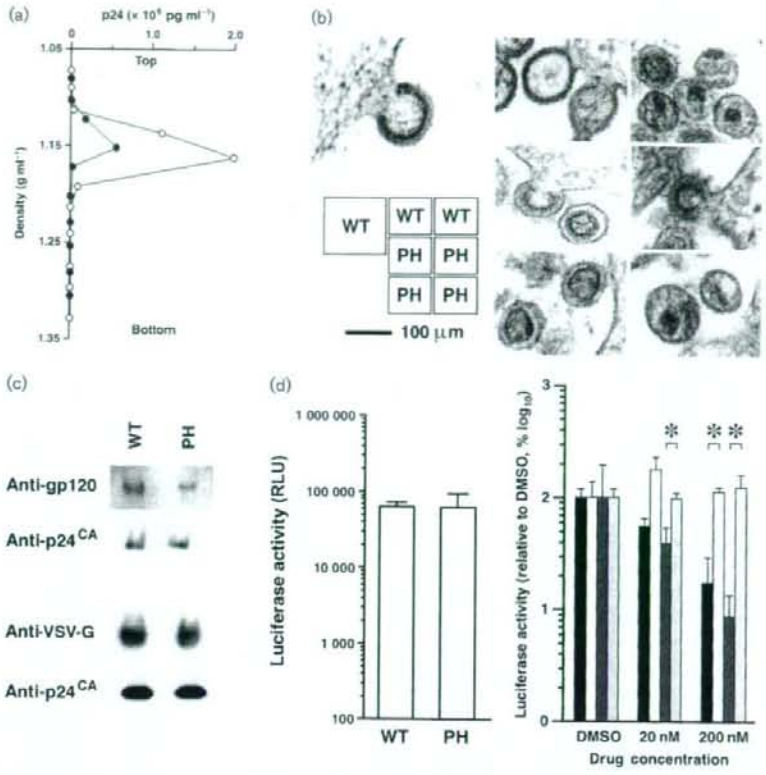

Fig. 2. Physical and biological properties of PH–Gag–Pol VLPs. (a) The virions produced by the p*gag-pol* (WT) and pPH-*gag-pol* (PH) expression vectors were analysed by equilibrium sucrose density-gradient centrifugation. The virion-containing fraction was determined by an ELISA detecting $p24^{CA}$. Representative data from four to five independent experiments are shown. In this experiment, WT (filled circles) and PH (open circles) VLPs migrated in the 1.152 or 1.162 g ml^{-1} density fractions, respectively. (b) Transmission electron microscopy images of 293T cells transfected with p*gag-pol* (WT) or pPH-*gag-pol* (PH). Representative images are shown. (c) Incorporation of HIV-1 Env (upper panel) and VSV-G (lower panel) into Gag–Pol (WT) and PH–Gag–Pol (PH) virions. The virion fractions were subjected to Western blot analysis detecting gp120, VSV-G and $p24^{CA}$. (d) The early phase of the HIV-1 life cycle is supported by PH–Gag–Pol. 293T cells were exposed to virus-containing culture supernatants with similar CA concentrations (270 and 220 ng ml^{-1} for Gag–Pol and PH–Gag–Pol, respectively), and luciferase activities were measured at 2–3 days post-infection as relative light units (RLU). The luciferase activities of PH–Gag–Pol (PH) and Gag–Pol (WT) virus-infected cells were almost identical (left graph). The luciferase transduction by WT (bars 1 and 2) and PH (bars 3 and 4) pseudovirions was performed in the presence of nevirapine (NVP, bars 1 and 3) or TAK-779 (bars 2 and 4). The luciferase signals decreased in the presence of NVP for both WT (bar 1) and PH (bar 3) but not in the presence of TAK-779 for both WT (bar 2) and PH (bar 4), respectively. Representative data from several independent experiments are shown. Asterisks indicate statistical significance ($P<0.01$, $n=3$, Student's *t*-test).

diameters were almost identical (Fig. 2b). Virion budding structures showed that the electron-dense layer, which represented multimerized Gag, of the PH–Gag–Pol VLP was slightly separated from the viral envelope compared with that of the Gag–Pol VLP (Fig. 2b). This indicated that the PH domain was positioned between the viral envelope and the electron-dense layer. In contrast, the morphologies of the mature PH–Gag–Pol and Gag–Pol virions were similar, suggesting that myristoylation is dispensable for mature virion morphology and that the PH–Gag–Pol virion may be infectious.

