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Acetylation of HIV-1 integrase by p300 regulates viral integration

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Integration of HIV-1 into the human genome, which is catalyzed by the viral protein integrase (IN), preferentially occurs near transcriptionally active genes. Here we show that p300, a cellular acetyltransferase that regulates chromatin conformation through the acetylation of histones, also acetylates IN and controls its activity. We have found that p300 directly binds IN both *in vitro* and in the cells, as also specifically demonstrated by fluorescence resonance energy transfer technique analysis. This interaction results in the acetylation of three specific lysines (K264, K266, K273) in the carboxy-terminus of IN, a region that is required for DNA binding. Acetylation increases IN affinity to DNA, and promotes the DNA strand transfer activity of the protein. In the context of the viral replication cycle, point mutations in the IN acetylation sites abolish virus replication by specifically impairing its integration capacity. This is the first demonstration that HIV-1 IN activity is specifically regulated by post-translational modification.

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Introduction

Integration of the proviral genome into host chromosomal DNA is a requirement for efficient viral replication. The virus-encoded integrase (IN) and other proteins that participate in the formation of the preintegration complex (PIC) bind specific sequences located at the ends of the viral cDNA (*att* sites) in order to successfully complete the integration reaction. In contrast, no strong primary sequence in the cellular genome has been shown as a preferential target site for integration. Notwithstanding the lack of sequence specificity, several reports suggest that integration preferentially occurs in nucleosomal rather than in naked DNA; in particular, hot spots for integration have been identified in portions of DNA bent around nucleosomal cores (Pryciak and Varmus, 1992; Muller and Varmus, 1994; Pruss *et al*, 1994). Several *in vivo* studies indicate that DNase I hypersensitive regions, which characterize decondensed chromatin, are favorable sites for retroviral integration (Vijaya *et al*, 1986; Rohdewohld *et al*, 1987), while heterochromatic regions are disfavored (Carteau *et al*, 1998). Recently, broad analyses on integration site selection have indeed revealed that retroviruses preferentially integrate near transcriptionally active genes (Schroder *et al*, 2002; Wu *et al*, 2003; Mitchell *et al*, 2004).

The level of chromatin organization is mainly determined by the covalent modification of histones. Histone acetyl transferases (HAT) is a family of proteins that function enzymatically by transferring an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of specific lysine residues within the histone basic N-terminal tail region (Roth *et al*, 2001). Histone acetylation facilitates the access of the transcription machinery to DNA by loosening the interactions between both adjacent nucleosomes and DNA, as well as by serving as a recognition site for the recruitment of accessory regulatory factors (Carrozza *et al*, 2003).

In addition to histones, HAT have been described to acetylate other proteins, of both cellular and viral origin, such as transcription factors (among them E2F1, p53, MyoD, HIV-1 Tat, and others) and non-histone chromatin-associated proteins (HMG I(Y), HMG-14, and HMG17), also including cytoplasmic proteins (α-tubulin) (Sterner and Berger, 2000). It has been demonstrated that acetylation modulates the activity of these factors by affecting their interaction with other proteins or with DNA, or by altering their cellular localization.

Based on the preferential integration of HIV-1 near the transcriptionally active regions of the chromatin, we set out to explore whether IN might associate with cellular proteins possessing HAT activity. Here we demonstrate that the carboxy-terminus (C-terminus) of IN specifically binds p300, one of the prominent HAT family members. This interaction determines the acetylation of three specific lysines of IN, a modification that increases the affinity of the protein to DNA as well as its enzymatic activity. The relevance of this finding *in vivo* is highlighted by the observation that proviral integration is specifically impaired by mutation of the residues acetylated by p300 and by the specific inhibition of p300 catalytic activity.

Results

HIV-1 IN binds p300

Given the relationship between retroviral integration and chromatin conformation, we set out to explore whether IN might associate with cellular proteins possessing HAT activity. A codon-optimized IN tagged with Flag (Limon *et al*, 2002) was expressed in 293T cells, followed by immunoprecipitation using an anti-p300 antibody and immunoblotting

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with an anti-Flag antibody. As shown in the upper-left panel of Figure 1A, Flag-IN, but not the Flag-Luc control, specifically co-precipitated with endogenous p300. In a consistent manner, endogenous p300 was found to co-immunoprecipitate with transfected Flag-IN, but not with Flag-Luc (Figure 1B, upper panel).

To better define the interaction between IN and p300, a glutathione *S*-transferase (GST)-pulldown assay was carried out between *in vitro* translated p300, labeled with ^{35}S-Met, and recombinant IN fused to GST (GST-IN). Decreasing amounts of GST-IN immobilized on beads were incubated with p300, the beads were then extensively washed and the proteins resolved by SDS–PAGE. Figure 1C shows the gel stained with Coomassie blue (upper panel) and after autoradiography (lower panel). Labeled p300 was found to specifically bind to GST-IN in a dose-dependent manner (up to 18% binding at the highest GST-IN concentration), but not to GST alone.

To understand which IN domain is involved in binding to p300, we performed the pulldown assays by using a series of recombinant IN mutants containing progressive deletions from the C-terminus of the protein. As shown in Figure 1D, beads loaded with both wild-type (wt) IN and the 1–272 truncation retained comparable amounts of labeled p300; in contrast, binding was abolished in all the recombinant proteins carrying further deletions toward the N-terminus. Thus, the region of IN responsible for the interaction with p300 lies between amino acid 264 and the C-terminus of the protein.

To assess whether the interaction between IN and p300 is direct, we employed the fluorescence resonance energy transfer technique (FRET), using the two proteins tagged with the

