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Identification of novel therapeutic targets for HIV infection through functional genomic cDNA screening

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Abstract

Despite decades of research, HIV remains a global health threat. Issues of multi-drug resistance and lack of an effective vaccine have recently led to the targeting of host factors for anti-viral drug development. While a few genome-wide screens for novel HIV co-factors have been reported, the promise of finding a therapeutic target has yet to be realized. Here, we report a screen of a cDNA library representing 15,000 unique genes in an infectious HIV system, and show that genomic screening can lead to the identification of novel proviral host factors. Mixed lineage kinase 3 (MLK3/MAP3K11) was identified as one of the strongest enhancers of infection and mutant studies show that its activity is dependent on its kinase function. Consistent with its known role in the activation of the AP-1 pathway through JNK kinase, MLK3 was able to enhance Tat-dependent HIV transcription in vitro thus leading to an increase in infection signal. RNA interference studies confirm the involvement of endogenous MLK3 in HIV infection, further implicating this kinase as a potential therapeutic target.
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Introduction

The advent of highly active anti-retroviral therapy (HAART), which combines several drugs against one or more viral targets, to treat HIV patients led to radically reduced viral load and maintained immune function in patients with access to therapy. However, the low fidelity of genomic replication mediated by reverse transcriptase generates mutations in the viral genes that render them resistant to anti-viral drugs (Parienti et al., 2004). Because of the small size of the viral genome, HIV relies heavily on the host cell machinery to complete its life cycle. The first efforts at targeting host factors for anti-viral therapy have focused on the receptor (CD4) and co-receptor (CCR5 and CXCR4) (Seibert and Sakmar, 2004) but compounds that block these proteins have not yet reached the clinic. Although we know about several other critical host factors involved in HIV infection, such as the proviral TSG101 (Demirov et al., 2002)

and anti-viral APOBEC3G (Harris and Liddament, 2004), there are likely many more that remain undiscovered.

On the heels of drug discovery, high-throughput genomic screening has emerged as the newest tool for rapidly identifying the individual players in a pathway of interest. The most widely used method for genome screening, the yeast-two-hybrid screen, quickly yields a pool of potential interacting partners. However, proving that the interaction is present and physiologically relevant in mammalian cells is more challenging. The nature of the yeast-two-hybrid system can also lead to many false negatives and false positives due to the hybrid protein structures and potentially altered behavior in the yeast cell nucleus (Colland and Daviet, 2004). Newer methods have employed screening of genomic libraries in mammalian cells, including both loss-of-function and gain-of-function screens. Pooled expression libraries have been used to isolate important immune receptors such as CD2 (Seed and Aruffo, 1987). Pathway dissection through disruption of gene expression has been achieved using siRNA/shRNA (Nguyen et al., 2006; Vanhecke and Janitz, 2004; Willingham et al., 2004), as well as by expression of genetic suppressor elements (GSEs) (Gudkov et al., 1993) and ribozymes

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(Suyama et al., 2003). While still mostly limited to those mammalian cell lines that are easily transfected with high efficiency, movement of genomic libraries to viral vector systems (Lorens et al., 2001) and the development of high-throughput electroporation (Yamauchi et al., 2004) will soon expand the potential for discovery to any pathway in any desired cell type.

Functional genomic screening offers the opportunity to rapidly find novel host factors involved in HIV biology that could be exploited as potential therapeutic targets. We therefore performed a high-throughput screen of a cDNA library of 15,000 unique genes to find novel proviral factors whose overexpression would lead to enhancement of HIV-IIIb infection (library details at http://function.gnf.org). From the pool of top enhancers chosen for follow-up, 13 host factors with no known connection to HIV infection continued to confirm with levels of enhancement from 2- to 8-fold over control. RNAi-mediated depletion of endogenous levels of several cDNA hits, including IFIT1, DNAJC14, and PITPN, inhibited HIV infection. Because of its clear role in pathways known to be utilized by HIV and potential for small molecule intervention, we chose to focus on the cDNA hit mixed lineage kinase 3 (MLK3/MAP3K11) for further follow-up. Studies with a kinase-inactive mutant form of MLK3 showed that kinase activity was necessary for HIV enhancement. Consistent with its role in AP-1 activation, MLK3 overexpression was shown to increase Tat-dependent transcription, providing a mechanism for its viral effects. RNA interference studies showed an inhibition of HIV infection following MLK3 depletion, confirming a role for endogenous MLK3 in the HIV life cycle.

