRET approach is a powerful technique for directly identifying a trimolecular complex in living cells, and may be applied to other protein–protein interactions.

Any application of FRET or BRET techniques to GPCR has to be done carefully, as GPCR often yield significant background signals.[41] The use of comparative studies with other GPCR, including CXCR2, CXCR3, and CCR5, as well as the careful monitoring of GFP and Rluc expression levels, allowed us to obtain compelling evidence for a specific homotypic interaction of CXCR4. The technical limitation of BiFC–BRET is that it is not possible to determine how many molecules are involved in the higher-order complex. To further characterize the molecular assembly, it will be necessary to develop a system that yields signals only when a target molecule tetramerizes.

We demonstrated that the higher-order clusterization of CXCR4 was not mediated by the amino- or carboxy-termini but by multiple TMD, consistent with the GPCR oligomerization models. However, we were unable to determine the TMD pairs that contribute most to CXCR4 multimerization. Consequently, we failed to isolate monomeric CXCR4. Treating cells with the CXCR4 ligand CXCL12/SDF-1α at cell migration-inducing concentrations did not increase BRET levels (data not shown), also suggesting the presence of higher-order CXCR4 clusters under steady-state conditions. This type of preformed receptor multimerization has been documented for receptors such as CCR5.[28] The biological advantage of CXCR4 assembly is its effective ability to 'radar' CXCL12/SDF-1α in the microenvironment for the induction of cell migration or targeted metastasis of cancer cells.[42,43] Steady-state multimerization is important because a molecular cluster would increase avidity for CXCL12/SDF-1α. We assume that high sensitivity for the ligand is critical for normal and malignant cells to be able to determine which direction to migrate to in in response to ligand stimulation.

CXCR4 does not display apparent punctate signals on the cell surface, as visualized by GFP tagging or immunostaining, even though our data predict that CXCR4 multimerizes. This suggests that only a limited number of CXCR4 molecules might participate in a multimeric unit. Bovine rhodopsin has been demonstrated to form a higher-order structure characterized by dimer arrays.[44] Given the structural similarities among GPCR, the basic unit of the higher-order cluster of CXCR4 may also be a dimer. It is possible that a pair of dimers forms a CXCR4 tetramolecular complex. These hypothetical models may be probed further using atomic force microscopy.

As shown in Figure 5b, the expression of CXCR4-mKGN and CXCR4-mKGC yielded green fluorescence at the cell periphery. This is compelling evidence that CXCR4 multimerizes at the cell surface. CXCR4 does not actively form a complex in the ER

but does so in the post-ER-to-Golgi compartments as suggested by the following data. When 293T cells expressing CXCR4-GFP and CXCR4-Rluc were treated with brefeldin A, which blocks the transport of proteins from the ER to post-Golgi compartments, the BRET levels dropped substantially to $63.7 \pm 15.2\%$ ($n = 7$) at 4 h after exposure. This drop in BRET level corresponded to the decrease in cell surface CXCR4 levels, even though the GFP and Rluc levels were unchanged (data not shown). Thus, it is unlikely that multimerization is required for CXCR4 to egress from the ER. CXCR4 may start to oligomerize in post-ER compartments and probably play a role at the cell surface, as hypothesized above. We believe that it will be informative to study how CXCR4 oligomerization is prohibited in the ER. By knowing this, we may be able to design novel CXCR4 inhibitors that disassemble the CXCR4 multimer. BiFC–BRET-based biophysical analyses would be a powerful tool for further studies.

Acknowledgments

We thank Dr Tsutomu Murakami for critically reading the manuscript. This work was partly supported by the Japan Health Science Foundation, the Japanese Ministry of Health, Labor and Welfare (H18-AIDS-W-003), and the Japanese Ministry of Education, Culture, Sports, Science, and Technology (18689014 and 18659136).

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doi: 10.1111/j.1349-7006.2008.00997.x

Research Letters

AIDS 2008, **22**:1081–1089

Cyclin K/CPR4 inhibits primate lentiviral replication by inactivating Tat/positive transcription elongation factor b-dependent long terminal repeat transcription

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The positive transcription elongation factor b complexes comprise CDK9 and a C-type cyclin, required for the efficient expression of both eukaryotic and primate lentivirus-encoded genes. Cyclin K/CPR4 is the least studied of the positive transcription elongation factor b-forming cyclins. Here, we demonstrate that cyclin K/CPR4-containing positive transcription elongation factor b complexes are unresponsive to Tat and HEXIM1-mediated inactivation. Enhancing expression of cyclin K/CPR4 inhibited the human and simian immunodeficiency viral replication. These data indicate that cyclin K/CPR4 functions as a natural inhibitor of primate lentiviruses.

