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HIV Nef Enhances Tat-Mediated Viral Transcription through a hnRNP-K-Nucleated Signaling Complex

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SUMMARY

Although dispensable in vitro, HIV Nef enables high-level viral replication in infected hosts by an as yet unexplained mechanism. Previously, we proposed that Nef functionally cooperates with the viral transactivator Tat by derepressing the viral promoter via a Nef-associated kinase complex (NAKC). Here, we demonstrate that hnRNP-K, a host factor thought to facilitate crosstalk between kinases and gene expression, interacts with Nef and, as part of NAKC, nucleates Nef-interacting kinases, including Lck, PKCδ, and PI-3 kinase, leading to Lck and Erk1/2 activation. This strongly increased HIV transcription, which depended on Tat and the NF-kB motif in the viral promoter, but not on NF-kB activation. Depletion of hnRNP-K in a Jurkat model of HIV latency increased Erk1/2 activity and greatly augmented HIV reactivating stimuli. We conclude that hnRNP-K coordinates membrane signaling with transcriptional derepression through Erk1/2 and is targeted by HIV to enable Tat-mediated transcription.

INTRODUCTION

In the infected host, the Nef protein of HIV is required for high-level viral replication; however, the molecular mechanism is still unresolved. *nef*-deleted virus mutants persist in vivo, but their replication activity is barely detectable in the peripheral blood (Deacon et al., 1995). Thus, it had been speculated that Nef influences viral transcription. Early studies, however, revealed that Nef has no or, rather, a negative influence on the viral promoter (Hammes et al., 1989; Kim et al., 1989). Therefore, it was assumed that the combination of various Nef effects increase the viral load by indirect means.

The well-documented Nef activity on the downregulation of T cell surface receptors, namely CD4, was thought to prevent multiple infections of a single cell and, thus, an overload of the cellular transcription and translation machinery (Lama, 2003). The same CD4 downregulation effect was found to increase the infectivity of viral particles by reducing CD4 incorporation into the viral coat (Lama et al., 1999; Ross et al., 1999). However, Nef also enhances infectivity in the absence of CD4 (Chowers et al., 1995). Besides enhancing infectivity, Nef increases the release of viral particles from infected cells in vitro. The latter correlated with the interaction of the Vav protein, but seemingly not with an increased transcriptional activity of the HIV promoter (Fackler et al., 1999; Simmons et al., 2005). Finally, Nef was shown to prolong the survival of the infected cell by activating antiapoptotic signaling pathways (Geleziunas et al., 2001; Wolf et al., 2001) and by avoiding CTL-mediated lysis through downregulation of MHC class I (Collins et al., 1998).

While the above listed Nef effects may increase the number of viral particles in vivo, they do not sufficiently explain the absence of a multilog viral load usually observed in HIV infection, but not in individuals infected with a *nef*-negative mutant virus (Learmont et al., 1999). Thus, the question remains whether Nef-mediated T cell signaling supports viral transcription in a manner that has not been elucidated yet.

Previously, we have described a multimeric signaling complex that associates with the N terminus of Nef and is required for optimal viral replication (Baur et al., 1997). Therefore, we hypothesized that it was functionally connected to viral transcription. Notably, the complex contained at least two kinase activities, namely Lck and a serine kinase, which we recently identified as a member of the nPKC subfamily (PKCδ/θ) (Wolf et al., 2008). Therefore, we termed the complex NAKC for Nef-associated kinase complex. In subsequent studies, we identified the polycomb protein Eed as a component of NAKC (Witte et al., 2004) (see also Figure 1A). Eed bound Nef directly and colocalized with Lck at the plasma membrane. Importantly, it stimulated Tat-dependent transcription in concert with Nef and supported our initial hypothesis that NAKC was linked to HIV transcription. However, Eed would not bind Lck or PKC directly, and, thus, its role in NAKC as well as the connection to both kinases was unclear.

hnRNP-K, or K protein, is a cytoplasmic and nuclear factor that seems to be involved in many cellular functions, including signal transduction and gene expression. It contains multiple protein—as well as RNA- and DNA-binding motifs—and is, therefore, believed to facilitate the crosstalk between kinases and factors involved in gene expression (Bomsztyk et al., 2004). In vitro, hnRNP-K was shown to interact with the tyrosine kinases Lck and Src, PKCδ, and, notably, also with Eed (Adolph et al., 2007; Bomsztyk et al., 2004; Denisenko and Bomsztyk, 1997; Ostrowski et al., 2000; Schullery et al., 1999). However, the relevance and function(s) of these associations are not known.

We asked whether hnRNP-K is a component of NAKC and a bridging factor between Eed and the kinases Lck and PKCδ.

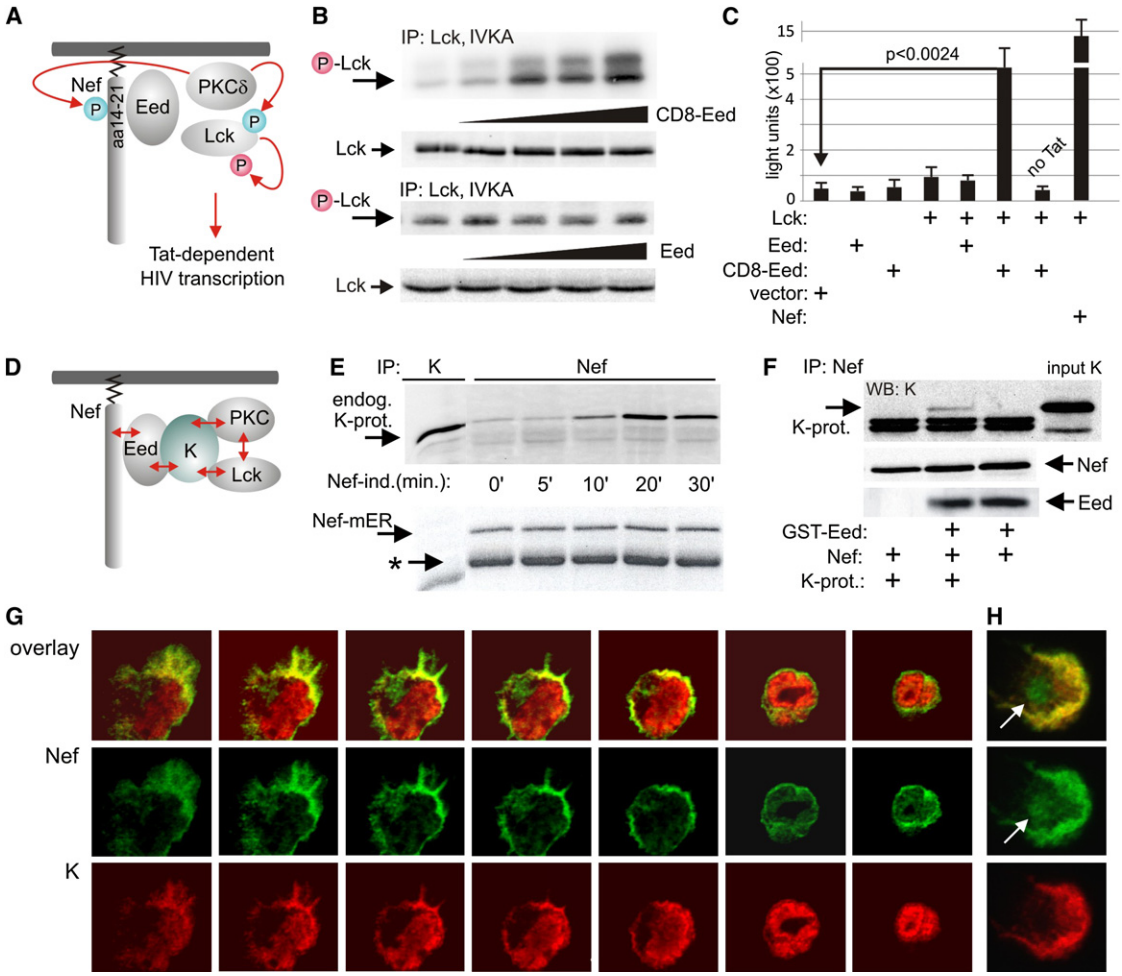

Figure 1. The Nef-Associated Kinase Complex Is Functionally Coherent and Contains hnRNP-K

(A) Nef-associated kinase complex (NAKC) composition and function as demonstrated previously (Baur et al., 1997). A short α helix in Nef (aa14–21) binds Eed and coprecipitates Lck and PKCδ/θ, causing phosphorylation events as indicated. NAKC formation correlated with increased Tat-dependent HIV transcription (Witte et al., 2004).