We examined HIV-1 Env incorporation into the PH–Gag–Pol virion. To do this, we used codon-optimized gp160 (p96ZM651gp160-opt; NIH AIDS Research and Reference Reagent Program). The PH–Gag–Pol virion incorporated HIV-1 Env less efficiently than the Gag–Pol virion as demonstrated by Western blot analysis detecting CA and

gp120 (anti-gp120 antibodies from Santa Cruz Biotechnology; Fig. 2c). This was presumably because PH interfered with the MA–Env interaction. Alternatively, PH may actively incorporate cellular proteins that block efficient Env incorporation into virions. We were unable to evaluate the entry efficacy of PH–Gag–Pol virions pseudotyped by HIV-1 Env because of the limit of detection. PH–Gag–Pol virion infectivity was re-examined by virions pseudotyped with vesicular stomatitis virus G glycoprotein (VSV-G). The incorporation efficiencies of VSV-G into Gag–Pol and PH–Gag–Pol virions were similar (anti-VSV-G antibody from Sigma; Fig. 2c). The lentiviral vector system was used to test this, as HIV-1 provirus gene modifications often fail to produce infectious virions, probably due to viral gene dysregulation. 293T cells were transfected with expression plasmids for Gag–Pol, VSV-G (Komano *et al.*, 2004), Rev and Vpu (a generous gift from Dr H. Göttlinger, University of Massachusetts Medical School, MA, USA), and with a packaging vector encoding a luciferase expression cassette. The HIV-1-based vector expressing firefly luciferase upon infection was recovered 2 days post-transfection. 293T cells were exposed to virus-containing culture supernatants with similar CA concentrations, and luciferase activities were measured at 2–3 days post-infection. When viral preparations with similar p24 concentrations were used, the luciferase activities of PH–Gag–Pol and Gag–Pol virus-infected cells were almost identical to each other (Fig. 2d, left graph). Luciferase expression was blocked by the non-nucleoside reverse transcriptase inhibitor nevirapine (NVP; Boehringer Ingelheim) but not by the CCR5 inhibitor (TAK-779; NIH AIDS Research and Reference Reagent Program), suggesting that gene transduction was mediated by viral infection (Fig. 2d, right panel). Similar results were obtained in several independent experiments. Thus, the PLCδ1 PH domain can functionally replace the HIV-1 Gag myristoylation signal to support both viral production and entry processes, and this myristoylation is dispensable for MA function in the early phase of the virus life cycle. This is the first report describing an infectious pseudovirion without myristoylated Gag. Given that PH–Gag can enhance virus production, HIV-1 with PH–Gag might have been expected to be selected in nature. This is not the case, presumably because the addition of PH to the HIV-1 genome would increase its genome size close to the upper limit that can be incorporated into the retroviral particle, leading to a decrease in genome uptake efficiency, which is clearly a growth disadvantage, despite the enhanced virus production with PH-Gag. More importantly, PH–Gag is unable to incorporate HIV-1 Env efficiently enough to support the production of fully infectious virions. Our data point to the selective advantage of myristoylated Gag in viral evolution.

The myristoylation-dependent Gag–PM association [maximal dissociation constant (K_d) of ~0.5–1.0 $\times 10^{-5}$ M] is presumably important for Gag multimerization at the PM (Provitera *et al.*, 2006). After the first contact of Gag with the PM, the membrane binding of Gag is assumed to be stabilized by the Gag–PI(4,5)P(2) interaction (Ono *et al.*, 2004; Saad *et al.*, 2006). The multimerization of Gag appears to induce a conformational change in MA to expose myristoyl groups to enhance the PM targeting of Gag. The higher-order Gag multimerization is probably facilitated by the increased local concentrations of Gag at the PM. Although Gag and PH–Gag are similar to the extent that PI(4,5)P(2) is involved in their PM association, Gag binds to one of the acyl chains of PI(4,5)P(2), as modelled previously (Saad *et al.*, 2006), whilst the PH domain binds the phosphorylated inositol group (Lemmon *et al.*, 1995). The K_d of binding between the PLCδ1 PH domain and PI(4,5)P(2) (~1–2 $\times 10^{-6}$ M; Lemmon *et al.*, 1995) suggests that the primary force driving PH–Gag to the PM is at least 2.5-fold stronger than that of myristoylation-mediated PM targeting of Gag. This might be one reason why PH–Gag–Pol was 3.2-fold more efficient at virion production than Gag–Pol. Our data suggest that the myristoyl group-dependent Gag–PM affinity is not a prerequisite for efficient Gag assembly at the PM or for viral production.