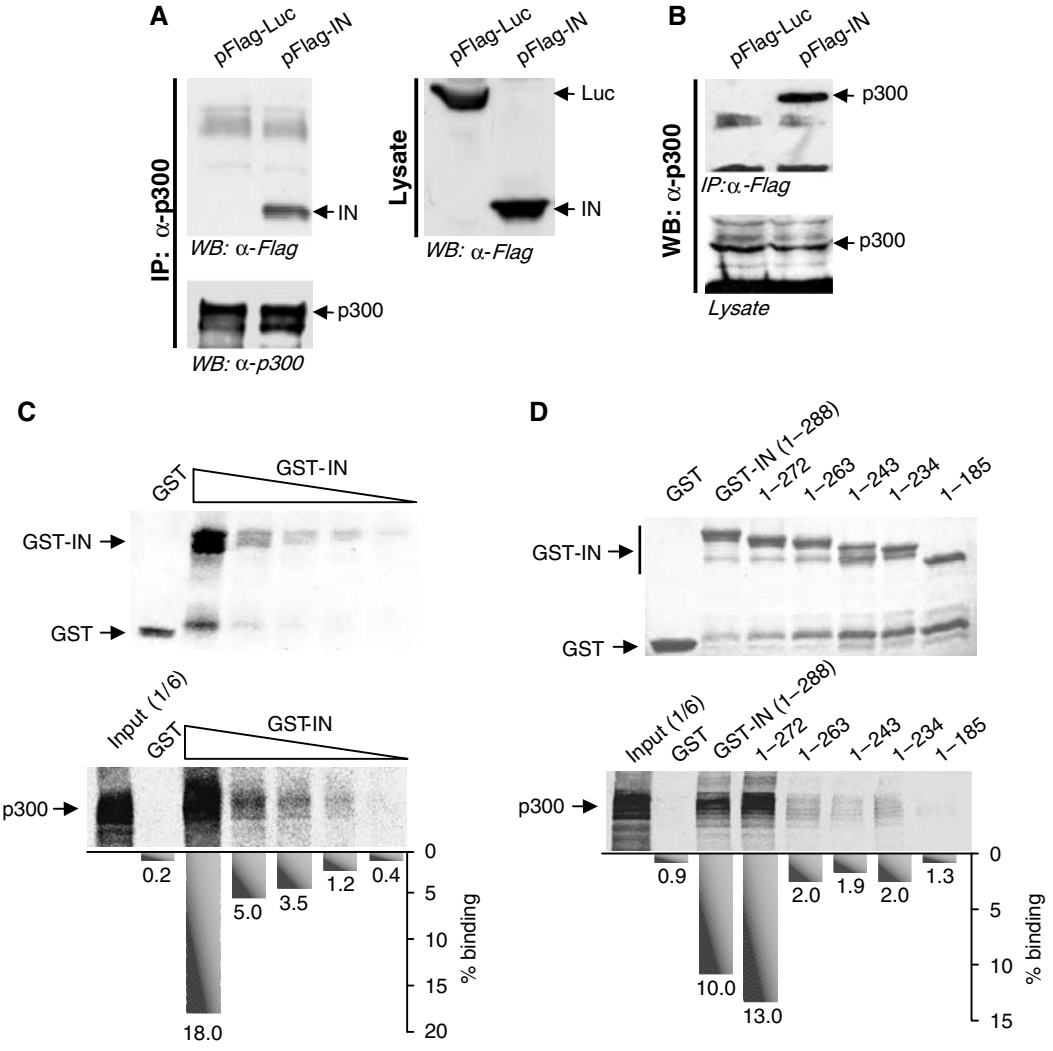

Figure 1 HIV-1 IN binds p300 *in vitro* and *in vivo*. (**A**) IN interacts with p300 *in vivo*. Extracts prepared from cells transfected with pFlag-IN or pFlag-Luc vectors were immunoprecipitated with an anti-p300 antibody and immunoblotted with an anti-Flag antibody (upper panel) or an anti-p300 antibody (lower panel). The same extracts were run on an SDS–PAGE gel and immunoblotted with an anti-Flag antibody (right panel). (**B**) Extracts as in (A) were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-p300 antibody (upper panel). The same extracts were analyzed by immunoblotting with an anti-p300 antibody (lower panel). (**C**) *In vitro* IN/p300 binding. Serial dilutions of GST-IN on agarose beads were incubated with fixed amounts of ^{35}S-p300 and analyzed on an SDS–PAGE gel. (**D**) GST-IN fragments were incubated with ^{35}S-p300 and analyzed on an SDS–PAGE gel. In (C) and (D), the upper panels show the Coomassie staining of the gels and the lower panel gels exposed to Cyclone screen. The graphs express the amounts of bound proteins as percentages of the input of radiolabeled protein.

EYFP:ECFP fluorescent protein pair. FRET consists in radiationless energy transfer between one fluorophore (the donor) in the excited state and another fluorophore (the acceptor) when in close proximity. Simple colocalization of two proteins is not sufficient to yield energy transfer, which requires the proximity of the two fluorophores at distances that are to the order of a few nanometers (Day *et al*, 2001). Thus, the presence of FRET between two proteins is an indicator of direct protein–protein interaction.

Human U2OS cells were transfected with a plasmid expressing codon-optimized IN fused to EYFP, together with a plasmid expressing p300 fused to ECFP. FRET image analysis of a representative cell expressing the two constructs is shown in Figure 2A. The panels on the left side show the EYFP-IN (upper) and ECFP-p300 (lower) fluorescence under excitation at 514 and 458 nm, respectively. FRET analysis was performed using the acceptor photobleaching technique, which consists in comparing the emission of the donor fluorophore (ECFP) before and after photobleaching of the acceptor fluorophore (EYFP) within a limited area (named the region of interest (ROI)). After EYFP photobleaching of the ROI (upper right panel), fluorescence of ECFP in the ROI was markedly enhanced (lower right panel), giving a clear indication of FRET between the two proteins. Since the *in vitro* experiments indicated that the deletion of the terminal 25 amino acids of IN abolishes its interaction with p300, FRET analysis was additionally performed by coexpressing ECFP-p300 and IN 1–263 fused to EYFP. As shown in Figure 2B, after EYFP photobleaching in the ROI, the intensity of ECFP fluorescence did not vary, indicating the lack of interaction between p300 and the IN-deleted mutant. Two irrelevant nuclear proteins not known to interact (EYFP-CDK9 and ECFP-Rb) were also negative for FRET (Figure 2C). Quantitative analysis (see Materials and methods) of at least 20 cells from three experiments is shown in Figure 2D. The efficiency of FRET between EYFP-IN and

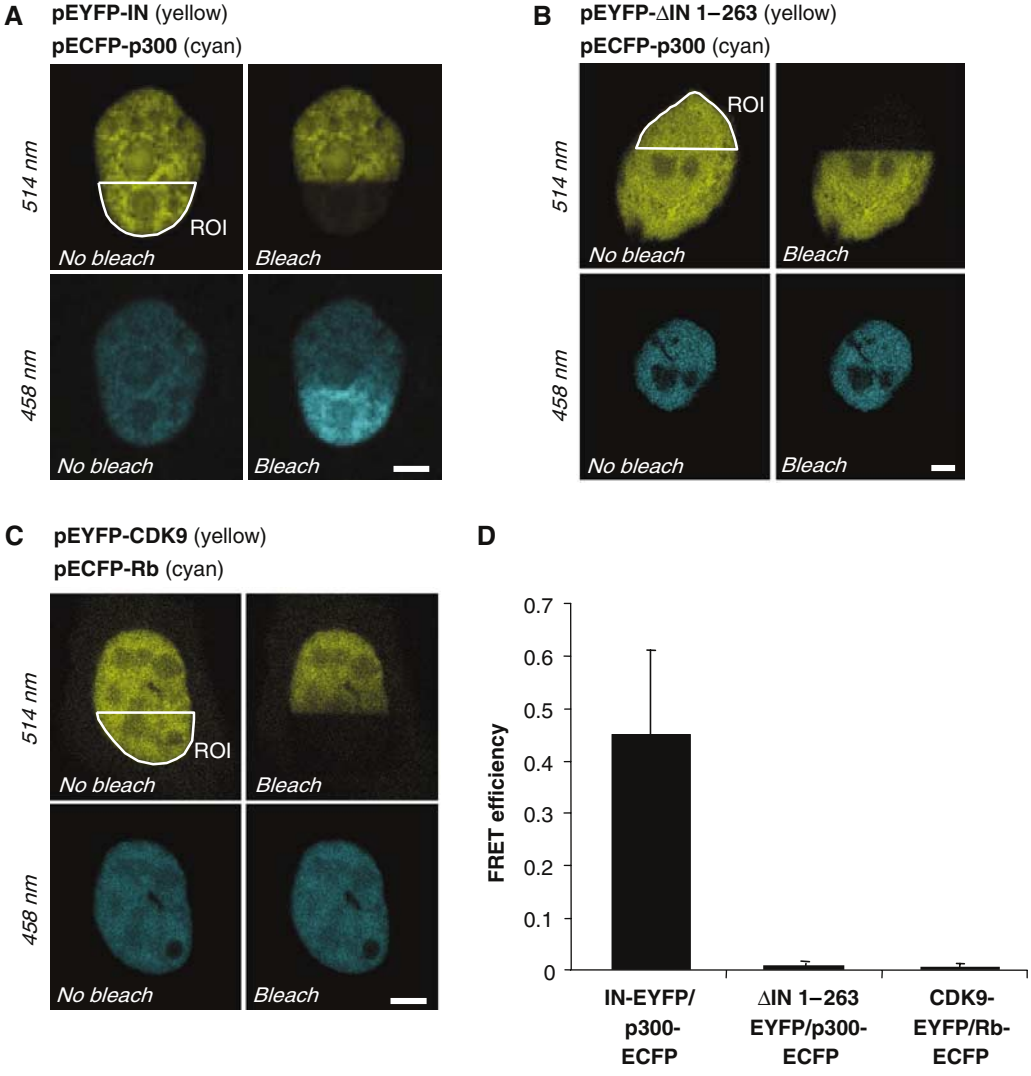

Figure 2 FRET analysis of protein–protein interaction. (**A**) Visualization of FRET. pEYFP-IN and pECFP-p300 vectors were transfected in U2OS cells and visualized by excitation at 514 nm showing EYFP fluorescence (upper panels), or at 458 nm showing ECFP fluorescence (lower panels). In the upper left panel, the region chosen for photobleaching (ROI) is indicated. (**B**) As in (A), following transfection with pEYFP-ΔIN 1–263 and pECFP-p300. (**C**) As in (A), following transfection with pEYFP-CDK9 and pECFP-Rb. (**D**) Quantification of FRET. The FRET efficiency was measured by comparing the fluorescence intensity of the donor ECFP before and after photobleaching of the acceptor EYFP within the ROI (see Materials and methods). Bar scale: 5 μm.