(B) CD8-Eed increases Lck activity. Lck (1 μg) was cotransfected with increasing amounts of CD8-Eed or Eed (0.5, 1, 2, 4 μg) into 293T cells, immunoprecipitated, and subjected to an in vitro kinase assay (IVKA). Lck expression was controlled in lysates of transfected cells.

(C) CD8-Eed stimulates Tat-dependent transcription. The indicated plasmids (2 μg) were cotransfected with an HIV-LTR luciferase reporter (0.5 μg) and suboptimal amounts of Tat (30 ng) into J.Cam.1 cells, which lack a functional Lck molecule, as described previously (Witte et al., 2004). After 16 hr, luciferase activity was determined. The p value is based on three experiments. Error bars show means ± SD from three independent experiments. The p value in the graph was calculated on the basis of the mean values of the indicated bars. $p < 0.0024$.

(D) Model of NAKC containing hnRNP-K. Red arrows depict the reported interactions in the literature.

(E) Nef recruits endogenous hnRNP-K. The Nef-mER Jurkat line was induced with tamoxifen for various time points as indicated before cells were immunoprecipitated for Nef-mER and blotted for hnRNP-K association. Subsequently, the NC filter was stripped and blotted for Nef-mER (lower panel). The star indicates the heavy chain.

(F) Nef does not bind hnRNP-K directly. Recombinant proteins (1 μg) derived from bacterial (GST-Eed, Nef) and baculovirus expression systems (hnRNP-K) were incubated as indicated at 4°C/1 hr before Nef was immunoprecipitated and blotted for hnRNP-K.

(G) Nef and hnRNP-K colocalize. Nef-mER Jurkat cells were induced for 30 min, stained for Nef and hnRNP-K, and analyzed by confocal microscopy.

(H) Nef and hnRNP-K colocalize in the cytoplasm, but not the perinuclear region. Same procedure as in (G). White arrows indicate the perinuclear region where Nef was found without hnRNP-K.

Here, we demonstrate that hnRNP-K interacts with Nef in the context of NAKC and recruits Lck, PKCδ, and PI-3 kinase. Formation of the complex was sufficient to activate Lck and Erk1/2, which, in T cells, caused a strong Tat-dependent increase of HIV transcription. Our study describes a signaling module that connects key T cell kinases with transcription and further unravels the complex but potent mechanism by which Nef supports HIV transcription.

RESULTS

One of the hallmarks of the Nef-associated kinase complex (NAKC) was the increased autophosphorylation of coprecipitated Lck in vitro (Baur et al., 1997) (Figure 1A). Lck, on the other hand, was crucial for the cooperation of Nef with Tat and its stimulating effect on HIV transcription (Witte et al., 2004). Therefore, we decided to determine the mechanism by

which Nef activates Lck in order to understand its role in HIV transcription.

First, we wanted to confirm that NAKC was sufficient to activate Lck, which would suggest that it was a functionally independent and coherent signaling complex. To verify this assumption, we asked whether Eed, which connects the Nef N terminus with NAKC (Figure 1A), would activate Lck similarly as Nef when targeted to the plasma membrane by a CD8 tag. We used the previously described CD8-Eed fusion-protein (Witte et al., 2004), which was transfected into Jurkat cells. As demonstrated in Figure 1B, increasing amounts of CD8-Eed increased the activity of endogenous Lck, whereas the same amount of nonfused Eed had no effect.

For further confirmation, we asked whether CD8-Eed would induce the assumed downstream effect of NAKC, namely an increase of Tat-dependent HIV transcription. Lck was cotransfected with Eed or CD8-Eed, suboptimal amounts of Tat, and an HIV-LTR-Luc reporter plasmid into J.CaM.1 cells, similar as described previously (Witte et al., 2004). As shown in Figure 1C, CD8-Eed, but not Eed, was able to stimulate HIV transcription in a Tat-dependent manner, albeit not as efficient as Nef itself. Together, these results confirmed that membrane-targeted Eed/NAKC was sufficient to activate Lck-and Tat-dependent HIV transcription.

In subsequent experiments, however, we were unable to demonstrate an association between Eed and Lck or PKCδ/θ (data not shown) and, therefore, assumed that an additional factor was involved. hnRNP-K seemed a likely candidate because it interacted directly with both kinases and also with Eed (Bomsztyk et al., 2004). Using recombinant proteins from bacterial and baculovirus expression systems, we were able to confirm these reported interactions, including the direct binding of Nef and Eed (Figures 1F and S1 available online). Based on these results, we hypothesized a model for NAKC as depicted in Figure 1D, which suggested a central role for hnRNP-K.

In order to confirm this model and clarify the role of hnRNP-K, we first wanted to demonstrate the coimmunoprecipitation of endogenous hnRNP-K with Nef. We used our previously described inducible Jurkat line expressing a Nef-mER fusion-protein (Witte et al., 2004). At various time points after induction, Nef-mER was immunoprecipitated and blotted for endogenous hnRNP-K. Recruitment of hnRNP-K was observed as early as 10 min and peaked after 20 min (Figure 1E). Notably, using the same cell line, we observed an identical kinetic for the recruitment of Eed by Nef (Witte et al., 2004). As predicted by our model (Figure 1D), Nef did not bind hnRNP-K directly but required Eed seemingly as a bridging protein. This was demonstrated in pull-down experiments with recombinant proteins, as described above (Figure 1F). Since hnRNP-K is found in the nucleus as well as in the cytoplasm, we analyzed the subcellular localization of Nef and hnRNP-K in Nef-induced Jurkat cells by confocal microscopy. In 60% of double-positive cells (Nef, hnRNP-K; $p < 0.0016$), cytoplasmic colocalization of both proteins was observed in a membrane/membrane-proximal (Figure 1G), but not perinuclear, area (white arrows in Figure 1H), where Nef is internalized after initial membrane targeting. In summary, we had established that Nef recruited hnRNP-K in T cells using Eed as a bridging molecule.

In a next step, we asked whether hnRNP-K was involved in Nef/NAKC-mediated Lck activation, which would confirm that it had a functional role in this complex and would give further insight into the Lck activation mechanism. Lck activation is a complex process and requires at least CD4/CD8, CD45, Csk, and Unc119 (Palacios and Weiss, 2004)—proteins that are only present in T cells. We reasoned that, in non-T cells and thus in the absence of these factors, Lck would not be activated unless by a different mechanism, as, for example, in the context of NAKC. Therefore, we chose 293T epithelial cells for our analysis. Lck and other NAKC components (Nef, Eed, hnRNP-K, PKCδ) were cotransfected in different combinations before Lck was immunoprecipitated and analyzed for in vitro kinase activity and tyrosine autophosphorylation. Cotransfection of Lck with one other NAKC component had no effect (Figure 2, lanes 2–5). However, the addition of two or more factors induced Lck activity (lanes 6–15). For example, the coexpression of hnRNP-K with Lck was ineffective (lane 2), whereas addition of Nef strongly increased Lck activity (lane 7). This included the typical mobility shift pattern of Lck caused by serine phosphorylation (Figure 2, third panel). Therefore, coexpression of Nef and hnRNP-K was sufficient for Lck activation in 293T cells, and this combination was used for subsequent experiments in 293T cells.