Results

Because of their ease of transfection, we employed HIV permissive HeLaCD4βgal cells for screening of a 15,000 gene human cDNA library using a reverse transfection protocol previously described (Chanda et al., 2003). Negative control (Sport6-gfp) and positive control (Tat-Sport6) cDNAs were spotted into wells of 384-well plates containing the library cDNA (one gene per well) followed by the addition of transfection reagent solution containing an LTR-luciferase reporter construct responsive to HIV Tat (Fig. 1A). Cells were added after complex formation, and after 24 h, each well was infected with HIV-IIIb. Infection was allowed to proceed for 3 days to allow for effects on all stages of infection from entry to release and spread throughout the culture to be observed. The use of this multi-day infection assay to investigate effects throughout the viral life cycle was demonstrated previously by the ability of siRNA against CXCR4 (early stage), Furin (middle stage) and TSG101 (late stage) to effectively decrease the infection signal (Nguyen et al., 2006). Infection in the cDNA screen was assessed by measuring the amount of luciferase produced via the viral LTR promoter using an equivalent input of Tat cDNA (40 ng) as the positive control. The entire screen was performed in duplicate in order to assess variability. The data for each cDNA were compared to the median signal of the entire plate and expressed as the ratio afa/mfa, which is the average fold activation (afa) divided by the adjusted standard deviation of the fold activation (mfa). The mfa in effect penalizes the value for fold activation if the standard deviation between the replicates is high, allowing hits to be identified as those that enhanced infection reproducibly (see Materials and methods for analysis of details). Over-expression of the positive control Tat showed a distribution of response with a mean approximately two standard deviations from that of the negative control, empty Sport6-gfp (data not shown). Hits were defined as those cDNAs whose enhancement of infection was at least 2-fold over the plate median, a level which was close to the mean of the Tat-positive control.
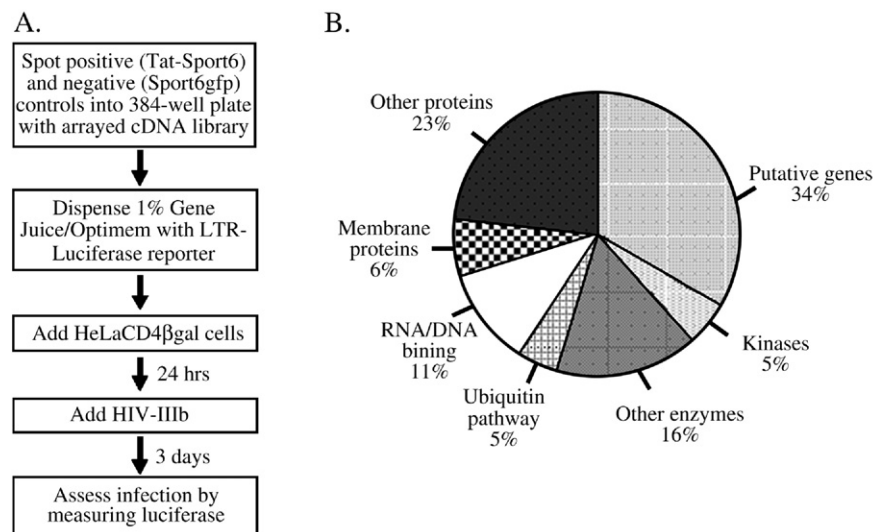

Fig. 1. High-throughput cDNA screen layout, performance of controls and families of hits. (A) Negative control (Sport6GFP) and positive control for enhancement (Tat-Sport6) cDNAs were spotted into the wells of a 384-well opaque plate containing one cDNA/well of the library followed by the addition of transfection reagent and Tat-responsive reporter plasmid LTR-Luc. After complex formation, HeLaCD4βgal cells were added and incubated overnight, after which HIV-IIIb (4 ng/well) was added. Three days later, levels of infection were assessed by measuring luciferase produced from the reporter using Brite Glo. (B) Families of the enhancing hits chosen for follow-up. Genes were chosen based on their screen performance and literature review.

Table 1
Gene ontology analysis of cDNA screen hits

Database ID[a]	Description	# of hits	Hits	LogP
GO:0046785	Microtubule polymerization	2	MAPT, CENPJ	−3.327
IPR012956	CBF, N-terminal	2	HNRPAB, HNRPD	−3.297
GO:0003746	Translation elongation factor activity	4	EEF1A2, EEF1G, TUFM, SUPT16H	−3.224
IPR002475	BCL2-like apoptosis inhibitor	3	BCL2A1, BCL2L1, BCL2L13	−2.921
IPR002048	Calcium-binding EF-hand	11	AIF1, POLR2C, PPEF1, S100A12, S100P, SLC25A12, RASGRP1, KCNIP1, USP32, REPS1, MRLC2	−2.905
GO:0003729	mRNA binding	4	HNRPAB, HNRPD, HNRPDL, TNRC4	−2.877
GO:0050773	Regulation of dendrite morphogenesis	2	DBN1, MT3	−2.856
IPR011992	EF-hand type	11	AIF1, PPEF1, S100A10, S100A12, S100P, SLC25A12, RASGRP1, KCNIP1, USP32, REPS1, MRLC2	−2.72
GO:0050767	Regulation of neurogenesis	3	DBN1, MAPT, MT3	−2.715
GO:0019867	Outer membrane	5	BCL2L1, TIMM23, NUP62, NUP133, TOMM22	−2.647
IPR003093	Apoptosis regulator Bcl-2 protein, BH4	2	BCL2L1, BCL2L13	−2.532
IPR000712	Apoptosis regulator Bcl-2, BH	3	BCL2A1, BCL2L1, BCL2L13	−2.468
IPR001751	Calcium-binding protein, S-100/ICaBP type	3	S100A10, S100A12, S100P	−2.468
GO:0042221	Response to chemical stimulus	12	CCR1, HSPA1L, HSP90AB1, PLP2, RNASE2, S100A12, CCL5, SPP1, ABCG2, NUP62, KCNIP1, DERL1	−2.428
GO:0009628	Response to abiotic stimulus	13	CCR1, FECH, HSPA1L, HSP90AB1, PLP2, RNASE2, S100A12, CCL5, SPP1, ABCG2, NUP62, KCNIP1, DERL1	−2.367
GO:0016358	Dendrite morphogenesis	2	DBN1, MT3	−2.346
GO:0008159	Positive transcription elongation factor activity	2	HMGN1, SUPT16H	−2.317

[a] Identification number of represented family in either the GO or Interpro (IPR) databases.