ECFP-p300 had an average value of 0.446 ± 0.161; in contrast, FRET efficiency was <0.005 (indicating no FRET) for the other two analyzed protein pairs.

Considered collectively, these results show that IN directly binds p300 in the nucleus and that this interaction requires the C-terminal domain of IN.

p300 acetylates the C-terminal domain of IN

Since IN forms a physical complex with p300, we investigated whether p300 might also acetylate IN. For this purpose, GST-IN was incubated with the HAT domain of p300 fused to GST, in the presence of ^{14}C acetyl-CoA. As shown in Figure 3A, GST-IN, but not GST or BSA, scored clearly positive for acetylation. In addition to IN, the HAT domain of p300 was also positive for acetylation, as expected, due to the auto-catalytic activity of the enzyme (Figure 3A, upper bands).

In order to determine which portion of IN was acetylated by p300, each individual domain of IN was fused to GST, as schematized in Figure 3B, and then analyzed by the *in vitro* HAT assay. In Figure 3C, lanes 3–5 indicate that, while the acetylation of the N-terminus and the core domain was below the detection levels, the C-terminus (aa 213–288) was highly acetylated. The C-terminus of the protein contains nine lysines that are potential targets for acetylation; therefore, a series of GST-IN fragments with progressive deletions starting from the C-terminus was produced (schematized in Figure 3B). The GST-IN fragment 1–272 was acetylated at a similar level as wt IN (Figure 3C, lane 6), while all the shorter fragments (1–263, 1–243, and 1–212) were not acetylated (Figure 3C, lanes 7–9). Thus, the region that is the target for p300-HAT acetylation lies between amino acids 264 and 272. Since this region contains three lysines at positions 264, 266, and 273 (Figure 3B), they were changed, either one at a time or in combination, into arginines (this amino acid is characterized by the same positive charge as lysine, but cannot be modified by acetylation). As shown in the two right panels of Figure 3C, lanes 12–17, all these recombinant proteins scored positive for acetylation by p300-HAT, except for the triple mutant (lane 18). From this set of experiments, we conclude that these lysines at positions 264, 266, and 273 are all substrates for acetylation.

Next, we investigated whether IN was also acetylated *in vivo*. Flag-IN was expressed in 293T cells, immunoprecipitated and analyzed by Western blotting with an anti-acetylated lysine antibody (Figure 3D, upper left panel). Compared to the control Flag-Luc protein, Flag-IN was clearly acetylated; in addition, when p300 was coexpressed, the levels of acetylation were markedly increased (compare lanes 2 and

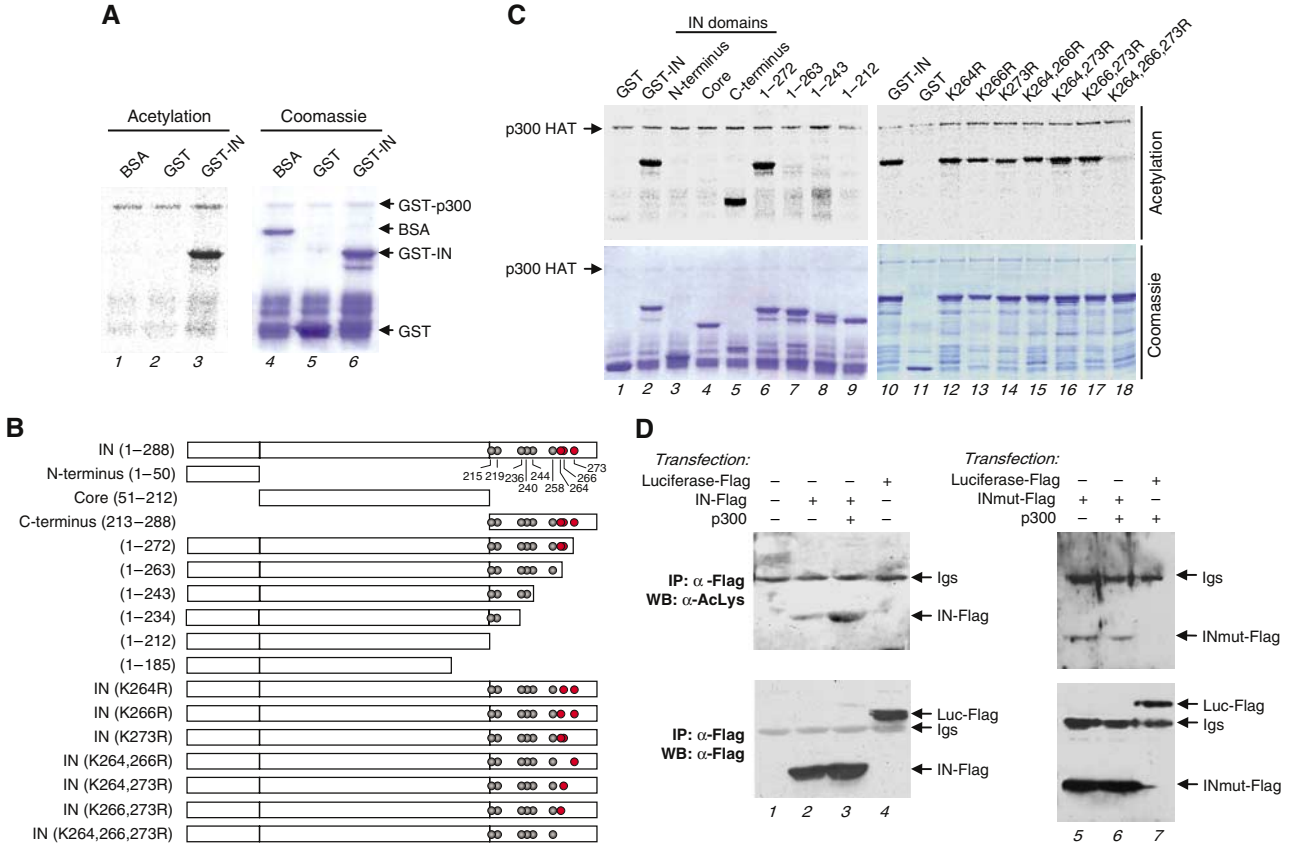

Figure 3 IN is acetylated by p300 both *in vitro* and *in vivo*. (**A**) *In vitro* IN acetylation. p300-HAT was incubated with GST-IN (lane 3) or controls (lanes 1 and 2) in the presence of ^{14}C acetyl-CoA; after incubation, the reaction mixture was resolved by SDS–PAGE and the gel exposed to Cyclone screen. Lanes 4–6 show Coomassie blue staining of the same gel. (**B**) Schematic representation of the GST-IN proteins used for pulldown and acetylation assays. The positions of the lysines in the IN C-terminal domain are indicated. The lysines positive for acetylation are shown in red. (**C**) The IN full-length protein (lane 2), the N-terminal, core, and C-terminal domains (lanes 3–5), a series of IN mutants with C-terminal truncations (lanes 6–9), and point mutants in the lysines in the C-terminal domain (lanes 12–18) were assayed for acetylation by p300-HAT. Upper panels: gels exposed to Cyclone screen. Lower panels: Coomassie-stained gels. (**D**) *In vivo* IN acetylation. Extracts of 293T cells transfected as indicated were immunoprecipitated with an anti-Flag antibody and immunoblotted using an anti-Ac-Lys antibody (upper panels). The same filters were incubated with an anti-Flag antibody (lower panels).