Positive transcription elongation factor b (P-TEFb) complexes comprise CDK9 and a C-type cyclin (cyclin T1, T2 or K) and play a role in transcriptional initiation in/or elongation of cellular genes [1–6]. The P-TEFb complex is critical for Tat-dependent transcription from the human and simian immunodeficiency virus (HIV-1 and SIV) long terminal repeat (LTR) promoter. By interacting with the P-TEFb complex, Tat recruits the P-TEFb complex to the trans-activation-responsive (TAR) viral RNA element, which is located close to the transcriptional start site to which Tat binds in complex with P-TEFb complex. CDK9 phosphorylates the carboxy-terminal domain of RNA polymerase II, which boosts transcription from the proviral genome [7]. Among the P-TEFb complex-forming cyclins, only CCNT1 has the Tat responsive motif (TRM). Given the structural and functional similarities between CCNT2 and CCNK [8], expression of CCNK has been assumed to negatively affect Tat-dependent transcription, thus primate lentiviral replication. This possibility, however, has not yet been examined. Recently, it was found that the P-TEFb complex containing CCNT1 or CCNT2 is inactivated by the 7SK RNA–HEXIM1 complex [9–12]. The HEXIM1-mediated inhibition of CCNK-containing P-TEFb complexes remains to be tested.

We constructed mammalian expression plasmids for wild type and N-terminal FLAG-tagged or green fluorescent protein (GFP)-tagged human CCNK (WT, fCCNK or GFP-fCCNK). The CCNK function is unaffected by N-terminal epitope tagging [2,13,14]. GFP-fCCNK was used to examine the intracellular localization of fCCNK in NP2 cells. GFP-fCCNK accumulated in the discrete nuclear speckle compartments, similar to GFP-CCNT1, known to be sites of RNA Pol II-mediated transcription and co-transcriptional premRNA processing [15] and exhibited co-distribution with hemaglutinin eipope tagged CDK9 (data not shown). These data suggest functional similarities between CCNK and CCNT1.

We examined the physical association between CCNK and the transcriptional regulators HEXIM1 and HIV-1 Tat. Under the same conditions, endogenously expressed HEXIM1 did not co-immunoprecipitate with fCCNK, but co-immunoprecipitated successfully with FLAG-tagged CCNT1 (fCCNT1; Fig. 1a). Similarly, FLAG-tagged Tat could not co-immunoprecipitate CCNK under conditions in which it did precipitate GFP-CCNT1 (Fig. 1a). These data suggest that the CCNK-containing P-TEFb complex is unresponsive to HEXIM1 or Tat and, thus, it would appear that CCNK is unique among P-TEFb complex-forming cyclins.

To test whether CCNK expression reduces HIV-1 production at the level of transcription, we performed a transient transfection assay to measure HIV-1 production in the presence of different levels of CCNK expression. HIV-1 proviral DNA was co-introduced into 293 T cells with increasing amounts of fCCNK expression plasmid. It was found that the viral production was decreased dramatically in a dose-dependent manner (Fig. 1b). Similarly, the production of SIV was reduced (data not shown). Western blot analysis revealed that the level of p24 antigen in cell lysates dropped significantly with increasing levels of fCCNK (Fig. 1b). By contrast, the level of the internal control Bip was unaffected (Fig. 1b). Real-time (RT)-PCR analysis revealed that viral mRNA levels fell with increasing fCCNK expression, whereas the internal control cyclophilin A mRNA levels were unaffected (Fig. 1b). The reduction in viral mRNA was comparable with that observed for viral protein. Specifically, RT-PCR demonstrated that 80 ng of pfCCNK exerted the maximum reduction in viral mRNA levels (5.1% relative to the control), a finding similar to the viral production levels quantified by enzyme-linked immunosorbent assay (3.3%, Fig. 1b). These data strongly suggest that CCNK inhibits HIV-1