Out of the entire library, 315 (2.1%) genes increased infection with an afa/mfa ≥ 2, the cutoff chosen for follow-up. Several genes already known to be involved in HIV infection, such as the enhancers S100A12 (Ryckman et al., 2002), PP2A regulatory subunit B (Hrimech et al., 2000), nuclear exportin CRM1 (Wodrich and Krausslich, 2001) and CDK2 (Ammosova et al., 2005), were identified as enhancers by the screen, serving as an internal validation. The list of screen hits was then analyzed for identification of statistically over-represented ($\log P \leq -2.3$) functional categories by comparing to the GO and Interpro databases of gene families (Table 1). Several families representing genes involved in enhancing cellular metabolism (mitochondrial genes), gene transcription and

translation enhanced HIV infection, as well as many proteins that inhibit cellular apoptosis. Because HIV relies heavily upon the host cell machinery in order to carry out its life cycle, those proteins involved in increasing basic cellular metabolic and replicative functions while preventing cell death would be expected to be identified as hits in this cDNA screen. HIV utilizes the microtubule network for particle entry and translocation into the nucleus, and chemicals that destabilize microtubules block infection (Matarrese and Malorni, 2005; McDonald et al., 2002). Interestingly, several families with proteins known to be involved in microtubule dynamics (MAPT, DBN1) were identified in the screen, which may also suggest additional roles for them in HIV infection. The families

Table 2
Top cDNA enhancers of HIV infection in HeLaCD4βgal cells

Gene hit	Accession #	Symbol	Function	Original hit afa/mfa[a]	Reconfirmation fold over control[b]
Phosphatidylinositol transfer protein alpha	NM_006224	PITPNα	Cytosolic lipid transport and vesicle trafficking	2.77	2.95
Extracellular link domain containing 1	NM_006691	XLKD1	Receptor for hyaluronan	2.60	2.7
Tripartite motif-containing 28	NM_005762	TRIM28	Transcriptional corepressor	2.67	5.8
Lysozyme	NM_000239	LYZ	Bacterial cell wall degradation	2.67	8.6
Interferon-induced protein with tetratricopeptide repeats 1	NM_001548	IFIT1	IFN alpha induced expression, unknown function	4.90	3.5
Coronin, actin binding protein, 1A	NM_007074	CORO1A	Phagosome formation, and motility	2.90	2.92
Clone PP902	AF218032		Unknown function	2.47	2.4
DnaJC14 protein	NM_032364		Chaperone	5.70	5.5
Aldehyde dehydrogenase 3 family, memberA1	NM_000691	ALDH3A1	Oxidizes aromatic aldehyde substrates	2.07	1.70
SET domain-containing protein 8	AY102937	SET8	Histone H4-lysine 20-specific methyltransferase	2.13	2.2
Mitogen-activated protein kinase kinase kinase 11	NM_002419	MAP3K11, MLK3	Regulator of JNK kinase, IKK alpha and beta	4.60	8.80
KIAA0247 gene product	NM_014734		Membrane protein, unknown function	3.90	2.01
Dual specificity phosphatase 18	XM_038481	DUSP18	Ser/Thr and Tyr phosphatase, unknown function	2.99	2.10

[a] Average fold activation (afa) divided by the adjusted standard deviation of the fold activation (mfa), which accounts for both the effect of each cDNA and the deviation between the replicates.
[b] Negative control Sport6gfp, same as used in screen.

of calcium EF-hand binding proteins, including the known HIV enhancer S100A12, were also statistically significantly represented in the hitlist, which may lead to a broader understanding of the involvement of this class of proteins as a whole in infection. In addition, several novel protein families, including those with protein methyltransferase activity (GO:0008276) and from the peptidase M24 family (IPR000994), were identified that could lend further insight into HIV biology.

The top enhancers of infection, as determined both by screen performance and literature review, were chosen for follow-up. The hits represented a variety of gene families including enzymes and transcription factors and genes from pathways known to be important in HIV infection, such as the ubiquitin pathway (Fig. 1C). Each cDNA hit was re-grown at mini-prep scale and re-tested in the original assay, and then the top performers were grown at maxi-prep scale, tested in the original assay, and sequenced to ensure proper hit identity. At this stage, 13 sequence-confirmed hits continued to show enhancement of activity compared to the negative control, ranging from 1.7-fold to 8.8-fold over Sport6-GFP-negative control (Table 2). Because the exact biological function of several of these factors is either unknown or inferred, investigation into their role in HIV biology will also allow insight into their cellular functions.

We next investigated whether the seven strongest cDNA enhancers (performing ∼3-fold or greater over control) were essential for HIV replication by depleting the endogenous proteins in HeLaCD4βgal cells using siRNA. For 6 of these we obtained validated siRNA SmartPools and evaluated their effects on HIV-IIIb infection in HeLaCD4βgal cells. Of those tested, siRNA against chaperone DnaJC14, interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and phosphatidylinositol transfer protein alpha (PITPNα) decreased HIV replication without significant toxicity (Fig. 2A) further strengthening their role in infection while the others had no effect on viral replication or cell viability (data not shown). Quantitative RT-PCR was used to ascertain the ability of these siRNAs to knock down the target gene of interest in this system (Fig. 2B). There are several reasons why host factors that were identified by cDNA screening may not be confirmed in siRNA studies. Because cDNA screening involves the introduction of genes normally expressed in a variety of tissues, the effects seen may not be supported by RNA interference due to lack of adequate expression or function of those genes in the cellular assay system. Identification of putative host factors through cDNA screening then serves as a starting point to focus further efforts in cellular co-factor studies. In addition, overexpression of any protein may lead to effects outside the normal scope of physiologic function. Thus, proteins such as lysozyme, whose normal activity is the breakdown of bacterial cell wall components as part of the innate immune system, may exhibit alternative activities such as enhancement of HIV infection when overexpressed, even if this function may not be physiologically relevant or mechanistically obvious.