3). Of interest, the IN mutant in which the three lysines target for p300-mediated acetylation were mutated (K264, K266, k273) was still found acetylated *in vivo*, but the levels of acetylation were unchanged upon p300 overexpression (Figure 3D, upper right panel). As a control, Flag-Luc was found not aceylated either in the presence or absence of p300 (Figure 3D, upper panels, lanes 4 and 7). In all these experiments, the levels of the immunoprecipitated proteins were controlled by immunoblot with an anti-Flag antibody (Figure 3D, lower panels).

Taken together, these experiments indicate that IN is acetylated by p300 both *in vitro* and *in vivo* at lysines 264, 266, and 273. The observation that IN mut is also acetylated *in vivo* is consistent with the conclusion that other cellular HATs, in addition to p300, are also able to acetylate the protein *in vivo* at different sites.

Acetylation increases the affinity of IN to DNA

Since the region of IN involved in acetylation and binding to p300 corresponds to the domain responsible for nonspecific binding with DNA (Vink *et al*, 1993; Woerner and Marcus-Sekura, 1993; Engelman *et al*, 1994; Lutzke *et al*, 1994), we investigated the effects of acetylation on the IN/DNA interaction.

Recombinant IN was incubated with p300-HAT either in the presence or absence of acetyl-CoA. Scalar amounts of the reaction mixtures were then mixed with ^{32}P-labeled double-stranded U5-LTR DNA, followed by UV-crosslinking and separation by SDS–PAGE electrophoresis. As expected, the amounts of bound DNA were proportional to the levels of IN in the reaction (Figure 4A, lanes 1–5). Interestingly, when IN was incubated with p300 in the presence of acetyl-CoA, the quantity of bound DNA was consistently higher at all IN concentrations (Figure 4A, compare lanes 6–10 with lanes 1–5). Quantitative analysis of three independent experiments indicated that, under these conditions, acetylation of IN determines an overall two-fold increase in affinity of the protein for its target DNA (Figure 4B). When pondering these results, it should be considered that the amount of IN that is acetylated *in vitro* under these conditions corresponds to $\sim$15% of the total IN quantity; therefore, the actual increase of affinity of acetylated IN to DNA is most likely underestimated.

To prove that enhanced IN binding to DNA specifically depends on the acetyltransferase activity of p300, the same experiment was performed using Lys-CoA, a molecule that specifically inhibits p300 catalytic activity (Lau *et al*, 2000). Figure 4C shows that scalar amounts of IN incubated with p300 and acetyl-CoA bound lower amounts of DNA when Lys-CoA was added to the reaction mixture (Figure 4C, compare lanes 7–9 with lanes 4–6). Figure 4D shows the average values obtained by the quantitation of DNA bound to IN in three independent experiments. Of note, the levels of DNA bound to IN when Lys-CoA was added to the HAT reaction were similar to those observed when acetyl-CoA was omitted, indicating that Lys-CoA fully reversed the effect of acetylation on the modulation of IN affinity to DNA.

In these experiments (Figure 4A and C), the DNA-binding affinity of IN in the presence of acetyl-Coa or Lys-CoA alone was tested to exclude any possible effect of these compounds (data not shown).

When the IN mutant K(264,266,273)R was tested, we observed that DNA binding was modestly reduced as compared to the wt protein and, most notably, that binding was insensitive to either acetyl-CoA or acetyl-CoA plus Lys-CoA addition (Figure 4C, lanes 10–18, and Figure 4D).

Finally, to verify that the effect of Lys-CoA on the IN/DNA interaction was indeed due to the inhibition of acetylation, we directly assessed the acetylation of IN by a HAT assay. As shown in Figure 4E, incubation with Lys-CoA almost abolished p300-mediated acetylation of IN, as well as the autoacetylation of the enzyme (lane 3). As expected, no acetylation of the mutant was observed in these experiments (lane 5).

These results indicate that the enhancement in the DNA-binding affinity of IN observed following incubation with p300 specifically depends on the acetylase activity of this enzyme, and that the integrity of lysines 264, 266, and 273 is an absolute requirement for this enhancement to occur.

IN strand transfer activity is enhanced by acetylation

Since the binding of IN to DNA is required for the integration reaction to occur, and given the increased affinity of IN for DNA following acetylation, we analyzed whether acetylation of the protein might affect its enzymatic function. For this purpose, IN was incubated with p300-HAT either in the presence or absence of acetyl-CoA prior to a strand transfer assay. This experiment evaluates the capacity of IN to produce DNA fragments of different sizes (P), as a result of multiple integration events, when a short oligonucleotide is used as a substrate (S) (Marchand *et al*, 2001). Figure 5A shows the result of an experiment in which the strand transfer assay was performed using wt and mutated IN after incubation with p300-HAT either with or without acetyl-CoA. In the presence of acetyl-CoA, the DNA strand transfer activity of wt IN was significantly enhanced (compare lanes 1 and 2). In contrast, the activity of the IN mutant, which was slightly diminished as compared to the wt protein, remained unaffected (lanes 3 and 4). The results obtained from the quantification of three independent experiments indicate that a two-fold increase in strand transfer products is obtained following wt IN acetylation, while no variation is observed with IN mut following acetylation treatment (Figure 5B). Again, these results were obtained after *in vitro* acetylation of $\sim$15% of the total amount of IN in the reaction, and thus parallel those obtained with the DNA-binding assays.

Another enzymatic activity of IN is measured by evaluating the excision of 2 nt at the 3′ end of a double-stranded DNA template (3′-processing activity) (Marchand *et al*, 2001). As shown in Figure 5C, this activity was not affected by acetylation; the same amounts of processed DNA substrate were obtained by using IN incubated with p300 either in the presence or absence of acetyl-CoA (lanes 1 and 2).

Mutations of lysines 264, 266, and 273 impair HIV-1$_{BRU}$ integration

In order to determine how the mutation of lysines 264, 266, and 273 of IN affects the virus replication cycle, the arginine substitutions were introduced into the HIV-1$_{BRU}$ viral clone, containing a Flag-tagged IN (Petit *et al*, 1999), to produce the HIV-1$_{BRU(K264,266,273R)}$ molecular clone (hereafter HIV mut).