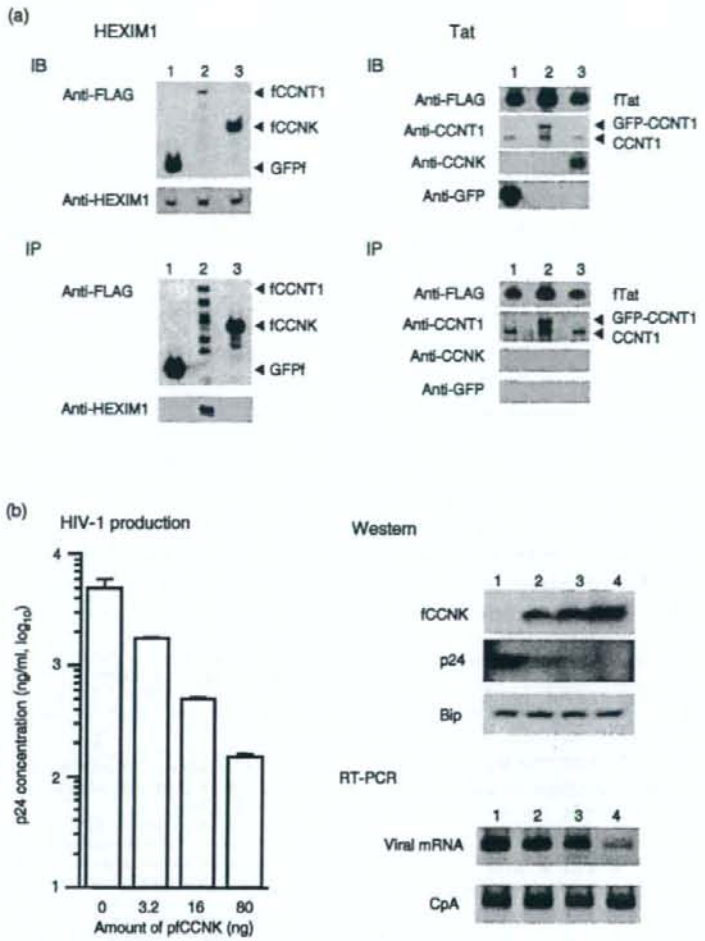

Fig. 1. Expression and functional characterization of cyclin K/CPR4. (a) Co-immunoprecipitation assay demonstrating that cyclin K/CPR4 does not interact with HEXIM1 or HIV-1 Tat. For the co-immunoprecipitation assay with HEXIM1, 293 T cells were transfected with 2 μg of expression vector and then grown in six-well plates. Expression vectors included FLAG-tagged green fluorescent protein (GFP) (lane 1, GFPf), FLAG-tagged CCNT1 (lane 2, fCCNT1) and FLAG-tagged cyclin K/CPR4 (lane 3, fCCNK). For the co-immunoprecipitation assay with Tat, 293 T cells were co-transfected with 1 μg FLAG-tagged Tat (fTat) and 1 μg of GFP (lane 1), GFP-CCNT1 (lane 2) or cyclin K/CPR4 (lane 3). Protein expressions from transfected plasmids were detected by western blot analysis (IB). Cell lysates were immunoprecipitated with agarose beads conjugated with anti-FLAG M2 antibody. Co-immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted using the antibodies indicated (IP). (b) fCCNK expression inhibited transcription of HIV-1. The 293 T cells were co-transfected with HIV-1 proviral DNA (200 ng) and increasing amounts of the fCCNK expression plasmid (0, 3.2, 16 and 80 ng per well in a six-well plate). Viral production was examined 48 h post-transfection by p24 enzyme-linked immunosorbent assay. Western blot analysis was used to examine expression levels of fCCNK, p24 antigen and Bip in transfected 293 T cells. Real-time polymerase chain reaction was used to examine viral and cyclophilin A mRNA expression in transfected 293 T cells. Lanes 1–4 correspond to the amount of pfCCNK 0, 3.2, 16, and 80 ng, respectively.

production by specifically limiting Tat-dependent LTR transcription.

Finally, we investigated the potential inhibitory activity of CCNK on HIV-1 replication in a physiologically relevant system. We established human T cell lines that constitutively overexpressed fCCNK or luciferase (Luc) by infecting MOLT-4, MT-4 and M8166 cells with murine leukemia viral vectors carrying fCCNK-IRES-GFP or Luc-IRES-GFP cassettes and GFP-positive cells were collected as described previously to examine the role of HEXIM1 in the primate lentiviral replication [16]. Expression of fCCNK was verified by western blot analysis using an anti-CCNK antibody and the expression

of cellular proteins such as CCNT1, CDK9, HEXIM1, Bip, and cell surface receptors for HIV-1 (CD4 and CXCR4) was comparable between fCCNK-expressing and Luc-expressing cells. In addition, no differences in rate of cell proliferation were detected between fCCNK-expressing and Luc-expressing cells (data not shown). We measured HIV-1 and SIV replication in these T cell lines and found that the viral replication was strongly inhibited in all the three fCCNK-expressing T cell lines (data not shown). These data directly demonstrate that CCNK functions as a negative regulator of primate lentiviral replication. In addition, we verified that constitutive fCCNK expression did not block the early phase of the viral life cycle by conducting a real-time Alu-LTR PCR assay to quantify the integrated viral genome in MOLT-4 and MT-4 cells infected with VSV-G-pseudotyped replication-incompetent HIV-1 vector (data not shown).

Taken together, our results indicate that CCNK is able to limit the replication of primate lentiviruses by competing with CCNT1 for CDK9, which results in a reduction of CCNT1-containing Tat-responsive P-TEFb complexes, thereby inactivation of Tat/P-TEFb-dependent transcription of the viral LTR promoter. Our report is the first demonstration that constitutive upregulation of a C-type cyclin CCNK limits primate lentiviral replication in human T cell lines without conferring any detectable effect on cell proliferation. The lack of any detectable effect on cell proliferation could be because CCNK-containing P-TEFb complexes can complement the role played by the CCNT1-containing P-TEFb complex in cellular gene transcription. These data highlight the importance of Tat-dependent LTR transcription for the efficient propagation of the primate lentivirus and the potential protective role of CCNK in the pathogenesis of primate lentiviruses.

Acknowledgements

This work was supported in part by the Japan Health Science Foundation, the Japanese Ministry of Health, Labor and Welfare (H18-AIDS-W-003) and the Japanese Ministry of Education, Culture, Sports, Science and Technology (18689014 and 18659136).