The MA has multiple functions throughout the virus life cycle (reviewed by Bukrinskaya, 2007; Fiorentini *et al.*, 2006; Hearps & Jans, 2007; Klein *et al.*, 2007). In the PH–Gag–Pol virion, approximately one-fifth of the PH–MA was unanchored from the PM as MA* (Fig. 1b), which might accompany the pre-integration complex to support nuclear targeting. Using PH–Gag–Pol might enable separation of myristoylation-dependent and -independent MA functions, particularly during the entry phase. PH–Gag–Pol might also be useful for producing high-titre lentiviral vectors or for studying Gag trafficking in cells that poorly support PM targeting of myristoylated Gag, such as rodent cells. Furthermore, functional assays comparing the virus production of Gag–Pol and PH–Gag–Pol might enable the identification of chemical inhibitors or cellular factors specifically targeting myristoylated Gag.

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Research Letters

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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

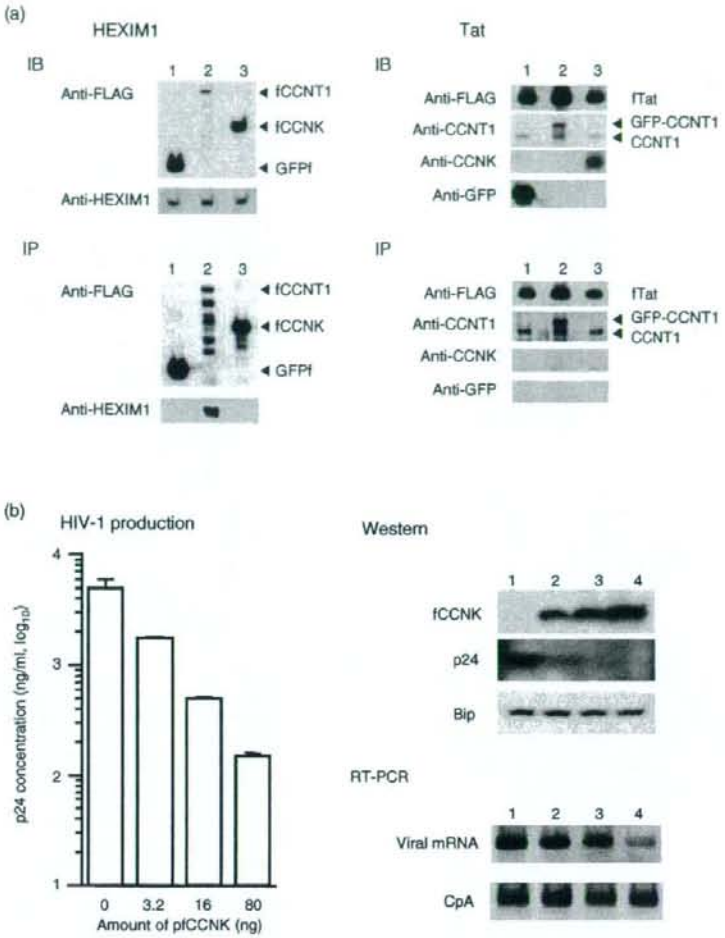

Fig. 1. Expression and functional characterization of cyclin K/CPR4. (a) Co-immunoprecipitation assay demonstrating that cyclin K/CPR4 does not interact with HEXIM1 or HIV-1 Tat. For the co-immunoprecipitation assay with HEXIM1, 293 T cells were transfected with 2 μg of expression vector and then grown 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.

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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