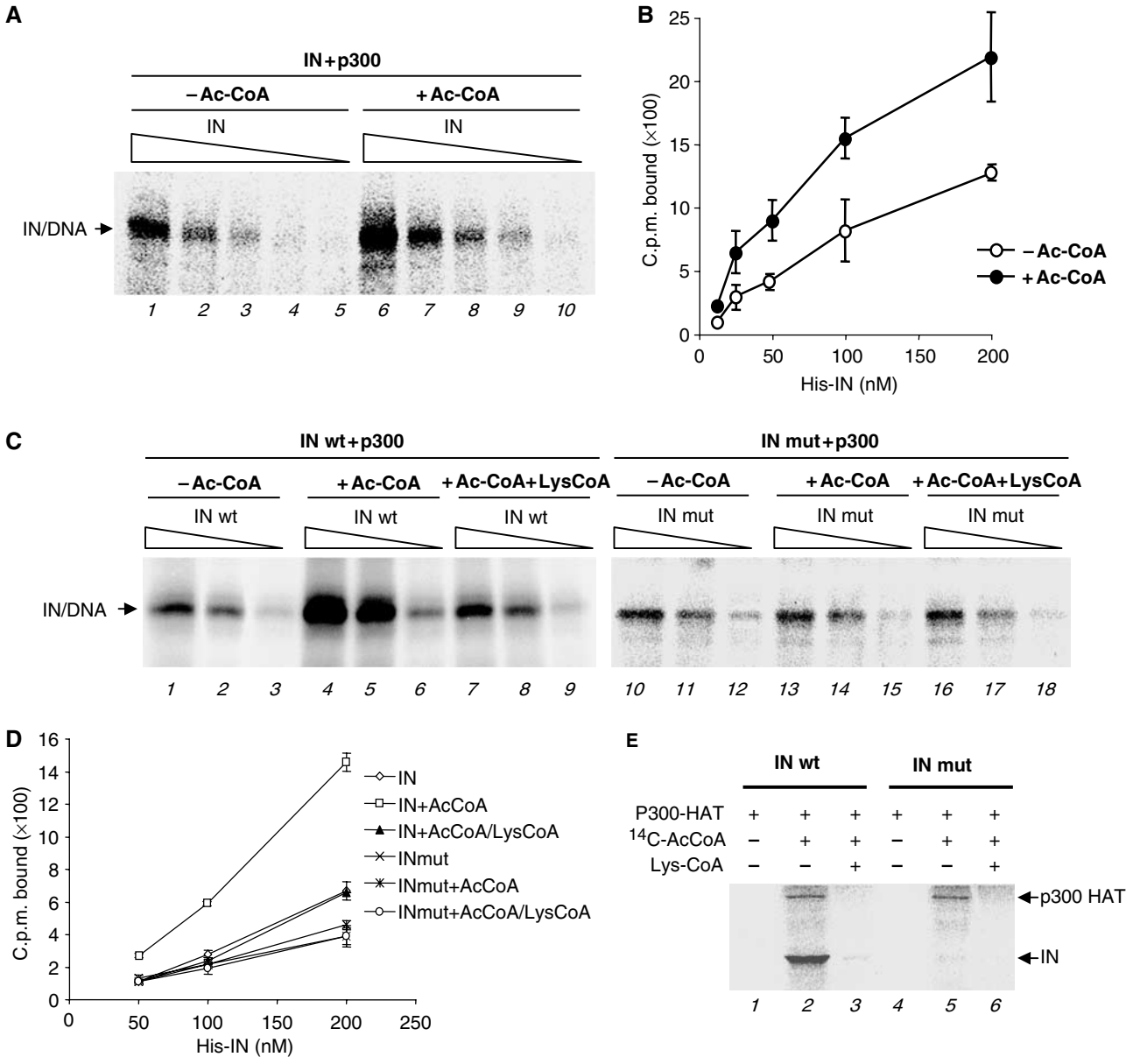

Figure 4 Acetylation of IN increases DNA binding. (**A**) Scalar amounts of His-IN were preincubated with p300 HAT either in the absence (lanes 1–5) or presence (lane 6–10) of cold acetyl-CoA, followed by UV-crosslinking DNA-binding analysis (see Materials and methods). The bands correspond to the complex between probe and IN. (**B**) The graph summarizes the results obtained from three independent experiments performed as in panel A; the average and s.d. of the amount of probe bound to IN are shown. To compare the different experiments, the c.p.m. values were normalized to the amount of probe bound by the lowest amount of IN in the absence of acetyl-CoA. (**C**) Scalar amounts of His-IN either wt (lanes 1–9) or mutated in K(264,266,273)R (lanes 10–18) were preincubated with p300-HAT either in the absence (lanes 1–3 and 10–12) or presence of cold acetyl-CoA (lanes 4–9 and 13–18) in addition to Lys-CoA (lanes 7–9 and 16–18), followed by UV-crosslinking DNA-binding analysis (see Materials and methods). (**D**) The graph summarizes the results obtained from three independent experiments performed as in panel (C) and indicated as in (B). (**E**) HAT assay performed with recombinant His-IN wt (lanes 1–3) or mutated in K(264,266,273)R (lanes 4–6) in the presence of ^{14}C acetyl-CoA (lanes 2, 3 and 5, 6) in addition to Lys-CoA (lanes 3 and 6).

Equal amounts of wt and mutated virus were used to infect CD4+ CEM T-cells and primary peripheral blood lymphocytes (PBLs) from two healthy individuals. Viral replication was monitored by RT activity assays over a period of 15 days. As shown in Figure 6A, CEM cells, as well as PBLs, infected with wt virus showed a peak of infection around day 8 post-infection. Conversely, the levels of replication of HIV mut were very low in CEM cells and almost undetectable in PBLs. The infectivity of both the wt and mutated virus was also tested in CEM cells at viral loads that were two- and five-fold higher. Both viruses showed a dose-dependent increase in viral replication; nevertheless, replication of HIV mut continued to be grossly impaired (Figure 6B).

To verify that the absence of viral replication observed using the mutated virus was not determined by a major alteration in viral structure, a Western blot analysis was performed on the viral stocks using an antibody recognizing the matrix (MA) protein and an antibody against the Flag epitope in IN. As shown in Figure 6C, left panel, the bands corresponding to the Gag precursor (p55) containing the MA

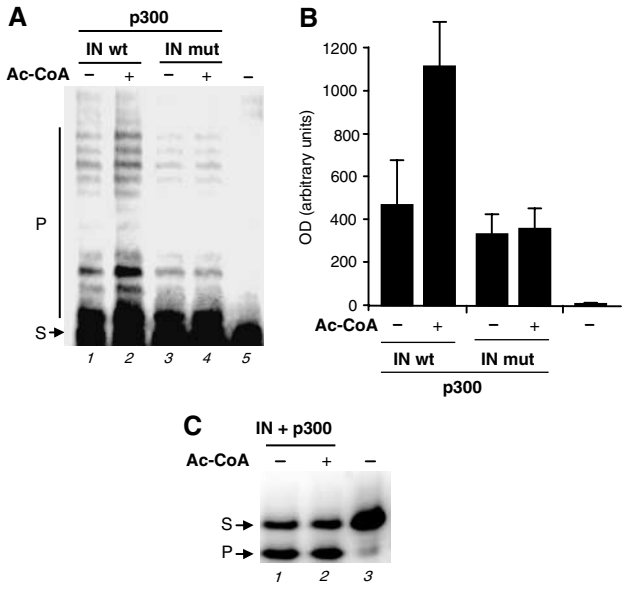

Figure 5 Acetylation increases strand transfer activity of IN. (**A**) Strand transfer activity of His-IN wt (lanes 1, 2) or mutated in K(264,266,273)R (lanes 3, 4) preincubated with p300-HAT either in the absence (lanes 1, 3) or in the presence (lanes 2, 4) of cold acetyl-CoA. Lane 5: substrate without His-IN. (**B**) Quantification analysis of the strand transfer activity obtained from three independent experiments performed as in (A); the average and s.d. of the summary of the intensity of bands that result as products of the strand transfer activity in each lane are shown. (**C**) 3′ processing activity of His-IN, after preincubation with p300-HAT either in the absence (lane 1) or presence (lane 2) of cold acetyl-CoA. Lane 3: substrate without His-IN. In (A) and (C), the DNA substrate (S) and catalytic products (P) are indicated.

epitope, as well as the MA protein (p17) as a product of proteolysis, are equally represented in both the wt and mutated viruses. Similarly, the anti-Flag antibody revealed IN at the same size and amount in both viruses (Figure 6C, right panel). These results clearly indicate that the structure and maturation of the mutated virus are not hampered by the mutations introduced. The similar amounts of HIV wt and HIV mut produced during viral stocks preparation, as measured by evaluating the RT/p24 ratios (data not shown), are also consistent with this conclusion.