In light of its clear physiologic role and high potential as a therapeutic target, we next focused our efforts on the strong cDNA enhancer mixed lineage kinase 3 (MLK3). This serine/threonine kinase is involved in the activation of downstream
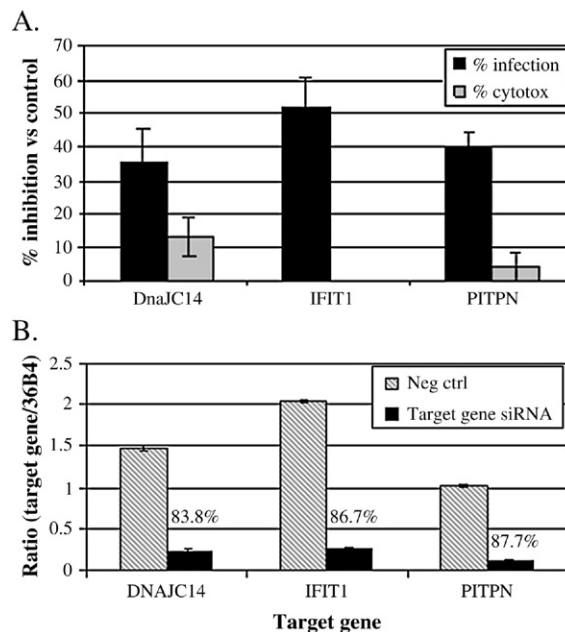

Fig. 2. Efficacy of siRNA targeting top non-druggable cDNA hits in HeLaCD4βgal cells. SMARTPool siRNAs against the top cDNA hits were transfected into HeLaCD4βgal cells and after 24 h, cells were challenged with HIV-IIIb (A). Infection was assessed after 3 additional days by measuring the beta-galactosidase produced from the stable LTR-βgal reporter within the cells using a chemiluminescent substrate (Gal Screen). Cytotoxicity of each siRNA was also determined by using Cell Titer Glo in parallel uninfected cultures. Of those SMARTPools tested, three showed inhibition of infection without significant toxicity compared to control and are shown here (PITPN, IFIT1, DNAJC14). (B) To ensure proper target knockdown, SMARTPools were transfected in parallel wells and RNA harvested at 24 h post-transfection. Levels of each gene were then determined using TaqMan quantitative RT-PCR and normalized to an internal rRNA component reference standard 36B4. Percentage of gene knockdown was calculated relative to the GL2-negative control and is representative of at least two experiments.

Map kinases, including JNK, p38, and ERK and has been shown to play a critical role in neuronal apoptosis including that mediated by HIV gp120 (Bodner et al., 2004; Gallo and Johnson, 2002). A compound inhibitor of MLK3, CEP-1347, was in clinical trial for the treatment of neurodegenerative diseases, highlighting its role in neuronal cell death and its applicability as a drug target (Bodner et al., 2004). To address whether the kinase activity of MLK3 was responsible for the enhancement of infection seen using the cDNA, we obtained a kinase-inactive mutant (MLK3 KI) and tested its effects on HIV infection. Unlike the wild-type protein, MLK3 KI was unable to increase infection, suggesting that the function of MLK3 is critical for enhancement (Fig. 3A). Because of its role in activating the AP-1 complex through JNK activation, we hypothesized that MLK3 could be acting by enhancing HIV transcription via the AP-1 site located in the HIV LTR (Cullen, 1991b). In support of this hypothesis, overexpression of wild-type MLK3, but not the kinase-inactive mutant, led to increases in overall JNK phosphorylation (data not shown). To test whether this could be responsible for enhanced HIV transcription, MLK3 was co-transfected with the LTR-luciferase construct either with a Tat expression vector or a control plasmid to assess both Tat-dependent and Tat-independent
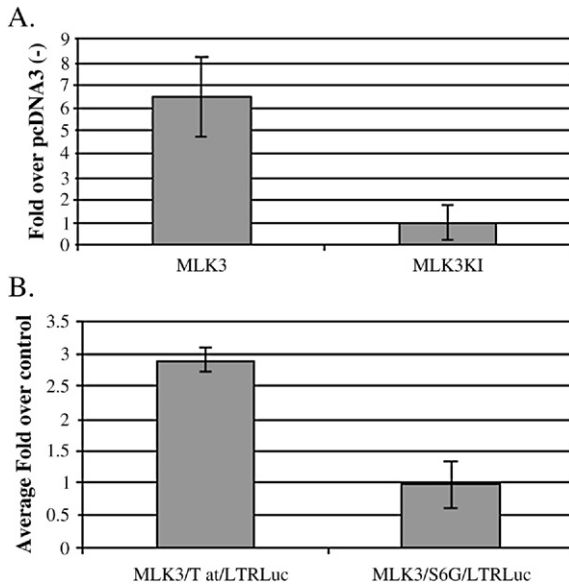
no effect on levels of early reverse transcripts or integrated provirus (data not shown). We further tested MLK3 siRNA in HIV-IIIb infection of Jurkat cells, and found that it decreased infection, although to a lesser extent than that seen in the HeLa-CD4-Bgal cells (Fig. 4C). Western blot analysis showed that the amount of protein depletion in the Jurkat cells was lower than that seen in the HeLaCD4Bgal cells, thus explaining the lower level of inhibition seen (Fig. 4D). Along with recent evidence showing a lack of gross defects in MLK3 knockout mice (Brancho et al., 2005), these results indicate that MLK3 is
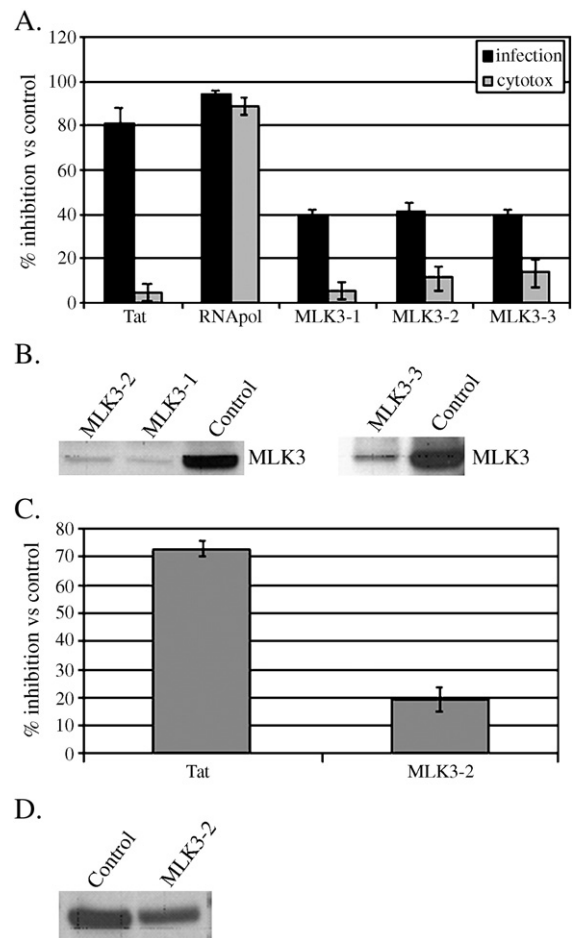