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Potential role of CD8+CD28− T lymphocytes in immune activation during HIV-1 infection

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As CD8+CD28− T cells have been associated with dendritic and T cell suppression, we analyzed whether an increase in CD8+CD28− T cell numbers during HIV-1 infection could lead to impaired T cell responses. In contrast to the in-vitro generated CD8+CD28− suppressors, peripheral blood CD8+CD28− T cells of both HIV-infected and

FEBS Letters 582 (2008) 4053–4058

Identification of the P-TEFb complex-interacting domain of Brd4 as an inhibitor of HIV-1 replication by functional cDNA library screening in MT-4 cells

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Received 26 August 2008; revised 10 October 2008; accepted 21 October 2008

Available online 7 November 2008

Edited by Ivan Sadowski

Abstract **We conducted a phenotypic cDNA screening using a T cell line-based assay to identify human genes that render cells resistant to human immunodeficiency virus type 1 (HIV-1). We isolated potential HIV-1 resistance genes, including the carboxy terminal domain (CTD) of bromodomain-containing protein 4 (Brd4). Expression of GFP-Brd4-CTD was tolerated in MT-4 and Jurkat cells in which HIV-1 replication was markedly inhibited. We provide direct experimental data demonstrating that Brd4-CTD serves as a specific inhibitor of HIV-1 replication in T cells. Our method is a powerful tool for the identification of host factors that regulate HIV-1 replication in T cells.**

Keywords: HIV-1 replication; Host factor; cDNA library; Brd4; P-TEFb complex; Tat-dependent LTR transcription

1. Introduction

The identification of specific molecular interactions required for efficient HIV-1 replication should provide clues towards improved understanding of the mechanisms of viral pathogenesis, as well as of host defence against HIV-1. In addition, this may help design highly specific inhibitors against HIV-1. Genome-wide screening for HIV-1 replication regulatory factors has been attempted by using various experimental approaches. Most of them were based on adherent epithelial cells, because these cells exhibit higher transduction efficiencies (by transfection or by viral vector transfer) when compared with T cell lines [1,2]; however, cells of epithelial origin are not relevant hosts for HIV-1 in vivo. Furthermore, viral vectors pseudotyped with vesicular stomatitis virus-G (VSV-G) are often used for screening purposes, instead of wild-type HIV-1. These vectors enter cells via the VSV-G-restricted route, which is different from the HIV-1 envelope-mediated entry pathway. These factors constitute potential caveats of these assays.

To overcome these potential problems, we carried out a phenotypic screen to identify human cDNAs that confer resistance to HIV-1 replication, without affecting cell proliferation. The assay was performed in a human T cell line, a physiologically relevant host, stably transduced with a human cDNA library. We isolated several potential HIV-1 resistance genes successfully, many of which were not known as HIV-1 regulatory factors. In this work, we studied Brd4 in detail to demonstrate the applicability of our phenotype screening. Our study of Brd4-CTD suggests the presence of a potential anti-HIV-1 drug target in the host transcription regulator cyclin T1 (CCNT1).

2. Materials and methods

2.1. Cells and transfection

Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen, Tokyo, Japan), and then incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

2.2. Plasmid construction

The Brd4-CTD was amplified from 293T RNA by reverse transcriptase PCR (RT-PCR) using the primers 5′-AGATCTCTCATCCGACCACCCCCTCCTCC-3′ and 5′-TCAGGATCCCGAAAAGATTTTCTTCAAATATTG-3′. The BglII–BamHI fragment of the PCR product was cloned into the corresponding restriction sites of the pEGFP-C2 (Clontech, Palo Alto, CA). The XhoI–MfeI fragment from the resulting plasmid was cloned into the corresponding restriction sites of the pCMMP KRAB vector, creating the pGFP-Brd4-CTD. The cDNA encoding firefly luciferase (Luc$^+$) was amplified by PCR from the pGL3-Basic (Promega, Madison, WI) using the primers 5′-ACCGGTCTCGAGGGCCACCATGGAAGACGCCAAAAACATAAAGAAAGG-3′ and 5′-GAATTCGGATCCTTACACGGCGATCTTTCCGCCCTTCTTGGCC-3′. The PCR product was digested with AgeI and BamHI, and cloned into the corresponding sites of the pCMMP GFP vector, generating the pCMMP Luciferase. The BamHI–XhoI fragment of pLenti6/V5-GW/lacZ (Invitrogen) was removed, and Luc$^+$ was inserted with BglII and SalI sites artificially attached at its extremities, creating the pLenti Luciferase. Other plasmids used in this study were described previously [3,4].

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.10.047

2.3. Selecting human cDNAs that confer resistance to HIV-1

The lentiviral vector carrying an hPBL cDNA library was described previously [5]. MT-4 cells (1×10^6) transduced with the cDNA library were infected with HIV-1_{HXB2} propagated in MT-2 cells, by resuspending MT-4 cells in a viral preparation containing 70–1250 ng/ml p24 viral capsid antigen in 20 ml of culture medium for 30 min at room temperature with continuous mixing. Anti-CD4 magnetic beads (0.5–1.0×10^7; Dynal, Oslo, Norway) were added to the cell suspension to prevent cell-to-cell contact, and the cells (1×10^5 cells per well in 200 μl of culture medium) were plated in flat-bottomed 96-well plates. At 3–4 weeks post-infection, cells from four wells positive for cell outgrowth were pooled and genomic DNA was extracted. The cDNA inserts were PCR-amplified and sequenced using primers described previously [5].

2.4. Generation of viruses and infection

Viruses were produced as described previously [3,4]. Human T cell lines (MT-4 and Jurkat cells; 1×10^5 cells) were incubated with 500–1000 μl of MLV preparations in the presence of 8 μg/ml polybrene for 1 h at 4 °C with continuous agitation. For HIV-1 infection, 1×10^5 cells were incubated with an HIV-1-containing culture supernatant (*ca.* 5–5000 pg p24), for 30 min at room temperature. HIV-1 replication was monitored as described previously [3,4].