To analyze whether this remarkable impairment in viral replication might be attributed to a defect in viral cDNA integration, total genomic DNA was extracted from the infected CEM cells and the amount of integrated virus was determined by Alu-PCR analysis. A representative PCR experiment, performed by the amplification of five-fold serial dilutions of DNA extracted from HIV wt- and HIV mut-infected cells, is shown in Figure 7A, along with the average results obtained from the densitometric analysis of five independent experiments. The results of these semiquantitative experiments clearly show that the amount of integrated HIV mut DNA is at least 15 times lower than that of wt HIV-1.

It has been reported that, following reverse transcription of viral RNA, not all linear DNA molecules containing an LTR at each end become integrated, and that a fraction of retroviral cDNA is circularized by host recombination enzymes in the nucleus. One circular DNA product, in particular, containing a tandem repeat of the LTR, has been extensively used as a marker for nuclear localization because its LTR–LTR junction is a convenient template for PCR (Engelman, 1999). Therefore, we quantified the levels of 2-LTR circles in the HIV wt- and HIV mut-infected cells to monitor cDNA nuclear import. Figure 7B shows the result of a representative PCR experiment using serial dilutions of total DNA along with the average values of three independent experiments. The results indicate that the amount of 2-LTR circles in HIV mut-infected cells is at least 15 times higher than the amount found in cells infected with HIV wt.

As a control for the total amount of DNA used for these quantifications, we amplified a cellular genomic sequence (lamin B2 gene), as previously reported (Lusic *et al*, 2003); amplification efficiency was comparable in both the HIV wt- and HIV mut-infected cells (Figure 7C).

In addition to integration reaction, IN mutations can affect reverse transcription (Engelman, 1999). We therefore analyzed the kinetics and the amounts of cDNA synthesized by HIV wt and HIV mut; the results obtained indicate that the mutations K264, 266, 273R do not significantly alter the viral reverse transcription steps (see Supplementary data for details).

From these experiments we conclude that the K264, 266, 273R IN mutation does not affect viral cDNA synthesis or nuclear transport, but markedly impairs viral cDNA integration.

Inhibition of p300 catalytic activity inhibits viral integration

Infection with HIV mut indicated that the lysines targeted for acetylation are necessary for efficient viral integration. To directly verify the effect of p300-mediated acetylation on viral integration, we performed HIV-1 infection in cells treated with the specific inhibitor of the enzymatic activity of p300 Lys-CoA. A human T-cell line (SupT1) was pretreated with sphingosylphosphorylcholine (SPC), a permeabilizing agent, and then infected with HIV-1_{BRU} either in the presence or absence of Lys-CoA. The efficiency of viral integration was then measured by Alu-PCR performed on five-fold serial dilutions of genomic DNA (controlled by amplification of a cellular genomic sequence). As shown in Figure 8A, no significant variation in viral integration was observed between infection with or without SPC. In contrast, the amount of integrated DNA was 12.2 ± 3.2-fold decreased in cells treated with SPC/Lys-CoA (mean $\pm$ s.d. of densitometric scanning from three independent experiments). To verify the efficacy and specificity of Lys-CoA treatment on p300 acetylation, cell lysates were incubated with antibodies specific for p300 or for GCN5, another cellular HAT. Immunoprecipitates were then tested in HAT assays using histones as substrates. As shown in Figure 8C, left panel, the activity of p300 in Lys-CoA-treated cells was significantly inhibited as compared to nontreated cells. Conversely, the histone acetyltransferase activity of GCN5 resulted unaffected. In the same experiments, the total levels of immunoprecipitated p300 and GCN5 proteins were monitored by Western blotting and shown to be unmodified by Lys-CoA treatment (Figure 8C, right panel). Finally, the viability of cells treated with Lys-CoA/SPC was monitored by the MTT assay and found to be unmodified as compared to untreated cells or cells treated with SPC alone (data not shown). Taken together, these results indicate that the enzymatic activity of p300 is required for efficient proviral integration *in vivo*.

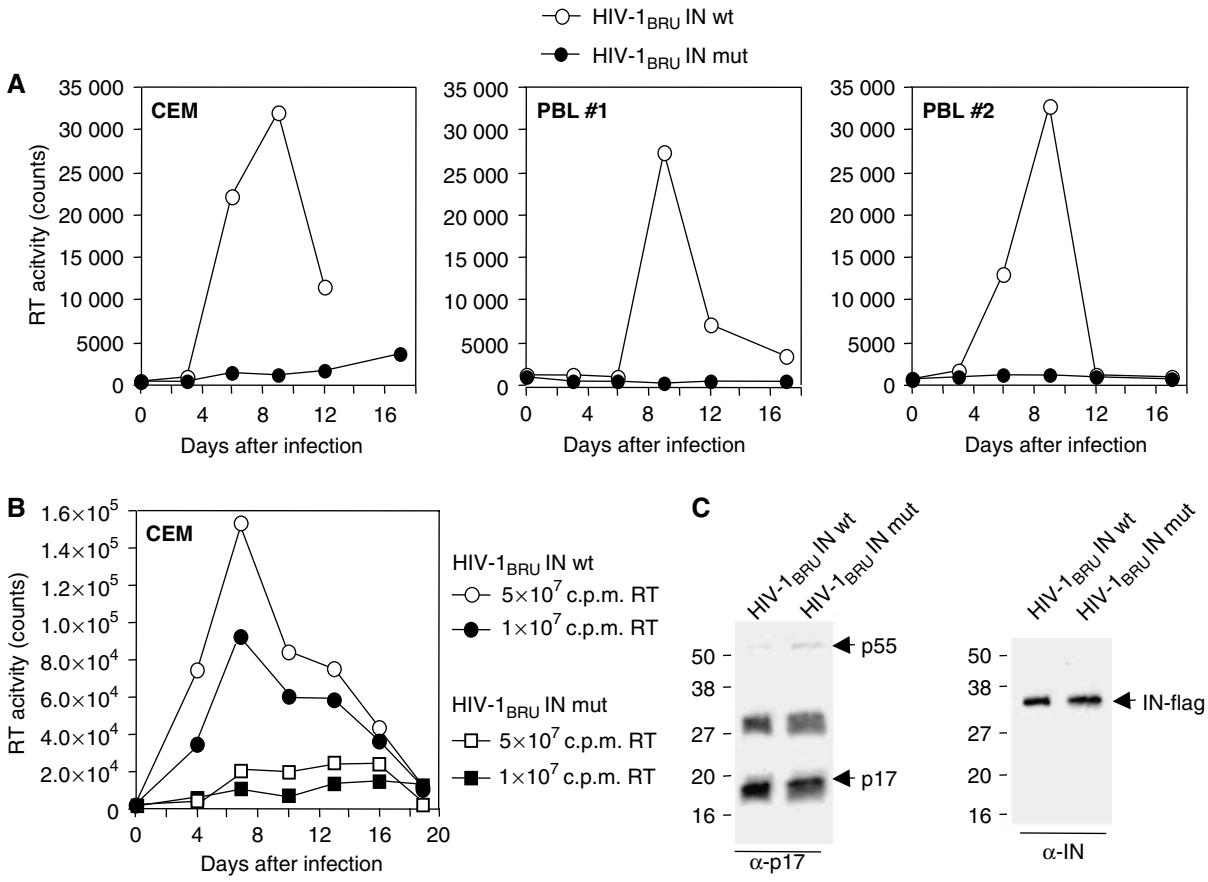

Figure 6 Replication of an HIV-1 viral clone carrying the IN K(264,266,273)R mutation is impaired. (**A**) Replication kinetics of wt HIV-1_{BRU} (HIV wt) and of an HIV-1_{BRU} viral clone carrying the IN K(264,266,273)R mutation (HIV mut). In both viral clones, the IN is Flag-tagged. CEM T-cells and PBLs from two healthy donors were infected with equal amounts (0.5×10^7 c.p.m. RT) of the two viruses; the RT activity of the supernatants was measured at different time points after infection. Each graph shows the average values from three independent experiments. (**B**) RT activity of the supernatants measured at different time points after infection of CEM T cells with 1×10^7 or 5×10^7 c.p.m. RT of HIV wt or HIV mut. (**C**) Western blot analysis of either HIV wt or HIV mut viral stocks using an anti-MA (left panel) or an anti-Flag (right panel) antibody.