Fig. 3. (A) Effect of kinase-inactive MLK3 (K144R) on HIV infection of HeLaCD4βgal cells. Cells were co-transfected with cDNA encoding wild-type MLK3, kinase-inactive MLK3 (K144R), or control plasmid along with a luciferase reporter under the control of the HIV-LTR followed after 24 h by infection with HIV-IIIb. Infection was assessed after 3 days by measuring luciferase activity using Brite Glo. The kinase-inactive mutant was unable to increase infection, highlighting the requirement of kinase function for the enhancement seen by wild-type MLK3. Data are shown as the fold enhancement over negative control plasmid and are the summary of four independent assays with two replicates in each assay. (B) Effect of MLK3 overexpression on HIV transcription. HeLaCD4βgal cells were co-transfected with cDNA encoding wild-type MLK3 or control plasmid (pcDNA3) along with a luciferase reporter under the control of the HIV-LTR (LTRLuc) and either a Tat expression vector (Tat) or control vector Sport6-GFP (S6G) to assess either Tat-dependent or Tat-independent transcription. Transcription was assessed after 2 days by measuring luciferase activity using Brite Glo. Expression of MLK3 enhanced Tat-dependent transcription of luciferase, but had minimal effect on Tat-independent transcription. Data are shown as the average fold enhancement with MLK3 (e.g., MLK3/Tat/LTRLuc) over negative control plasmid (e.g., pcDNA3/Tat/LTRLuc), and are the summary of two independent assays with two replicates in each assay.

effects on LTR-mediated transcription. Expression of MLK3 enhanced Tat-dependent transcription by approximately 3-fold over control but had no significant effect on Tat-independent transcription (Fig. 3B), suggesting that MLK3 enhances infection through viral-specific transcription. The level of Tat plasmid used in the co-transfection studies (40 ng) was the same level used as a positive control for the screen and showed maximal possible activation within this cellular system in a dose response study (data not shown). This indicates that over-expression of MLK3 can enhance viral-specific transcription above and beyond that seen with Tat alone in this system.

Considering the overexpression data, we finally investigated whether siRNA against MLK3 would inhibit HIV infection. We obtained three unique siRNA sequences targeting MLK3 and evaluated their efficacy against HIV in HeLa-CD4-βgal cells. All three siRNAs were effective at depleting MLK3 protein (Fig. 4B) and decreased HIV infection by approximately 40% (Fig. 4A). Consistent with its proposed mechanism of affecting Tat-dependent transcription, MLK3 depletion had

Fig. 4. Efficacy of siRNA targeting MLK3 against HIV infection. Individual MLK3 siRNAs were transfected into HeLaCD4βgal cells (A and B) or electroporated into Jurkat cells (C and D) and after 24 h, cells were challenged with HIV-IIIb. For HeLaCD4βgal cells (A), infection was assessed after 3 additional days by measuring the beta-galactosidase produced from the stable LTR-βgal reporter within the cells using a chemiluminescent substrate (Gal Screen). For Jurkat cells (C), input virus was removed 24 h after infection, and supernatants were collected after an additional 48 h and tested for levels of p24 by ELISA. Cytotoxicity of each siRNA was also determined by using Cell Titer Glo in parallel uninfected cultures. Data are shown as the percent infection inhibition or cytotoxicity compared to control GL2 siRNA deviation and are the average±standard deviation of at least three independent experiments with twelve replicate wells per experiment. To evaluate depletion of target proteins, HeLaCD4βgal cells (B) or Jurkat cells (D) were harvested after 72 h and for MLK3. All three siRNAs were able to significantly deplete endogenous MLK3 levels. The extent of protein depletion in the Jurkat cells is less than in the HeLaCD4βgal cells, explaining the lower level of infection inhibition seen.

a novel proviral host factor that could serve as a therapeutic target for HIV infection.

Discussion

The increasing incidence of transmission of multi-drug resistant virus and lack of an effective vaccine has led to a search for novel targets for anti-HIV therapy. The hope is that candidates can be identified that are necessary for viral replication but dispensable for overall cellular function, leading to a therapeutic window that allows infection inhibition without significant host toxicity. There are many reports in the literature using yeast-two-hybrid screening and microarray analysis to identify new cellular co-factors, however, little genome-scale screening within the context of a productive infection with live HIV has been reported. Because it involves the introduction of genes, cDNA screens like the one presented here allow for the identification of cellular co-factors that would be utilized in the physiologic cellular targets for HIV in cell types more amenable to screening, such as HeLaCD4βgal cells. In reviewing the data from the screen and choosing hits for follow-up, we focused on genes whose expression profiles showed at or above median expression in T cells or monocytes/macrophages, the normal cellular targets for HIV. However, overexpression of a gene outside of its physiologic environment can lead to false positives, where the factor may not actually be involved in the course of natural infection. Additional support through RNA interference, mutant studies, and pharmacologic intervention is thus valuable for the determination of true potential targets.