2.5. Western blotting

Western blotting was performed according to techniques described previously [4]. The following antibodies were used: anti-CCNT1 (ab2098, Abcam, MA), anti-Brd4 (ab46199, Abcam), anti-GFP (MAB3580, Chemicon International, Temecula, CA or 632381, Clontech), anti-p24 (183-H12-5C, NIH AIDS Research and Reference Reagent Program), anti-HEXIM1 (ab25388, Abcam), anti-Bip/GRP78 (clone 40, BD Transduction Laboratories), and EnVision+ system (Dako, Glostrup, Denmark).

2.6. Reporter assay

The 293T cells grown in 48-well plates were co-transfected with 20 ng of pLTR-Luc or pCMMP Luciferase, together with pGFP-Brd4-CTD. The total amount of transfected DNA was adjusted by pCMMP GFP. To measure the effect of Tat, cells were co-transfected with 100 ng of pSVtat in addition to the above-mentioned plasmids. Cells were replated in 96-well plates in triplicate at 2–4 h post-transfection. Luciferase activity was measured 48 h after transfection using the Dual-Glo assay kit (Promega).

2.7. RT-PCR

RT-PCR was performed as described previously [4]. For amplification of HIV-1 mRNA, forward (5′-CTCGACGCAGGACTCGGCTTGC-3′) and reverse (5′-AGTTCCACTCCTGCCCAAGTATCC-3′) primers were used. The mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using the primers 5′-GTGGAAGGACTCATGACCACAGTC-3′ and 5′- CATGTGGGCCATGAGGTCCACCAC-3′.

2.8. Quantitative real-time PCR

The real-time PCR reaction was performed as described previously [4]. Amplifications were performed using the QuantiTect SYBR Green RT-PCR/PCR Kit (QIAGEN). To estimate the amount of integrated HIV-1 DNA, Alu-LTR PCR was performed as described previously [6] using the following primers: first PCR reaction, 5′-AACTAGGGAACCCACTGCTTAAG-3′ and 5′-TGCTGGGATTACAGGCGTGAG-3′; and second PCR reaction, 5′-AACTAGGGAACCCACTGCTTAAG-3′ and 5′-CTGCTAGAGATTTTCCACACTGAC-3′.

3. Results

To isolate cDNA clones that confer resistance to HIV-1 without negatively affecting cell proliferation, we performed phenotype screening using MT-4 cells stably transduced with a lentiviral vector carrying a cDNA library from human peripheral blood lymphocytes (hPBL). The complexity of the lentiviral cDNA library was on the order of 10^6. The lentiviral vector encoded a GFP expression cassette. Approximately 70% of the MT-4 cells became GFP-positive after infection of the lentiviral vector, suggesting that a portion of the cells were infected with multiple lentiviral vectors. The GFP-positive cells were collected using a FACS sorter and subsequently exposed to replication-competent HIV-1. The surviving cell clones were propagated and their transduced cDNAs were examined. The average length of hPBL cDNA in the lentiviral vector was ~0.7 kbp, which is shorter than the average human cellular mRNA length (~2 kbp). A gene was considered an innate

Table 1
Summary of cDNAs recovered in an HIV-1-resistant phenotype screening in MT-4 cells.

Gene category	# of independent clones	Frequency (%)	Frequently isolated genes[a] (# of independent clones)
Metabolism	16	24.6	Haemoglobin (7)
			Pyridoxal kinase, PDXK (3)
Transcription	7	10.8	Bromodomain containing 4, Brd4 (3)
			Zinc finger protein 26, ZNF26 (3)
Ribosomal proteins	7	10.8	Ribosomal protein L14, RPL14 (3)
Signal transduction	7	10.8	Zinc finger protein 36, ZFP36L2 (2) transducin beta-like 1X-linked, TBL1X (1)[c]
			Chromosome 22 open reading frame 5, C22orf5 (1)[c]
Trafficking	6	9.2	Chromosome 9 open reading frame 86, C9orf86 (1)[b]
			Chromosome 1 open reading frame 142, C1orf142 (1)[b]
			Nedd4-binding partner 3, N4BP3 (1)
Immunology	2	3.1	MHC class II, DR alpha (1)
Oncogenesis	2	3.1	AXIN1 up-regulated, AXUD1 (1)[c]
Glycosylation	2	3.1	Hyaluronan and proteoglycan link protein 3, HAPLN3 (1)
Differentiation	2	3.1	Jumonji AT rich interactive domain 2, JARID2 (1)
Cytoskeleton	2	3.1	Beta actin (1)
Cell cycle control	1	1.5	CWF19-like 1, CWF19L1 (1)
Apoptosis	1	1.5	Chromosome 2 open reading frame 28, C2orf28 (1)
DNA repair	1	1.5	Non-SMC element 1 homolog, NSMCE1 (1)[b]
Non-ORF coding	9	13.8	-
Total	65	100.0	-

[a]All the clones isolated more than three times are listed. A representative clone is shown for categories with a few candidates.
[b]These genes exhibited regulatory functions on HIV-1 production.
[c]These genes exhibited no effect on HIV-1 production.