Discussion

This is the first study demonstrating that IN undergoes a post-translational modification by acetylation both *in vitro* and *in vivo*. Moreover, we show that IN specifically binds p300 *in vitro* and in the cell.

What could the relevance of these findings be in the context of new emerging evidence indicating that HIV-1 preferentially integrates in close proximity to expressed cellular genes? p300 is a transcriptional coactivator with a broad activity on gene transcription through its interaction with a number of cellular transcription factors and components of the basal transcriptional machinery (reviewed in Chan and La Thangue, 2001). In several instances, including the HIV-1 LTR promoter, it has been demonstrated that transcriptional activation occurs following acetylation of the nucleosomes through specific recruitment of p300, as well as of other acetyltransferases at the gene promoter region (Lusic *et al*, 2003). Thus, it might be conceivable that the specific interaction of IN with p300 might be one of the determinants that mediate the association of the viral PICs to actively transcribed genomic regions. Further investigations are required to substantiate this hypothesis.

Most interestingly, the same IN domain that binds p300 is also specifically acetylated by its acetyltransferase activity, resulting in the specific modification of lysines 264, 266, and 273. Acetylation of DNA-binding proteins often increases their binding affinity to DNA. This is the case for several transcription factors, including p53, E2F, MyoD, c-Myb, and, possibly, GATA1 (Sterner and Berger, 2000). In our study, we demonstrate that acetylation of IN by p300 also increases IN affinity to DNA. This conclusion is supported by the observations that the enhanced DNA-binding affinity of IN in the presence of p300 HAT is strictly dependent on the addition of acetyl-CoA and that this effect is completely reversed by Lys-CoA, a compound that specifically inactivates the catalytic activity of p300. In addition, the binding is not increased by p300-HAT when the three lysines targeted for acetylation are substituted into arginines. Consistent with these results, lysines 264, 266, and 273 are located in the C-terminal domain of IN, which binds DNA in a nonspecific manner (Vink *et al*, 1993; Woerner and Marcus-Sekura, 1993; Engelman *et al*, 1994; Lutzke *et al*, 1994). It has been reported that the minimal region of IN required for DNA binding comprises residues 220–270; in particular, lysine 264 appears to be essential in binding the protein to DNA (Lutzke *et al*, 1994). In addition, a footprint analysis has revealed that lysine 273 is also among the amino acids involved in DNA binding (Dirac and Kjems, 2001).

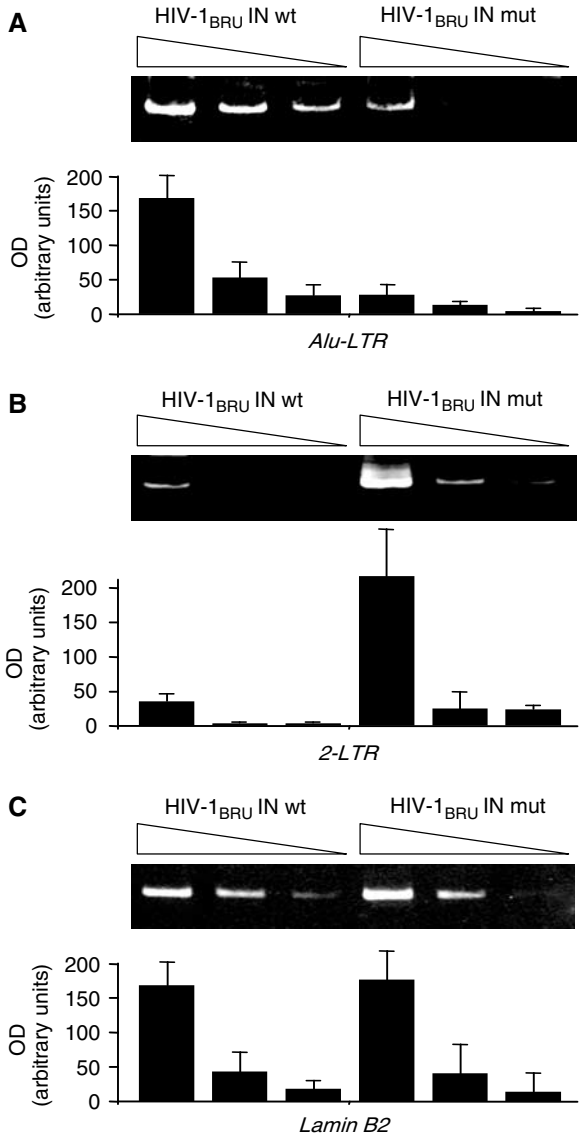

Figure 7 DNA integration of an HIV-1 viral clone carrying the IN K(264,266,273)R mutation is impaired. Serial dilutions of DNA extracted from CEM cells following infection with HIV wt or HIV mut were amplified with primers specific for (**A**) the Alu-LTR DNA fragment (to analyze the fraction of integrated HIV DNA), (**B**) the 2-LTR circles (to assess the levels of unintegrated HIV DNA), and (**C**) the lamin B2 cellular gene (to standardize the total amount of extracted DNA). The graphs were obtained by densitometric analysis of the PCR products obtained from 3–5 independent experiments.

In agreement with the DNA-binding results, the strand transfer activity of IN was also induced by p300-mediated acetylation. Of interest, both the DNA-binding data and the strand transfer assays indicate that the IN mutant, although still active in both assays, is functionally not sensitive to acetylation. These data demonstrate that modification of lysines 264, 266, and 273 by acetylation is a specific requirement for the enhancement of IN activity. It has been shown that, even though both 3′ end processing and strand transfer are catalyzed by a common active site, mutations at positions other than the catalytic site may differentially affect these activities. Deletion mutants involving the C-terminus of IN, in which acetylation occurs, have been reported to either maintain the same level of activity for both reactions (Engelman *et al*, 1993; Vink *et al*, 1993) or not (Drelich *et al*, 1992). Similarly to the last study, we observed that, in contrast to the strand transfer, the 3′-end processing reaction remained unaffected by acetylation. Thus, acetylation differentially affects only one of the enzymatic reactions carried out by the IN C-terminal domain.

In the context of the viral replication cycle, the catalytic activity of p300 appears essential for efficient integration, as indicated by results showing impaired proviral integration following p300 inactivation by Lys-CoA. In addition, *in vivo* data indicate that the integrity of lysines 264, 266, and 273 appears essential to establish productive infection, as demonstrated by the impairment of viral replication observed using the mutated viral clone. Quantification of the amount of integrated provirus, lower in the HIV mut-infected cells, suggests that the impairment of viral replication is at the level of provirus integration. This conclusion is validated by the observation that the episomal 2-LTR circles produced by the HIV mut are higher than those detected in cells infected with wt HIV. In fact, viruses containing IN mutations that specifically block the integration step, as opposed to IN mutations with pleiotropic effect, produce higher levels of unintegrated DNA circles (reviewed in Engelman, 1999). Of note, the experiments on infectious virus replication *in vivo* indicate that this mutation has an even more dramatic effect than expected from the *in vitro* assay. *In vitro*, this mutant shows a modest reduction in DNA-binding and strand transfer activities as compared to the wt enzyme, while the mutated viral clone is severely impaired in viral replication even at the highest multiplicity of infection. Although we cannot exclude that the lysine to arginine substitution of these residues alters other functions of IN inside the cells, a conceivable interpretation of this observation is that acetylation of IN might be essential for the proper activity of the protein *in vivo*, once in the nucleus.