Mixed lineage kinase 3 (MLK3) was one of the strongest enhancers of HIV infection identified in the cDNA screen. Through activation of downstream effectors such as JNK, p38, and ERK, MLK3 mediates the response of cells to various stimuli including cytokines and mitogens as well as other stress signals (Gallo and Johnson, 2002). Activation of MLK3 occurs as a result of Rac/Cdc42 interactions, which lead to proper stimulatory phosphorylation and membrane targeting. The outcome of MLK3 signaling varies depending on the cell type and stimulus. In neuronal cells, stimulation of MLK3 has been shown to induce apoptosis through JNK activation, which has led to the design of inhibitors for the treatment of neurodegenerative disorders (Xu et al., 2001). In non-neuronal cells, overexpression of MLK3 has been shown to promote cell proliferation and transformation via the Map kinase cascade as well as mediating morphological changes through microtubule dynamics (Cha et al., 2006). In the context of T cells, TCR signaling along with CD28 co-stimulation activates Vav-1, whose subsequent stimulation of Rac leads to MLK3 activation of JNK (Hehner et al., 2000a). Along with the CD28-mediated induction of NFκB through MLK3, this cascade leads to induction of IL-2 transcription via AP-1 and NFκB binding sites in the IL-2 promoter and subsequent proliferation (Hehner et al., 2000b). Early studies on the HIV promoter in the LTR region showed the presence of binding sites for a variety of transcription factors also involved in T cell activation, including AP-1, NFκB, and NF-AT (Cullen, 1991a). While activation of NFκB has been proven to enhance basal transcription from the

HIV-LTR, there are conflicting reports on the ability of AP-1 or its components (fos and jun) to enhance Tat-independent transcription. Studies in colon cell lines showed that over-expression of fos, but not jun, was able to increase basal LTR activity only in those cells that were susceptible to virus infection (Roebuck et al., 1993). However, later studies in HeLa cells indicated that the ability of fos and jun to enhance Tat-independent transcription was dependent upon the presence of activated NFκB (Yang et al., 1999). The ability of MLK3 to stimulate only Tat-dependent transcription within the HeLa cell background used here is consistent with the AP-1 data in HeLa cells. It is also possible that MLK3-mediated enhancement of transcription is through other Tat-dependent transcription partners, such as pTEFb, although a link between MLK3 signaling and pTEFb function has not been identified (Zhou et al., 1998). We recently described our data supporting the involvement of another kinase activated by Rac/Cdc42, Pak1, on HIV infection (Nguyen et al., 2006). Pak1 has many of the same downstream effectors as MLK3, including JNK, ERK, and p38 (Bokoch, 2003); however, Pak1 depletion with siRNA was shown to inhibit early events in the viral life cycle while MLK3 depletion did not (data not shown). Despite being involved in similar signaling cascades, these two kinases exhibit effects on different stages in the HIV life cycle, demonstrating the capacity of HIV for utilizing cellular co-factors in multiple capacities.

Interestingly, one of the other top enhancers was the interferon-alpha induced protein IFIT1, which has been shown to be upregulated in a variety of viral infections such as hepatitis C (HCV) (Wang et al., 2003) as well as in HIV infection of astrocytes (Kim et al., 2004). While the biological function of IFIT1 is unknown, it has been reported to be able to interact with Rho/Rac guanine nucleotide exchange factor, and thus may help in the activation of Rho/Rac proteins (Ye et al., 2003). HIV transcription in T cells is induced by Rac activation downstream of CD28 co-stimulation (Cook et al., 2003), and recent studies have suggested that statins are able to inhibit HIV infection by blocking Rho activation (del Real et al., 2004). The idea that HIV may use an interferon-induced protein to help facilitate its replication is intriguing and worthy of further study, especially considering the conflicting reports on the efficacy of interferon alpha as an anti-HIV agent. Less is known about the other cDNA hits whose siRNA effects confirmed their involvement in HIV infection. DNAJC14 is a member of the J-domain chaperone family involved in protein folding (Chen et al., 2003), and has been shown to associate with dopamine receptor in the ER, potentially preventing its cell surface localization (Bermak et al., 2001). In this capacity, DNAJC14 overexpression could lead to the downregulation of surface receptors whose natural function inhibits HIV infection or replication. PITPN is a member of a family of small proteins involved in the transport of phosphatidylinositol and phosphatidylcholine between cell compartments (Morgan et al., 2004). Because of the involvement of phosphatidylinositol in a variety of cell signaling cascades, PITPN is poised to regulate those signaling pathways involving PLC activity: phosphorylation of PITPN by PKC has been shown to prevent its PI exchange

capabilities and attenuate PLC activity (Morgan et al., 2004). Thus, overexpression of this factor could enhance some signaling pathways beneficial to the HIV life cycle. Considering the lack of data in the literature on the biological role of several of the top cDNA enhancers found in our screen, further investigation on how these genes impact HIV infection could also allow the elucidation of their cellular function.

Materials and methods

Cells lines and maintenance

HeLaCD4βgal cells from Dr. Michael Emerman were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Kimpton and Emerman, 1992), and were maintained in DMEM supplemented with 10% FBS, 1× penicillin/streptomycin/L-glutamine, 0.2 mg/ml G418 and 0.1 mg/ml Hygromycin B. Jurkat cells were maintained in RPMI-1640 supplemented with 10% FBS and 1× penicillin/streptomycin/L-glutamine. All cell culture reagents were obtained from Invitrogen.

cDNA screening in HeLaCD4βgal cells

High-throughput cDNA retro-transfection of HeLaCD4βgal cells was carried out essentially as described (Chanda et al., 2003). Briefly, individual cDNA of a sub-genomic library encompassing 15,000 genes (collection details at http://function.gnf.org), negative control Sport6GFP cDNA and positive control Sport6-Tat plasmid were spotted at 40 ng/well in 55 white opaque 384-well plates (Greiner). A solution of 1% Gene Juice (Novagen) containing 1 μg/ml of an HIV-LTR-Luciferase reporter plasmid (derived by PCR amplification of the LTR sequence of strain HxB2) in serum-free Opti-MEM media (Invitrogen) was added to each well (10 μl) using a Multidrop apparatus (Titertek) and complexes were allowed to form for 10 min. HeLaCD4βgal cells (1000 cells/30 μl/well in DMEM/ 10% FBS) were then added and the plates were incubated overnight, followed by the addition of 20 μl of DMEM/10% FBS with 200 ng/ml p24 of HIV-IIIb (Advanced Biotechnologies Inc.). After 72 h, infection was assessed by measuring luciferase production using Brite Glo (Promega) and reading on the CLIPR apparatus (Molecular Devices). The entire library was run in duplicate. Hits were reconfirmed after growing up additional cDNA by testing in the original assay and were sequenced to confirm their identity (Eton Bioscience).