negative factor for HIV-1 replication if the full-length open reading frame (ORF) was recovered. Alternatively, if a portion of a gene was recovered, the full-length gene was considered a potential positive factor for HIV-1 replication. We recovered 65 independent cDNA clones (43 genes, Table 1). A number of cDNAs encoded abundant cellular transcripts, including haemoglobin. In addition, cDNAs encompassing non-ORFs were isolated. The isolation of these cDNAs was likely due to the infection of a single cell with multiple cDNA-transducing lentiviral vectors, one of which encoded an HIV-1 resistance gene. If we disregard these cases, 26 genes were potential HIV-1 regulatory gene candidates, of which seven were examined for a potential HIV-1 regulatory functions as shown in Fig. 2. Four genes exhibited HIV-1 regulatory phenotypes (4/7 genes, 57.1%; Table 1). In addition to Brd4-CTD, C9orf86 and NSMCE1 scored as positive factors for HIV-1 replication, and C1orf142 was scored as a negative factor. This suggested that our screening was successful in selecting for HIV-1 regulatory genes. While each candidate gene will be studied in detail in future studies, here we focused on Brd4.

Brd4 was chosen for three reasons: (1) three independent Brd4 cDNAs were recovered; (2) Brd4 binds to the CCNT1/T2-bearing P-TEFb complex [7,8]; and (3) 13 independent Brd4 cDNA clones (13/42 clones, 31.0%) were isolated from a similar experiment in which the cDNA library from an *Oryctolagus cuniculus* kidney derived cell line was used. All the three Brd4 cDNA clones encoded Brd4-CTD: two encoded amino acids (aa) 1260–1362 and the third encoded aa 1209–1362 (Fig. 1A). The first two clones were translated using the Met-encoding codon at Brd4 nucleotide position 3778–3780, and the third was translated from the aberrant start codon in the primer upstream of the Brd4 ORF 3628 nt. To our surprise, aa fragment 1209–1264 of Brd4 was recently reported as an interactor of CCNT1 that inhibits Tat-dependent LTR-driven transcription [9]; however, the specific effect of this region on HIV-1 replication in human T cells was not fully investigated.

We hypothesized that the repression of HIV-1 replication in MT-4 cells may be due to the selective inhibition of viral gene transcription by Brd4-CTD. To test this, we cloned Brd4-CTD spanning aa 1209-1364 into a retroviral plasmid and fused GFP to its carboxy-terminus (GFP-Brd4-CTD, Fig. 1B). Confocal microscopy revealed that GFP-Brd4-CTD was localized mainly in the cytoplasm of MT-4 cells, with some signal found in the nucleus (Fig. 1C). A transient transfection assay revealed that the expression of GFP-Brd4-CTD modestly enhanced the luciferase expression driven by both the LTR and CMV promoters (Fig. 1D). In the presence of Tat, LTR

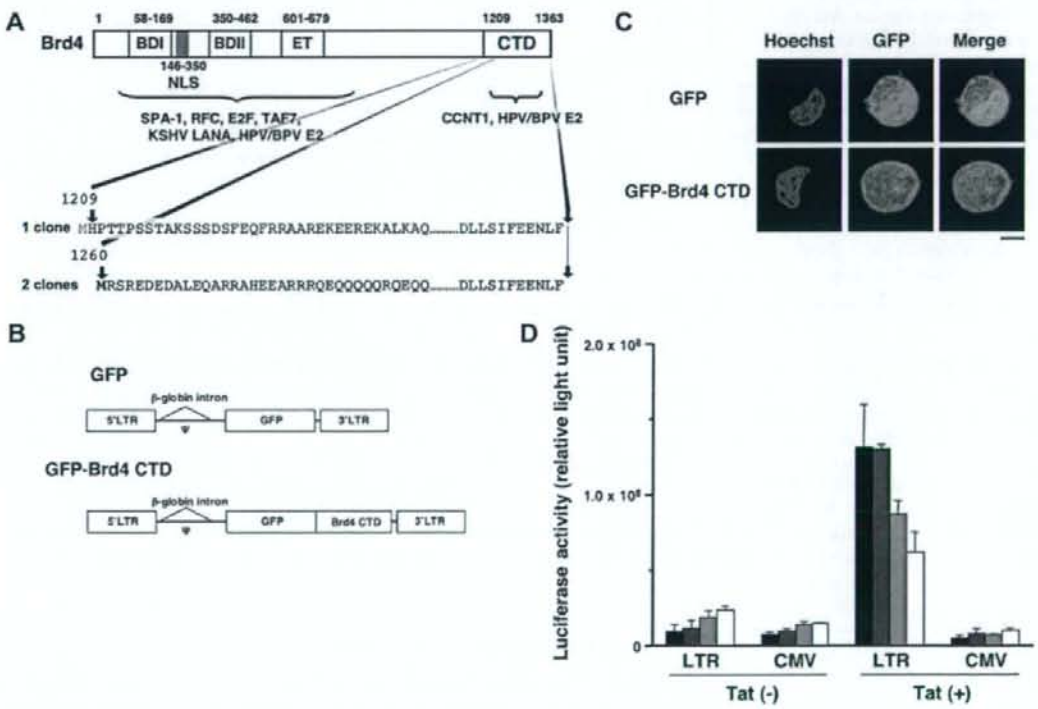

Fig. 1. Specific inhibition of Tat-dependent LTR transcription by GFP-Brd4-CTD. (A) Functional properties of the Brd4 domains and isolated Brd4 cDNAs. (B) Construction of MLV vector-based mammalian expression plasmids encoding GFP or GFP-Brd4-CTD. (C) Confocal microscopy images of MT-4 cells stably expressing GFP or GFP-Brd4-CTD. Green and blue represent GFP and the Hoechst 33258-stained nuclei, respectively. Magnification, 630×; scale bar, 5 μm. (D) Effect of GFP-Brd4-CTD on LTR and CMV promoter activities in the absence or presence of the Tat expression plasmid. Cells transfected with 0, 16, 80, and 400 ng of pGFP-Brd4-CTD correspond to black, dark gray, light gray, and white bars, respectively. Representative data from five independent experiments are shown.