The contribution of each NAKC factor toward Lck activity was not always additive and sometimes inhibitory (e.g., Nef/K, Figure 2, lane 7 versus Nef/K/Eed, lane 12). Potentially, this was a consequence of disproportionate expression levels that caused the squelching of endogenous NAKC factors. Importantly, however, the strongest Lck activity, characterized by four tyrosine phosphorylated bands generated by in vitro Lck autophosphorylation (56–63 kDa, see arrows) and an Lck mobility shift of more than 50% generated by in vivo serine phosphorylation (63 kDa), was seen after cotransfection of all NAKC proteins (lane 15). This implied that all factors were required for optimal Lck activation.

In order to understand the molecular mechanism leading to Lck activation, we investigated the hnRNP-K-Lck interaction in greater detail. hnRNP-K contains two tyrosine residues ($Tyr^{234,236}$), which are preferentially phosphorylated by Lck (Ostrowski et al., 2000), and a proline-rich region (aa289–315), which was shown to interact with and activate Src family kinases (Adolph et al., 2007; Bomsztyk et al., 2004). This suggested that hnRNP-K recruited and activated Lck by a PxxP-SH3 interaction mechanism depicted in Figure 3A. To test this assumption, we deleted the proline-rich region (KΔPxxP; aa289–315) and first determined the ability of this mutant to bind Lck. hnRNP-K and KΔPxxP were cotransfected along with Lck into 293T cells and, subsequently, immunoprecipitated and blotted for Lck association. While the association of hnRNP-K with Lck was weak but reproducible (Figure 3A, lane 2), we could not coprecipitate KΔPxxP with Lck (lane 3). This indicated that the proline-rich domain was indeed required to recruit Lck. Surprisingly, however, deletion of the proline domain did not abolish tyrosine phosphorylation of hnRNP-K by Lck. This was revealed after a similar transfection experiment blotting immunoprecipitated hnRNP-K and KΔPxxP with an antiphospho-tyrosine antibody (Figure 3B). This result suggested that, contrary to our expectation, the PxxP-SH3 interaction was not a prerequisite for hnRNP-K tyrosine phosphorylation and did not stimulate or increase the Lck kinase activity.

To verify this conclusion, Lck activity was determined in the presence of KΔPxxP. After cotransfection with KΔPxxP or hnRNP-K

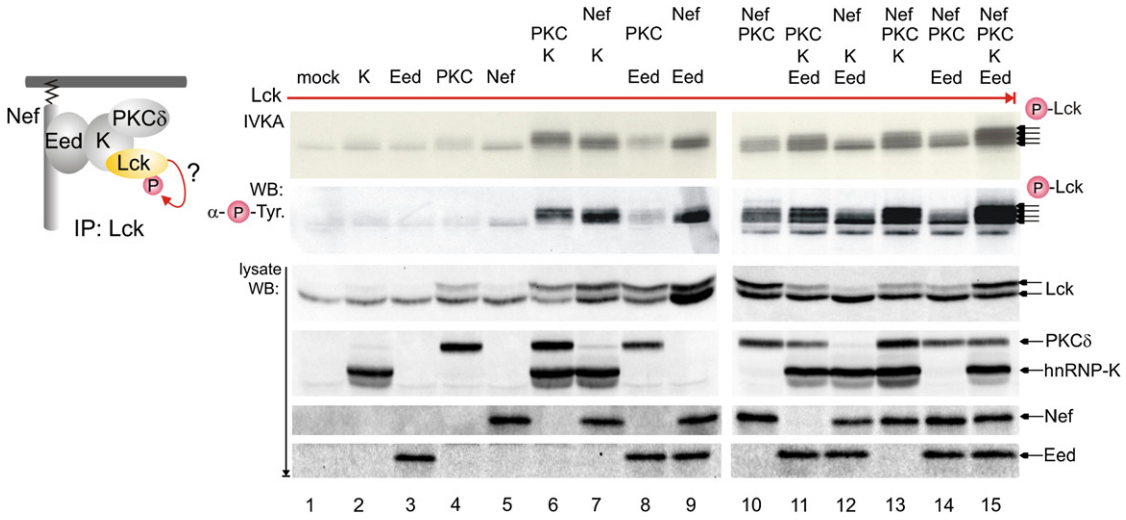

Figure 2. Activation of Lck by NAKC Factors

Lck was cotransfected with additional NAKC factors in various numbers and combinations into 293T cells as indicated. After 24 hr, Lck kinase activity was assessed by in vitro kinase assay (IVKA) (upper panel) and antiphospho-tyrosine western blot (second panel). Four different forms of tyrosine-phosphorylated Lck (autophosphorylation in vitro) were distinguished as indicated by the arrows. In parallel, lysates of the transfected cells were blotted for Lck expression and other NAKC factors, revealing an Lck mobility shift pattern (third panel).

and Nef, Lck was immunoprecipitated followed by an in vitro kinase assay and an antiphopho-tyrosine western blot. In agreement with the last experiment, deletion of the proline-rich domain did not reduce the kinase activity of Lck (Figure 3C, upper panel). However, the typical mobility shift pattern of Lck, which is unrelated to tyrosine and solely caused by serine phosphorylation, was inhibited (lower panel). The residual shift activity (lanes 6 and 7) was likely due to the presence of endogenous hnRNP-K. These findings led to several conclusions (see also detailed model in Figure 7): (1) Lck recruitment by the proline-rich domain of hnRNP-K was not required to activate the kinase but for its serine phosphorylation, and (2) there had to be an associated serine kinase that phosphorylated Lck.

Before identifying the Lck-phosphorylating serine kinase, we wanted to clarify whether hnRNPK directly activated Lck. The TCR-mediated signaling pathway that activates Lck is well defined (Palacios and Weiss, 2004). Thus, an alternative mechanism involving hnRNP-K could potentially be triggered by a different surface receptor. Alternatively, hnRNPK may simply recruit TCR-activated Lck and, subsequently, assemble a signaling complex, similar as suggested previously (Bomsztyk et al., 2004). To test this possibility, endogenous hnRNP-K was immunoprecipitated from Jurkat cells that had been stimulated/not stimulated with anti-CD3/anti-CD28 for 30 min. The immunoprecipitates were blotted for antiphospho-tyrosine and Lck. As demonstrated in Figure 3D, in activated—but not in resting—T cells, hnRNP-K coimmunoprecipitated a number of tyrosine phosphorylated proteins (upper panel), which included Lck (middle panel). In addition, hnRNP-K itself seemed to be tyrosine phosphorylated as a subfraction of the protein shifted in mobility (see arrow in lower panel). Taken together, this suggested that hnRNP-K became tyrosine phosphorylated by TCR-activated Lck early after T cell activation and, subsequently, recruited the kinase to form a signaling complex. This result further implied that hnRNP-K did not directly activate Lck but potentially stabilized the active kinase through crossphosphorylation events within NAKC (see Figure 7 and Discussion).