cDNA screen data analysis

For data analysis, the raw signal values are first normalized by dividing the raw value of a well by the median of all raw values on the plate where it comes from. When computing the median, wells designated as control wells are excluded. The normalized value then represents a fold change above or below the median of the plate. Prior to averaging or performing other computations such ratios are log transformed to yield symmetric ranges between fold changes above and below the median.

The remaining computations aim to provide a measure for each transcript's fold-change value to which degree it is an outlier, i.e., different from the average fold-change, in a way that is robust against the high amount of noise present in typical screening results. The average fold activation or 'afa' of the normalized values $x_1, x_2, ..., x_n$ of the n replicates of a particular well is defined on the basis of a geometric mean of the replicate values:

$$\text{afa} = \begin{cases} \exp(\mu) & \text{if } \mu \geq 0 \\ \dfrac{-1}{\exp(\mu)} & \text{if } \mu < 0 \end{cases}, \text{ with } \mu = \frac{\sum\limits_{i=1}^{n} \log(x_i)}{n}.$$

In this manner fold activation ($\mu > 0$) and fold repression ($\mu < 0$) are represented on the intervals $[1, \infty)$ and $(-\infty, -1]$ respectively. Hence, the dynamic range of activator and repressor screens can be compared on an equivalent scale.

Next afa is penalized by the standard deviation between replicates. Specifically, the penalized average fold activation (pafa or afa/mfa) for the normalized values $x_1, x_2, ..., x_n$ of the n replicates of a particular well is defined as

$$\text{pafa} = \frac{\text{afa}}{\text{mfa}}, \text{ with } \text{mfa} = \exp\left(\sqrt{\frac{\sum\limits_{i=1}^{n} (\log(x_i) - \mu)^2}{n - 1}}\right).$$

Note that when the standard deviation is zero, mfa equals 1 and thus in this case afa/mfa = afa. Wells in the generated hitlist are ranked by their afa/mfa score.

Statistical analysis of gene family enrichment

Screening hits were analyzed for identification of statistically over-represented functional categories. Among our cDNA screening collection of N genes with any piece of functional annotations, a subset of n genes were hit picked. If a certain functional category of size M matches m hits and $M-m$ non-hits, the probability that m or more genes in the category of interest occurs in our hitlist by chance in a random hit selection can be represented by the accumulative hypergeometric distribution (Zar, 1999):

$$P(N,n,M,m) = \sum_{i=m}^{\min(n,M)} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}.$$

Functional categories with smaller P-value therefore are more likely to reflect true underlying molecular processes. In this study, the entire gene ontology tree obtained from the GO database (http://www.geneontology.org), as well as the InterPro protein family database (http://www.ebi.ac.uk/interpro) was recursively evaluated. A P-value cutoff of 10^{-2} was used to define relevant gene families.

cDNA testing of kinase-inactive MLK3

MLK3 and a kinase-inactive mutant of MLK3 (K144R) in pcDNA3.1 vector were generously provided by Dr. Zhiheng Xu (Xu et al., 2001) (Columbia University) and were tested alongside the MLK3 Origene collection hit in 12-well plates using a scaled up version of the screen assay. Briefly, 1.28 µg cDNA/0.32 µg LTR-Luc/32 µl was spotted per well followed by 320 µl of 1% gene juice/Opti-MEM and then 6×10^4 HeLaCD4βgal cells in 1 ml of DMEM/10% FBS. After 24 h, cultures were infected with 90 ng p24 of HIV-IIIb, incubated for 3 additional days, and assessed for infection levels using Brite Glo (Promega) and reading on the CLIPR apparatus (Molecular Devices).

siRNA

GL2 luciferase siRNA (catalog #D-001100-01-20), and PITPNá (accession # NM_006224), TRIM28 (accession # NM_005762), IFIT1 (accession # NM_001548), LYZ (accession # NM_000239), CORO1A (accession # NM_007074), and DnaJC14 (accession # NM_032364) SmartPool siRNAs were obtained from Dharmacon. In the case of GL2 siRNA, additional amounts of the same sequence were also obtained from Qiagen. Two siRNA against Tat were synthesized and pooled for use as a positive control for inhibition of HIV infection: (1) CUGCUUGUACCAAUUGCUA-d(TT) which has been previously published (Coburn and Cullen, 2002) and (2) GCCUUAGGCAUCUCCUAUG-d(TT) which was designed using the Dharmacon siDESIGN center. MLK3-1 and MLK3-2 siRNA were designed using an algorithm previously described (Aza-Blanc et al., 2003) and had the following sequences: (MLK3-1) CCCTGAAGATCACC-GACTT-d(TT), (MLK3-2) GCTGTTAACAAGCTCACAC-d(TT). MLK3-3 siRNA was obtained from Dharmacon (catalog #D-003577-03).