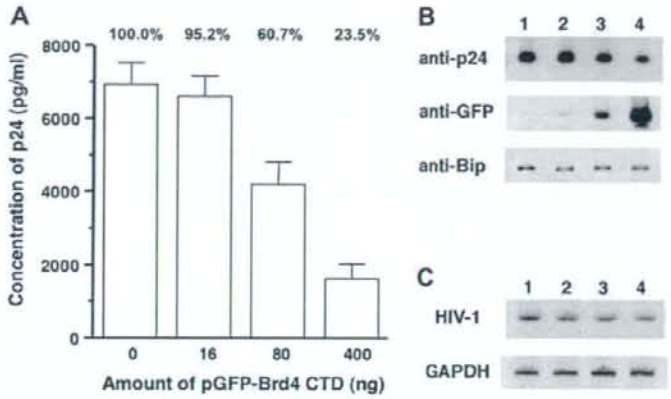

Fig. 2. Inhibition of HIV-1 production by GFP-Brd4-CTD. (A) Viral production was quantified by ELISA detecting p24 viral antigen. The relative decrease in viral levels are indicated on the top of the graph. (B) The viral protein levels in transfected cells were analyzed by Western blotting using the antibodies indicated. (C) The viral spliced transcript and GAPDH mRNA were amplified by RT-PCR. Lanes 1–4 in B and C correspond to the amount of pGFP-Brd4-CTD (0, 16, 80, and 400 ng, respectively). Quantification of these data is summarized in Table 2.

activity was markedly enhanced. When the GFP-Brd4-CTD expression vector was co-transfected, the Tat-dependent enhancement of LTR promoter-driven luciferase expression decreased. A similar trend was not observed for the CMV promoter. These data suggest that the Brd4-CTD specifically limits Tat-dependent LTR transcription.

We also investigated the effect of GFP-Brd4-CTD expression on HIV-1 production by using a proviral DNA mimicking the late phase of the viral life cycle. Consistent with the results described above, transfection of HIV-1 proviral DNA together with an increasing amount of the GFP-Brd4-CTD expression vector led to a decrease of viral yield, as well as of the levels of viral protein and mRNA in the transfected cells (Fig. 2). The viral RNA levels dropped in parallel with the protein levels, as demonstrated by real-time RT-PCR analysis (Fig. 2C and Table 2). These data suggest that GFP-Brd4-CTD inhibits HIV-1 production by blocking viral transcription.

To confirm the blockage of HIV-1 replication by Brd4-CTD, GFP-Brd4-CTD was transduced into MT-4 and Jurkat cells using an MLV-based vector (Fig. 3A). Green fluorescence indicated that the efficiency of MLV-mediated gene transduction in MT-4 cells was >90%, with a lower transduction efficiency observed in Jurkat cells, as estimated by FACS analysis. The GFP-positive Jurkat cells were collected using a FACS sorter. The expression of GFP and GFP-Brd4-CTD was verified by Western blot analysis (Fig. 3B). The expression levels of transcription-related gene products were not detectably affected by the constitutive expression of GFP-Brd4-CTD (Fig. 3B). In addition, there was no detectable difference in the levels of cell-surface HIV-1 receptors (CD4 and CXCR4), cell morphology, and cell proliferation rates between GFP- and GFP-Brd4-CTD-expressing cells (Fig. 1C and Supplementary data). We found that HIV-1 replicated less efficiently in GFP-Brd4-CTD-expressing cells than in GFP-expressing cells, in both cell lines tested, which confirms the HIV-1-resistant phenotype of MT-4 cells (Fig. 3C). The efficiency of viral genome integration into GFP-Brd4-CTD-expressing cells was indistinguishable from that of GFP-expressing cells (103.2 ± 24.1%) as examined by Alu-LTR PCR, suggesting that the early phase of the viral life cycle was not blocked by GFP-Brd4-CTD.

4. Discussion

Our phenotype screening method proved to be a powerful tool because a human T cell line was subjected to HIV-1 resistance screening by stable and non-transient introduction of a human cDNA library, and because wild-type HIV-1 was used; thus, the effect of candidate gene expression on cell proliferation was less of a concern in this system when compared with transient assay systems. In addition, HIV-1 inhibitory genes were isolated at a frequency of ~15% (4/26 genes), 75% of which were novel. We therefore believe that our system is remarkable in selecting genes that confer HIV-1 resistance in T cells. By applying this assay to other cDNA libraries, we

Table 2
Effect of GFP-Brd4-CTD on viral production examined by quantitative real time RT-PCR and ELISA.

pGFP-Brd4-CTD (ng)	HIV-1 mRNA (copy)[a]	GAPDH mRNA (copy)[a]	Ratio (HIV-1/GAPDH)	Normalized (%)[b]	p24 ELISA (%)[c]
0	274250	261750	1.048	100.0	100.0
16	221600	228850	0.968	92.4	95.2
80	138050	311450	0.443	42.3	60.7
400	120850	347750	0.348	33.2	23.5

[a]Copy per 100 ng total cellular RNA.
[b]Relative reduction of HIV-1 mRNA considering pGFP-Brd4-CTD 0 ng as 100%.
[c]See Fig. 2.