Residues 59 and 42 of Lck are the major serine phosphorylation sites after PMA treatment or TCR stimulation. The phosphorylated serines apparently do not modulate the kinase activity of Lck but change its substrate specificity (Joung et al., 1995; Park et al., 1995). Phosphorylation is mediated by Erk1/2 (S59) and PKC (S42), and only phosphorylation of S59 by Erk1/2 causes a visible shift in Lck mobility (Schroder et al., 2000; Watts et al., 1993; Winkler et al., 1993). In order to identify the Lck phosphorylating serine kinase, we tested both Lck serine point mutants (LckS59A and LckS42A) for their shift pattern upon NAKC formation. The mutants were cotransfected with hnRNP-K and Nef into 293T cells, and their cytoplasmic lysates were blotted for Lck. As demonstrated in Figure 4A, the S59A, but not the S42A mutant, failed to shift in mobility upon NAKC formation. This implied that NAKC-associated Lck was phosphorylated by Erk1/2.

Next, we asked whether NAKC formation caused the activation of Erk1/2, which then phosphorylated Lck. Lysates of 293T cells, which had been transfected with Lck, Nef, and hnRNP-K, or KΔPxxP instead of hnRNP-K, were blotted for activated endogenous Erk1/2 using a phospho-specific antibody. As demonstrated in Figure 4B (upper panel), cotransfection of wild-type hnRNPK, Lck, and Nef was sufficient to activate Erk1/2. Conversely, deletion of the proline-rich region in hnRNP-K abolished this effect (Figure 4C, upper panel) as well as serine phosphorylation of Lck (Figure 4C, lower panel). Control western blots revealed a mobility shift of a small amount of hnRNP-K, probably representing the Lck-phosphorylated subfraction, as revealed by an antiphospho-tyrosine western blot (Figure 4B). In summary, we had demonstrated that NAKC activated Erk1/2.

Since activation of Erk1/2 is an important downstream event that links membrane signaling with transcription, we aimed to analyze its activation and interaction with Lck/NAKC in greater

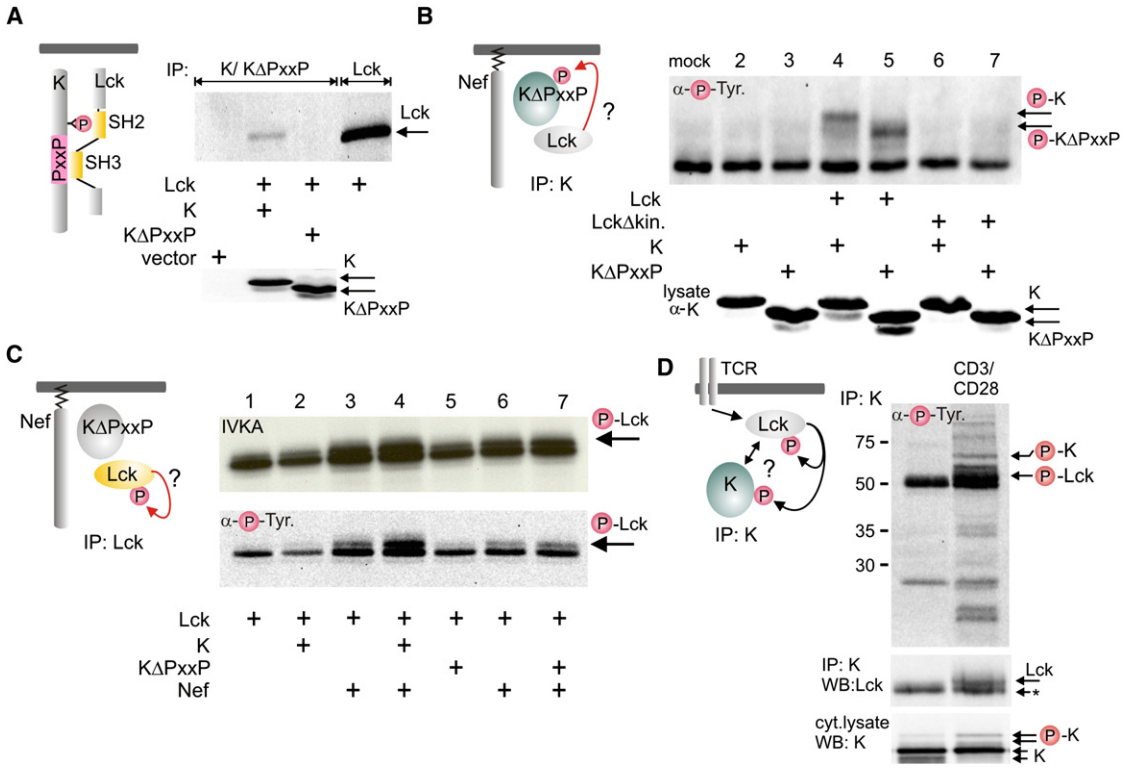

Figure 3. The Proline-Rich Domain of hnRNP-K Recruits Activated Lck

(A) Deletion of the proline-rich region in hnRNP-K abolishes association with Lck. hnRNP-K or a deletion mutant (KΔPxxP) were cotransfected with Lck. At 24 hr later, hnRNP-K and KΔPxxP were immunoprecipitated and blotted for Lck.

(B) Deletion of the proline-rich region in hnRNP-K does not abolish tyrosine phosphorylation by Lck. Same procedure as in (A); however, the immunoprecipitated K proteins were blotted with an antiphosphotyrosine antibody (4G10) while lysates of the transfected cells were blotted for hnRNP-K/KΔPxxP (lower panel).

(C) Deletion of the proline-rich domain in hnRNP-K inhibits the mobility shift of Lck. Lck was cotransfected with Nef and hnRNP-K or KΔPxxP into 293T cells, immunoprecipitated, and subjected to an in vitro kinase assay (IVKA, upper panel). In addition, the NC filter was probed with an antiphosphotyrosine antibody (lower panel). Note that, while tyrosine phosphorylation visualizes the mobility shift of Lck, the shift is actually caused by serine phosphorylation.

(D) hnRNP-K is tyrosine phosphorylated and associates with Lck following TCR activation. Jurkat cells were activated/nonactivated by anti-CD3/CD28 for 30 min before endogenous hnRNP-K was immunoprecipitated and blotted with an antiphosphotyrosine antibody (upper panel). The same NC filter was stripped and reprobed with an anti-Lck antibody (middle panel). In parallel, lysates of the stimulated cells were blotted for hnRNP-K (lower panel).

detail. Unexpectedly, we were unable to coimmunoprecipitate Erk1/2 with hnRNP-K or any other NAKC factor (data not shown). Therefore, we wondered where Nef-activated Erk1/2 was localized. Nef, hnRNP-K, and Lck were cotransfected into 293T cells, which were subsequently stained for activated Erk1/2 and Nef and analyzed by confocal microscopy. As demonstrated in Figure 4D (upper panels), activated Erk1/2 was found mainly in the cytoplasm and colocalized with Nef at the plasma membrane (in 64% of Nef-transfected cells, $p < 0.0015$), suggesting that the kinase was recruited to the NAKC complex despite negative coimmunoprecipitation results. Without cotransfection of hnRNP-K and Lck, Nef did not activate or colocalize with Erk1/2 (middle and lower panels), implying a recruitment of Erk1/2 to Nef after NAKC had formed.

Since we could not establish a link between the described NAKC factors and Erk1/2, we asked whether PI-3 kinase was involved. We have previously demonstrated that the Nef N terminus recruits PI-3 kinase (Wolf et al., 2001), and the kinase was found to be involved in Erk1/2 activation (Bondeva et al., 1998; Duckworth and Cantley, 1997). Performing 293T transfection experiments, we found that overexpression of p85, the regulatory subunit of PI-3 kinase, completely blocked NAKC-mediated activation of Erk1/2 (Figure 5A, lane 3). Conversely, coexpression of a transdominant-negative PDK (PH-domain deleted), a downstream effector of PI-3 kinase, or a transdominant-negative Pak kinase (PAK-R) (Wolf et al., 2001), an activator of the MAP kinase pathway and Nef interactor, or a transdominant-negative (kinase dead) PKCδ had no effect (Figure 5A). Confirming this finding, we could demonstrate that hnRNP-K precipitated p85 in cotransfection experiments (Figure 5B, lane 5). The association did not depend on the proline-rich region since KΔPxxP was still able to bind p85 (lane 6). In summary, these results suggested that PI-3 kinase was physically and functionally part of NAKC and involved in Erk1/2 activation.