siRNA in HeLaCD4βgal cells

siRNA retro-transfection of HeLaCD4βgal cells was carried out essentially as described (Aza-Blanc et al., 2003). Briefly, individual siRNAs were spotted at 14 ng/well in white opaque or white clear-bottom 384-well plates (Greiner) containing one siRNA sequence per well. A solution of 2% oligofectamine (Invitrogen) in serum-free Opti-MEM media (Invitrogen) was added to each well (10 µl) and complexes were allowed to form for 15–20 min. All 384-well dispenses were done using a Multidrop apparatus (Titertek). HeLaCD4βgal cells (1000 cells/ 30 µl/well in serum-free Opti-MEM) were then added and the plates were incubated overnight, followed by the addition of 20 µl of 30% FBS/DMEM with 200 ng/ml of HIV-IIIb (Advanced Biotechnologies Inc.). After 72 h, infection was assessed by measuring beta-galactosidase production using an equal volume of Gal Screen (Applied Biosystems). All 384-well plate reading was done using the CLIPR apparatus (Molecular Devices). Twelve replicates were run per 384-well plate and the data were expressed as percent inhibition compared to the

negative control GL2 siRNA. Cytotoxicity of the siRNA was measured at 96 h post-transfection by adding equal volume of a 1:4 dilution of Cell Titer Glo (Promega) and reading luminescence on the CLIPR apparatus, with the data again expressed as a percent inhibition of viability signal compared to negative control GL2 siRNA.

siRNA validation by quantitative RT-PCR

siRNA (800 ng) was spotted in 250 µl of serum-free Opti-MEM (Invitrogen) in 6-well plates followed by the addition of 250 µl of 1.5% Lipofectamine 2000 (Invitrogen) in serum-free Opti-MEM. Plates were incubated at room temperature for 20 min to allow for complex formation. HeLaCD4βgal cells (3×10^5 in 1.5 ml of serum-free Opti-MEM) were then added and incubated overnight followed by the addition of 1 ml of 30% FBS/DMEM. Cells were harvested 24 h post-transfection and RNA isolated using the RNeasy kit from Qiagen. Following normalization, RNA was used as template in one-step quantitative RT-PCR using reagents from Invitrogen and performed on the ABI Prism 7900 instrument (Applied Biosystems Inc.) using the following primer and probe sets designed with Primer Express software; (1) DNAJC14 forward: CTTTTCTTTCCATTCCATCTGCTT, DNAJC14 reverse: TCCCTCAGAGTAAGGCCCTTCT, DNAJC14 probe: 6FAM-CAACTGCCAGGGAACACCTGGAATTC-TAMSp; (2) IFIT1 forward: GCCTCCTTGGGTTCGTCTATAA, IFIT reverse: TCAAAGTCAGCAGCCAGTCTCA, IFIT1 probe: 6FAM-AGCCCTGGAGTACTATGAGCGGGCC-TAMSp; (3) PITPN forward: CGTGGAGGTCCTGGTGAATG, PITPN reverse: TGCAGGTGGTAGATCTTATGTGTGT, PITPN probe: 6FAM-CCCTACGAGAAGGACGGTGAGAAAGGC-TAMSp.

siRNA validation by Western blot

siRNA (800 ng) was spotted in 250 µl of serum-free Opti-MEM (Invitrogen) in 6-well plates followed by the addition of 250 µl of 1.5% Lipofectamine 2000 (Invitrogen) in serum-free Opti-MEM. Plates were incubated at room temperature for 20 min to allow for complex formation. HeLaCD4βgal cells (3×10^5 in 1.5 ml of serum-free Opti-MEM) were then added and incubated overnight followed by the addition of 1 ml of 30% FBS/DMEM. Cells were harvested 72 h post-transfection by scraping and lysed in cell lysis buffer (20 mM HEPES pH 7.2/10 mM KCl/1 mM EDTA/1% Triton X-100/1× protease inhibitors; Sigma Chemical Co.) for 1 h on ice. Total protein concentration of the lysates was measured using the Micro-BCA kit (Promega) and equal protein amounts were loaded onto 4–12% NuPage Bis–Tris gel (Invitrogen) and subjected to electrophoresis as suggested by the manufacturer. Following transfer to nitrocellulose, blots were blocked with 5% nonfat milk in PBST (phosphate-buffered saline with 0.05% Tween-20) and then subjected to immunoblotting with the following antibodies; rabbit polyclonal anti-MLK3 and goat–anti-tubulin antibody from Santa Cruz Biotechnology, HRP-conjugated goat-anti-rabbit secondary antibody from Sigma Chemical Co.,

HRP-conjugated donkey–anti-goat secondary antibody from Promega. All antibodies were used at dilutions suggested by the manufacturer and were diluted in 5% nonfat milk in PBST. Bands were visualized using ECL-plus detection reagent (Amersham).

siRNA transfection of Jurkat T cells

Jurkat T cells were washed once in PBS, resuspended in serum-free Opti-MEM (Invitrogen) at high density (2.4×10^8/ml) and 50 µl was added to 1 nmol of siRNA (50 µl of 20 µM) in an 0.2 cm gap cuvette. The mixture was subjected to electroporation using the BioRad Gene Pulser Xcell module using conditions suggested by the manufacturer (140 V, 1000 µF, exponential decay) and then transferred to 12 ml of RPMI supplemented with 10% FBS without antibiotics for 24 h. Cells were then pelleted, viable cells counted by trypan blue exclusion, and resuspended at a density of 1.7×10^6/ml in RPMI supplemented with 10% FBS and 1XPen/Strep/glutamine. For infection studies, 300 µl of siRNA-treated cells was added to wells of a 48-well plate and either 2 ng or 0.5 ng of HIV-IIIb, corresponding to MOIs of 0.0005 and 0.000125 respectively based on viral titer provided by the manufacturer, was added to each well. After 3 additional days, the cells were harvested, washed 3× in PBS, lysed, and infection was measured by p24 ELISA of the cell lysates. For cytotoxicity, 300 µl of siRNA-treated cells was added to wells of a 48-well plate and after 3 days, cell viability was measured using Cell Titer Glo (Promega) and reading on the CLIPR (Molecular Devices) or using Alamar Blue (TREK systems) and reading on the Acquest (LJL Biosystems). To determine siRNA efficacy, cell lysates were prepared and analyzed as in HeLaCD4βgal siRNA validation studies at 72 h post-electroporation.

Acknowledgments

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