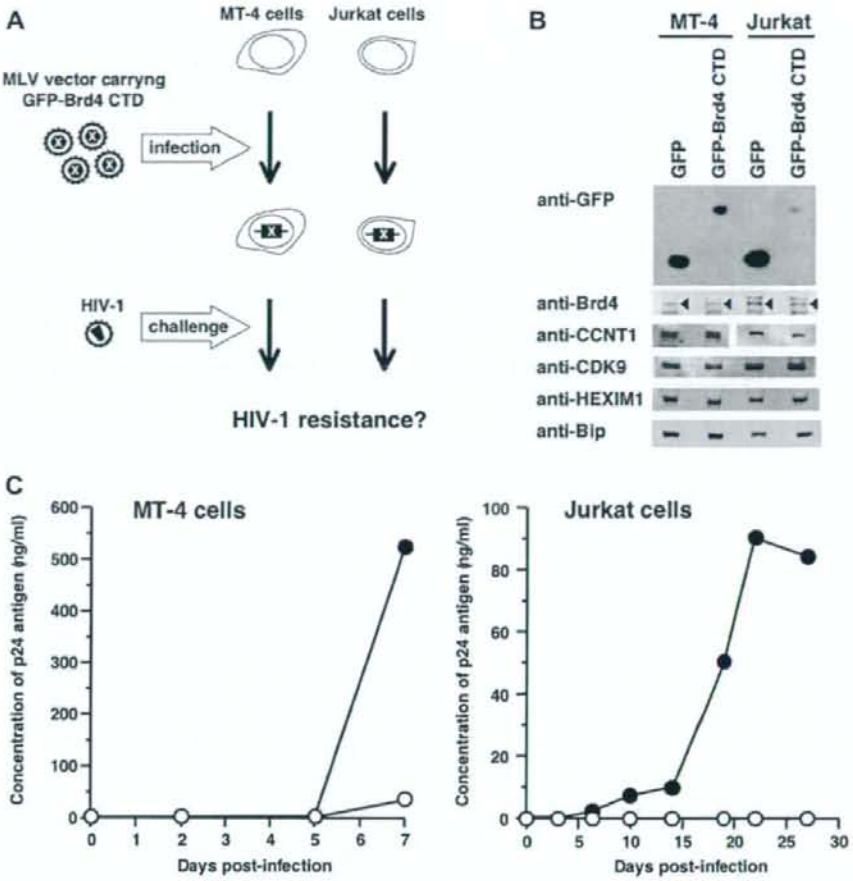

Fig. 3. Constitutive expression of GFP-Brd4-CTD limited the replication of HIV-1. (A) Experimental design. MT-4 and Jurkat cells were transduced with an MLV vector expressing GFP-Brd4-CTD. Cells were challenged by HIV-1 and the efficiency of viral replication was examined. (B) Western blot analysis of the expression levels of GFP, GFP-Brd4-CTD, Brd4 (arrowhead), CCNT1, CDK9, HEXIM1, and BiP in established MT-4 and Jurkat cells. (C) HIV-1 replication kinetics in MT-4 and Jurkat cells constitutively expressing GFP (black circles) or GFP-Brd4-CTD (white circles). Representative data from two independent experiments are shown.

may be able to isolate novel cellular factors that regulate HIV-1 replication.

The assessment of the selective impact of altered candidate gene expression or function on HIV-1 replication (without the alteration of cell proliferation) is critical to the identification of cellular molecular targets for novel anti-retroviral drugs. We demonstrated that Brd4-CTD was a specific silencer of HIV-1 replication, and verified that it effectively blocked HIV-1 replication in multiple human T cell lines without affecting cell proliferation. Our data indicate that primate lentiviral replication is more heavily dependent on the CCNT1-containing P-TEFb complex than cellular gene transcription, which is consistent with previous findings [4,10–11]. This implies that HIV-1 replication can be controlled by selectively restricting the CCNT1-containing P-TEFb complex. Our transcription assay indicated that the Brd4-CTD is not an inhibitor of the P-TEFb complex, but is rather a functional Tat inhibitor. Previous biochemical studies have suggested that Brd4-CTD and Tat bind to CCNT1 in a reciprocally exclusive fashion [7,9]. Given that the binding regions of these two proteins do not overlap, Brd4-CTD may be an allosteric inhibitor of the Tat-CCNT1 interaction. Taken together, our results indicate that the Brd4-interacting region of CCNT1 is a potential molecular target for the development of a novel HIV-1 inhibitor.

Acknowledgements: This work was supported by the Japan Health Science Foundation, the Japanese Ministry of Health, Labor and Welfare (H18-AIDS-W-003) and the Japanese Ministry of Education, Culture, Sports, Science and Technology (18689014 and 18659136).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.10.047.

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