The activation of Erk1/2 by NAKC was also demonstrated in Jurkat T cells. For this, NAKC components (Nef, Lck, PKC, Eed, and K or KΔPxxP) were cotransfected in increasing numbers followed by an analysis of the cellular lysates by western blot. As demonstrated in Figure S2, all NAKC components were required to activate Erk1/2 and, similar as in the 293T transfection experiments, the KΔPxxP mutant abolished this effect. As shown above, a small proportion of hnRNP-K

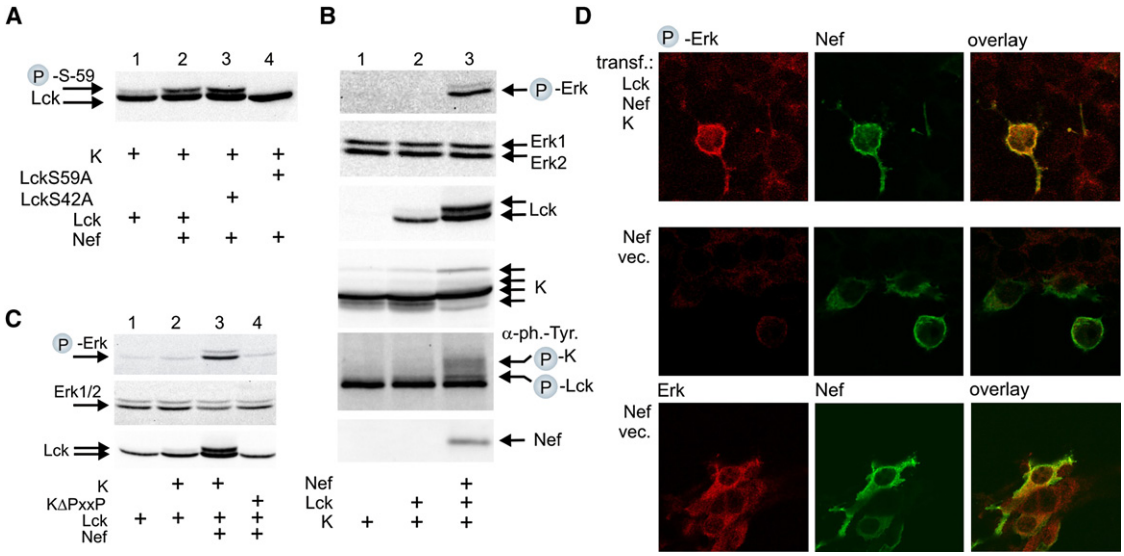

Figure 4. NAKC Activates Erk1/2

(A) Serine 59 of Lck is phosphorylated upon NAKC assembly. Lck and two point mutants (LckS59A and LckS42A) were cotransfected with Nef and hnRNP-K into 293T cells. At 24 hr later, lysates of the transfected cells were blotted for Lck.

(B) Nef, hnRNP-K, and Lck are sufficient to activate Erk1/2. Nef, hnRNP-K, and Lck were cotransfected into 293T cells. After 24 hr, cell lysates were blotted for activated Erk1/2 (phospho-Erk1/2) and controls. Note that a fraction of hnRNP-K shifted in mobility (see arrows), which seemed to correlate with tyrosine phosphorylation.

(C) Deletion of the proline-rich domain in hnRNP-K abolishes Erk1/2 activation. Nef, Lck, and hnRNP-K or KΔPxxP were cotransfected into 293T cells as described above. Cell lysates were blotted for phospho-Erk1/2, Lck, and controls.

(D) Nef and phospho-Erk1/2 colocalize at the plasma membrane. Nef, Lck, and hnRNP-K or controls were cotransfected into 293T cells as indicated. Subsequently, Nef was costained with phospho-Erk1/2 or Erk1/2 and analyzed by confocal microscopy.

shifted in mobility, possibly representing a tyrosine phosphorylated subfraction.

Next, we asked whether NAKC-mediated activation of Erk1/2 would correlate with the activation of Tat-dependent HIV transcription as shown for CD8-Eed in Figure 1. First, we determined the relative importance of individual NAKC factors. We transfected Lck and various combinations of NAKC components into J.CaM.1 cells along with suboptimal levels of Tat and an HIV-LTR-Luc reporter as described above. In summary, transfection of all five NAKC components gave the highest fold increase of transactivation, which was up to 60-fold over background (Figure 6A, lane 12). Conversely, the combination of only two to four factors had a significantly reduced effect (lanes 6–11). Thus, all NAKC components were essential for optimal stimulation of transcription. However, in the absence of only one kinase (Lck or PKC), transactivation was significantly

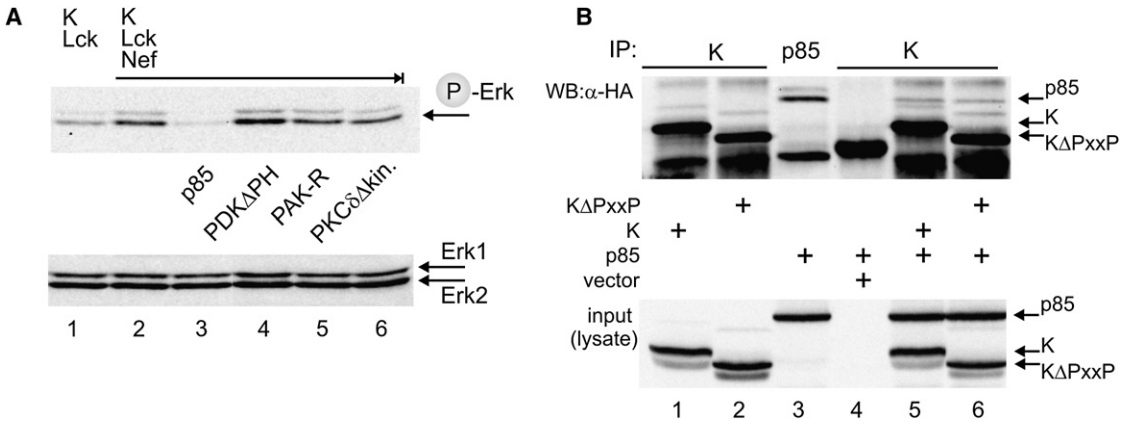

Figure 5. PI-3 Kinase Is Functionally and Physically Involved in NAKC

(A) Overexpression of PI-3 p85 inhibits NAKC-mediated Erk1/2 activation. Nef, Lck, and hnRNP-K were cotransfected into 293T cells along with transdominant-negative mutants of PDK (PDKΔPH), PAK (PAK-R), and PKCδ (PKCδΔkinase). At 24 hr later, cell lysates were blotted for phospho-Erk1/2 and Erk.

(B) hnRNP-K associates with PI-3 p85. hnRNP-K or KΔPxxP and p85 were cotransfected into 293T cells in different combinations as indicated. At 24 hr later, the K proteins were immunoprecipitated and blotted for p85.

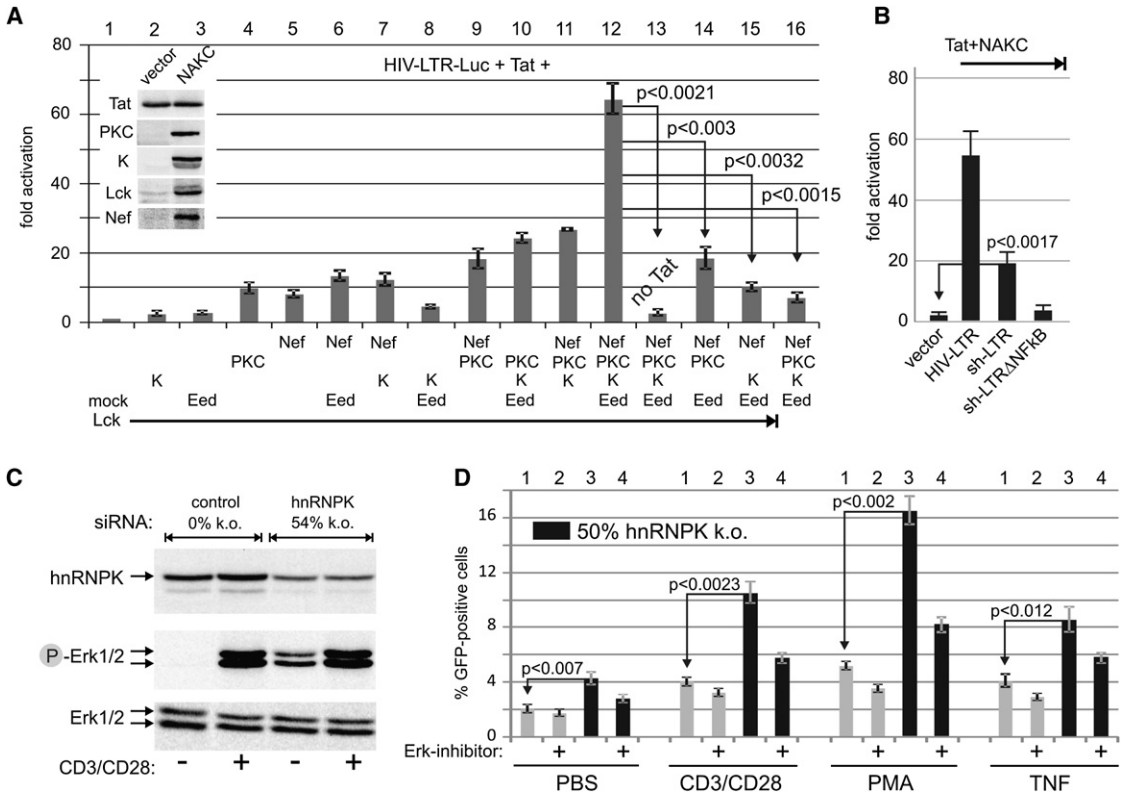

Figure 6. NAKC Activates Tat-Dependent HIV Transcription by Transcriptional Derepression

(A) Lck was cotransfected with NAKC factors in various combinations into J.Cam.1 cells along with suboptimal amounts of Tat and an HIV-LTR luciferase reporter as indicated. At 16 hr later, cells were analyzed for luciferase activity indicated in fold activation above background (Lck and mock). Control western blots in 293T cells showed that NAKC did not increase Tat expression in the transcription assays (insert). Error bars show means ± SD from three independent experiments. The p values in the graph were calculated on the basis of the mean values of the indicated bars.

(B) The NF-kB-motif in the HIV promoter is required for transcriptional effects of NAKC. Different HIV-LTR luciferase reporter constructs were cotransfected with NAKC (Nef, Lck, PKC, Eed, hnRNP-K) and Tat and analyzed for luciferase activity. HIV-LTR, wild-type HIV-promoter; sh-LTR (nts 345–531), promoter sequence upstream of the NF-kB element deleted; sh-LTRΔNF-kB, same as sh-LTR plus mutation of the NF-kB motif. Error bars show means ± SD from three independent experiments. The p value in the graph was calculated on the basis of the mean values of the indicated bars. $p < 0.0017$.

(C) siRNA knockdown of hnRNP-K activates Erk1/2. Jurkat cells were transfected three times with siRNA probes specific for hnRNP-K and analyzed for hnRNP-K expression and phospho-Erk1/2 activation before and after stimulation with anti-CD3/CD28.

(D) siRNA knockdown increases rescue of latent HIV by T cell activation stimuli. J.Lat Jurkat cells were transfected three times with hnRNP-K-specific siRNA as in (C), resulting in a roughly 50% reduction of hnRNP-K expression (data not shown). Then, cells were stimulated with anti-CD3/CD28, PMA, or TNF and analyzed for GFP expression by FACS after 12 hr. Cell culture aliquots were treated with the Erk1/2 inhibitor U0126 (Promega) as indicated. Error bars show means ± SD from three independent experiments. The p values in the graph were calculated on the basis of the mean values of the indicated bars.

Error bars in (A), (B), and (D) were calculated on the basis of three experiments.

reduced. Likewise, the omission of hnRNP-K had a greatly reduced effect (lane 14). Notably, the increase in transcription was not due to an increase of Tat expression through NAKC, as shown by an anti-Tat western blot after NAKC transfection into 293T cells (Figure 6A, insert). Together, these results suggested that (1) NAKC was a coherent signaling module that stimulated Tat transactivation, and (2) the activities of both kinases, Lck and PKC, as well as their docking platform hnRNP-K were essential for this effect.

In order to map the NAKC transcriptional effect on the HIV promoter, we repeated the transcription assay using a minimal promoter lacking sequences upstream of the NF-kB site (sh-HIV-LTR, nts 345–531). In addition, we used the same construct with a mutated NF-kB site (sh-HIV-LTRΔNFkB). First, we found that NAKC still increased Tat transactivation using the minimal promoter, albeit not as efficiently as with the wild-type promoter (Figure 6B, $p < 0.0017$). Mutation of the NF-kB site, however, completely abolished transactivation, implying that NAKC directly targeted NF-kB. However, despite numerous different experimental approaches, we were never able to demonstrate an effect of Nef or NAKC on NF-kB (V.W. et al., unpublished data and data not shown). Therefore, we assumed that NAKC targeted (a) so far unknown inhibitory factor(s) that occupied the NF-kB recognition motif and inhibited Tat transactivation (see Discussion).

Erk1/2 is an important regulator of transcription factors and chromatin-modifying proteins and, thus, the likely mediator of NAKC effects on Tat transactivation. Therefore, we hypothesized that depletion of hnRNP-K by siRNA would reduce TCR-mediated activation of Erk1/2 and HIV transcription. First, we investigated TCR-mediated activation of Erk1/2 after hnRNP-K depletion. Despite different approaches and multiple transfections,

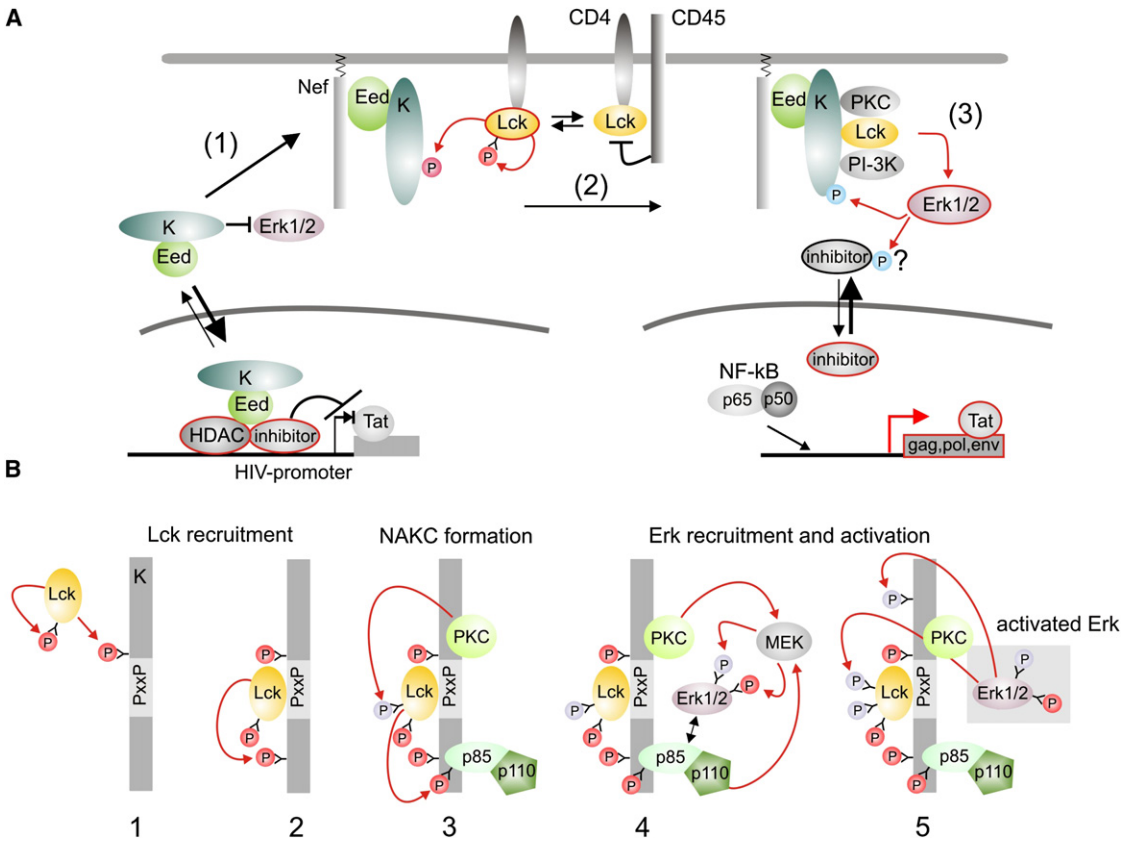

Figure 7. Model of NAKC Formation and Downstream Events

(A) NAKC formation and transcriptional derepression. (1) Eed and hnRNP-K are shuttle proteins. In resting cells, they exert a repressive effect on HIV transcription and Erk1/2. Upon Nef expression, their shuttle equilibrium shifts to the cytoplasm. (2) Targeted to the membrane, hnRNP-K recruits active Lck out of an equilibrium between active and nonactive molecules. (3) NAKC formation leads to the activation of Erk1/2, phosphorylation of hnRNP-K (Habelhah et al., 2001), and potential inhibitors affecting their shuttling equilibrium.

(B) NAKC formation and Erk1/2 activation. (1) Activated Lck phosphorylates hnRNP-K (Figure 3 and Ostrowski et al., 2000). (2) Upon Lck binding, additional tyrosines are phosphorylated, creating SH2 docking sites (Bomsztyk et al., 2004; Ostrowski et al., 2000). (3) Both events (1) and (2) lead to the recruitment of PKCδ (Schullery et al., 1999) and PI-3 kinase (Figure 5) and, thus, to NAKC formation. This induces the crossphosphorylation of Lck by PKC (Baur et al., 1997) and potentially the tyrosine phosphorylation of p85 (von Willebrand et al., 1994). (4) After NAKC has formed, Erk1/2 is recruited, potentially in a PI-3 kinase-dependent manner (Figure 5). The next phosphorylation steps leading to Erk1/2 activation are not clear; however, PKC and PI-3 kinase are likely involved as documented in the literature. (5) Activated Erk1/2 finally phosphorylates Lck and hnRNP-K.

we could not deplete more than roughly 50% of endogenous hnRNP-K in Jurkat cells using commercial probes (Figure 6C, upper panel). Nevertheless, we tested these cells for Erk1/2 activation. To our surprise, reduction of hnRNP-K greatly induced Erk1/2 activity, whereas stimulation through the T cell receptor did not show a difference (Figure 6C, middle panel). This suggested that, in resting T cells, hnRNP-K inhibited Erk1/2 activity, whereas this inhibitory function was reversed upon NAKC assembly.

To solidify this assumption, we asked whether a depletion/reduction of hnRNP-K by siRNA had a stimulating effect on HIV transcription using a previously described Jurkat latency model of HIV (J.Lat) (Jordan et al., 2003). These cells contain a single full-length HIV provirus in which the Nef gene is replaced by GFP. In our hands, treatment of these cells with CD3/CD28, PMA, or TNF for 12 hr only slightly increased the number of GFP-expressing cells (from 2% to 4%–5%) (Figure 6D, no. 1 bars). Conversely, simultaneous reduction of hnRNP-K by siRNA (~50%) roughly doubled (PBS, CD3/CD28, TNF) or tripled (PMA) the number of GFP-expressing cells (Figure 6D, no. 3 bars), whereas addition of the Erk1/2 inhibitor (U0126, Promega) reversed this effect (no. 4 bars). Taken together, these results were in agreement with our previous experiments (Figure 6B) (Witte et al., 2004), suggesting that the NAKC/Erk1/2 effect on Tat transactivation was caused by derepression and not by direct stimulation of the HIV promoter. Potentially, this enabled transcription factors like NF-kB and Tat to act at lower concentrations (Figure 7A).

DISCUSSION

The present study is a continuation of our previous work intended to determine the signaling mechanism by which Nef supports HIV transcription. With the current report, we have reached three conclusions. First, hnRNP-K is a docking platform for several key kinases in T cells. Second, the activation of Erk1/2 is a main consequence of this hnRNP-K-nucleated complex.

Third, NAKC/Erk1/2-mediated signaling leads to the derepression of the HIV promoter, enabling suboptimal amounts of Tat and transcription factors like NF-kB to initiate transcription.

Supporting our model, we have now identified the factor that regulates the accessibility of the NF-kB element and is removed by NAKC signaling (V.W. et al., unpublished data). We assume that, under conditions with limited availability of positive transcription factors, promoter derepression is essential for HIV to initiate replication. These conditions likely exist in nonactivated primary T cells and at the beginning of HIV transcription, which may even start before the virus is integrated (Wu and Marsh, 2001). Moreover, viral promoters, integrated or not, are rapidly occupied by repressive factors as implied by ChIP assays with transiently transfected HIV promoters, demonstrating the presence of Eed (Witte et al., 2004). Experiments using fast replicating tumor cells may not adequately reflect this situation, although we demonstrate here an up to 60-fold increase of Tat transactivation in the presence of NAKC. Taken together, promoter derepression by Nef may be a crucial step to initiate transcription in the repressive nuclear environment of a primary T cell.

hnRNP-K is a predominant nuclear protein that associates with RNA and DNA like its direct interactor, the polycomb protein Eed. In this function, both proteins may cooperatively act as inhibitors of transcription—Eed by recruiting HDAC proteins (van der Vlag and Otte, 1999) and hnRNP-K by binding repressive factors like Zik1 or Eed (Denisenko and Bomsztyk, 1997; Denisenko et al., 1996) and/or by inhibiting Erk1/2 activity as implied by the data shown here (Figure 7A). Possibly upon translocation to the plasma membrane and posttranslational modification, both proteins exchange this inhibitory for an activating role in transcription by serving as a docking platform for activated cytoplasmic kinases. This functional conversion may be induced upon NAKC formation or other activating stimuli. In the course of this transformation, Erk1/2 is activated and may induce transcriptional derepression by activating histone-modifying kinases and phosphorylating hnRNP-K, which leads to its cytoplasmic translocation (Habelhah et al., 2001). A comparable mechanism has been described for the transcriptional repressor Yan, which, upon Erk1/2 phosphorylation, is shuttled to the cytoplasm while, simultaneously, Erk1/2 phosphorylates the transcription factor Ets-AP1, which replaces Yan and enhances gene transcription (O'Neill et al., 1994). For the results presented here, we would also assume that the HIV promoter and NF-kB motif are occupied by a transcriptional repressor, which needs to be replaced before transcription factors are able to bind. Aside from its repressive function, the role of Eed in this scenario is unclear; however, it has been established that hnRNP-K is methylated on two arginines, which modulates its function (Ostareck-Lederer et al., 2006). The latter is potentially induced by Eed and associated Suz12/Ezh2 proteins.

Our results imply that NAKC directly activated Lck by an alternative mechanism: (1) NAKC-dependent activation of the kinase in a non-T cell environment, (2) the involvement of multiple T cell kinases, (3) the presence of a signaling docking platform (hnRNP-K, Eed), and (4) the strong autophosphorylation activity of Lck after coprecipitation through Nef argue for this assumption. However, we were unable to demonstrate a plausible mechanism—for example, a classical PxxP-SH3 interaction (Figure 3)—that would displace the Lck SH3 domain from its intramolecular lock as demonstrated for Nef and HCK (Moarefi et al., 1997). Rather, hnRNP-K appeared to be an immediate downstream effector of the T cell receptor that associated with TCR-activated Lck. In view of our results, we suggest that membrane-targeted hnRNP-K recruits activated Lck molecules out of a CD45/Csk-controlled equilibrium with inactive Lck (Palacios and Weiss, 2004) (Figure 7A). We would further speculate that assembly of the kinases at the hnRNP-K docking platform stabilizes active Lck molecules by crossphosphorylation. This would still qualify hnRNP-K as an activator of Lck similar, as demonstrated for c-Src (see Figure 7B). In summary, our findings do not provide enough evidence to propose an alternative mechanism of direct Lck activation through hnRNP-K; however, such a mechanism cannot be ruled out completely.

Although Nef is clearly a stimulating accessory protein of HIV, it has been very difficult to demonstrate its positive effects on signaling molecules/pathways and/or transcription. Results from different labs often have been contradictory, and positive effects of Nef in general require T cell costimulation (Schrager and Marsh, 1999). For example, an increased Erk1/2 activation by Nef was demonstrated upon costimulation with anti-CD3 (Schrager et al., 2002). Our study presented here may provide an explanation for this conundrum. The formation of a multimeric signaling complex requires all factors to be available in a balanced amount. Overexpression of one factor may lead to the precipitation of a cofactor in lower quantities and ultimately causes a squelching effect. We envision such a mechanism for some of the results in Figure 2 when not all NAKC factors were cotransfected. We would assume that the Nef signaling complex forms early in the replication cycle when Nef levels are below those of the interacting partners. Such conditions exist at the very beginning of viral replication. After several rounds of transcription, Tat is the more important factor for viral replication, and Nef is dispensable and/or required for additional functions like CD4 downmodulation. This model would explain why the Nef effect on transcription is entirely Tat dependent but at the same time absolutely essential in a short time frame of the viral life cycle.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Recombinant Proteins

Jurkat cells and their derivatives, J.CaM1, J.Lat, and the inducibe Nef-mER cell line, were cultured in RPMI 1640, 10% FCS; 293T cells in DMEM, 10% FCS. Sf9 insect cells were maintained in Sf-900 II SFM media. The Nef-inducible cell line was established using Jurkat "Tet-On" cells as described previously (Witte et al., 2004), expressing a Nef-(tamoxifen-sensitive) estrogen receptor fusion protein (mER) controlled by a Tetracycline-responsive element. Addition of doxycycline overnight caused expression of an inactive Nef protein, which was rendered active by addition of tamoxifen.

The sheep α-Nef serum was a gift from M. Harris and used at a dilution of 1:5000. The polyclonal α-Eed serum was raised in rabbits as described previously (Witte et al., 2004). The antibodies α-myc (clone 9E11), α-AU-1, and α-HA were purchased from Covance; the monoclonal antibodies α-hnRNP-K (clone D-6), α-Lck (clone 3A5), and α-PKCδ (clone C17), from Santa Cruz; α-Erk and α-phospho-Erk (Thr 202/204), from Cell Signaling; the α-phospho-tyrosine antibody (4G10), from Upstate; the α-CD3 (Clone HIT 3a) and α-CD28 (clone CD28.2 L293) for stimulation, from Becton Dickinson. The secondary α-mouse- and α-rabbit-HRP conjugated antibodies were from Promega; the α-sheep-HRP, from Sigma. The AlexaFluor488-conjugated goat α-sheep, AlexaFluor568/647 goat α-mouse, and AlexaFluor568 goat α-rabbit were from Molecular Probes.

The myristoilated recombinant Nef protein was a generous gift from Mathias Geyer, Dortmund, Germany. Recombinant GST-Eed was obtained and purified after bacterial expression. Recombinant proteins for hnRNP-K, Lck, and PKCδ were generated using the Baculovirus Expression System from Invitrogen according to the manufacturer's instructions.

Plasmids

The CD8-Nef fusion proteins (SF2 full-length and truncated forms) as well as Nef-AU1 were generated as described previously (Baur et al., 1997). Human isoforms of PKCδ and PKCθ, including PKCθ-K/R, were obtained from Gottfried Baier, Innsbruck, Austria. A HA tag was added as described previously (Wolf et al., 2008). The expression plasmid for p85 was kindly provided by A. Klippel. Lck and Hck expression plasmids were obtained from Arthur Weiss, San Francisco and Kalle Saksela, Helsinki. The S59A and S42A as well as K273R (kinase dead) mutations in Lck were introduced by site-directed mutagenesis. The expression plasmid for hnRNP-K was a kind gift from Karol Bomsztyk. The deletion of the PXXP region (aa 289–315) was introduced by overlapping PCR. The transdominant-negative PAK (PAK-R; aa1-225) expression plasmid was obtained from Matija Peterlin, San Francisco. PDKΔPH (deleted PH domain) was obtained from Alex Toker, Philadelphia. Tat, HIV-LTR-Luc (pGL2basic), sh-HIV-LTR-Luc (pEV284), and sh-HIV-LTR-LucΔNF-kB (pEV285) were obtained from Melanie Ott, San Francisco.

Transfections into T and 293T Cells

Transient transfections into 293T cells were performed as described previously (Wolf et al., 2001). For transient transfection of Jurkat cells, 10×10^6 cells were electroporated using up to 50 μg of DNA. J.CaM.1 cells for Luciferase reporter assays were transfected using DMRIE-C according to the manufacturer's instructions. For delivery of the siRNA (purchased from Dharmacon), cells were electroporated using a square-wave protocol. To obtain an ~50% knockdown of hnRNP-K, the electoporation was repeated three times within 3 days.

Protein Assays

Immunoprecipitations, western blots, and in vitro kinase assays were performed as described previously (Baur et al., 1997; Witte et al., 2004; Wolf et al., 2001). For the luciferase reporter assays, J.CaM.1 cells were harvested 16 hr after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer's instructions.

Immunofluorescence and Confocal Microscopy

293T cells were grown and transfected on glass coverslips before staining. T cells were plated on Poly-L-Lysine-coated (Becton Dickinson) glass coverslips for 10–20 min at 37°C to adhere before staining. Subsequently, cells were fixed with 3% paraformaldehyde, permeabilized using 1% Triton-X, blocked with 1% BSA, and stained with the corresponding antibodies. Slides were analyzed on a Zeiss confocal LSM 510 microscope (Carl Zeiss, Thornwood, NY) using the corresponding software (Zeiss LSM Image Browser). Confocal scanning settings were not changed nor was the staining intensity enhanced by subsequent picture processing for each individual antibody.

SUPPLEMENTAL DATA

The Supplemental Data include two figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/4/4/398/DC1/.

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