Further support for the importance of the conservation of lysines at these positions emerges from the comparative analysis of this IN region among retroviruses. While the C-terminus of the protein is highly variable among lentiviral INs, nevertheless, lysines 264 and 266 are highly conserved in most HIV-1 strains, as well as in HIV-2 and SIV (Cannon *et al*, 1996) (Table I). Additionally, lysine 266 is also conserved in several other human and animal retroviruses, belonging, or not, to the lentiviral family. This extent of conservation (albeit with some notable exceptions) indirectly highlights the role of these residues in preserving IN function. Whether these lysines are also acetylated in retroviruses other than HIV-1 is an important topic for future investigation.

Materials and methods

In vitro acetylation assay and cellular treatments with Lys-CoA

HAT assays were performed as previously reported, with minor modifications (Marzio *et al*, 1998, 2000). GST fusion proteins were incubated with the GST-HAT domain of p300 and ^{14}C-acetyl-CoA in HAT buffer (50 mM Tris, pH 7.5, 5% glycerol, 0.1 M EDTA, 50 mM KCl, and 2 mM sodium butyrate) in a final volume of 30 μl for 45 min at 30°C. Acetylated proteins were visualized by phosphoimaging (Cyclone) after separation by SDS–PAGE. The percentage of *in vitro* acetylated recombinant IN was calculated according to the moles of protein incubated for each reaction and the moles of incorporated ^{14}C-acetyl-CoA, by taking into account the specific activity of ^{14}C-acetyl-CoA. For this purpose, a conversion scale

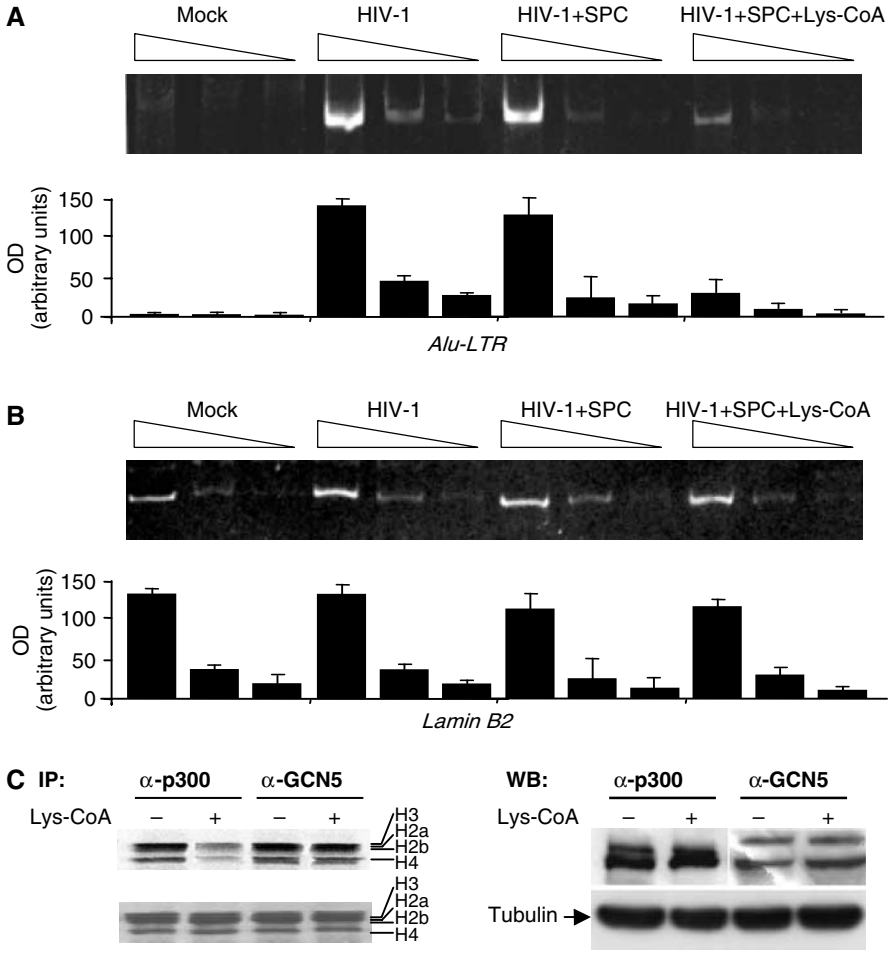

Figure 8 Inactivation of p300 catalytic activity inhibits HIV-1 integration. Serial dilutions of DNA extracted from SupT1 cells treated as indicated were amplified with primers specific for (**A**) the Alu-LTR DNA fragment and (**B**) the lamin B2 cellular gene. The graphs in (A) and (B) were obtained by densitometric analysis of the PCR products obtained from three independent experiments; the means ± s.d. are shown. (**C**) Left panels: HAT assay performed on histones using immunoprecipitates obtained by incubating antibody anti-p300 or anti-GCN5 with cell extracts of SupT1 treated with SPC alone or together with Lys-CoA (upper panel: gels exposed to Cyclone screen; lower panel: Coomassie-stained gels). Right panels: SupT1 cell extracts were analyzed by immunoblot with anti-p300 or anti-GCN5 antibody (upper panels), the same blot was then incubated with anti-tubulin antibody (lower panel).

between c.p.m. and moles was simply constructed by spotting scalar amounts of ^{14}C-acetyl-CoA on a filter and measuring the levels of radioactivity by phosphoimaging.

The cellular treatment with Lys-CoA (synthesized at the ICGEB Peptide Synthesis Core Facility) was performed as in Bandyopadhyay *et al* (2002), with minor modifications. Briefly, 3.5×10^6 exponentially growing SupT1 cells were incubated with the ICB solution (10 mM HEPES, pH 7.0, 0.14 M KCl, 0.01 M NaCl, and 2.4 mM $MgCl_2$) containing Lys-CoA (0.75 mM) and SPC (1.2 mg/ml) at 37°C for 10 min. Cells were then infected with viral stocks (1×10^7 c.p.m. RT) of HIV-1_{BRU} for 2 h. At 10 h after infection, cells were lysed for either DNA extraction or for immunoblot analysis and HAT assay.

IN activity

A recombinant histidine-tagged IN (His-IN) protein (400 ng) was incubated with 60 ng of the GST-HAT domain of p300 in a buffer containing 20 mM Hepes (pH 8.0), 7.5 mM $MnCl_2$, 0.05% NP40, 10 mM DTT and 2 mM sodium butyrate with or without the addition of 65 μM of cold acetyl-CoA.

The IN strand transfer reaction was performed in the same buffer used for the acetylation assay, while the 3′ end-processing assay was performed in 25 mM MOPS, 5% PEG-8000, 5% DMSO, 0.05% NP40, 30 mM NaCl, 10 mM $MgCl_2$, and 10 mM DTT. For both reactions, 32 ng of acetylated or nonacetylated IN and 1 pmol of radiolabeled oligonucleotide (see below) were used in a total volume of 15 μl. The reaction mixture was incubated at 37°C for 1 h and then analyzed on a 15% polyacrylamide gel in Tris-borate-EDTA, pH 7.6, and 7 M urea, and visualized by phosphoimaging (Cyclone).

The oligonucleotides used to perform the strand transfer assays were: 71 (5′-GTGTGGAAAATCTCTAGCA-3′) and 72 (5′-ACTGCTAGA GATTTTCCACAC-3′); the oligos used for the 3′ processing assays were 72 and 70 (5′-GTGTGGAAAATCTCTAGCAGT-3′) (Parissi *et al*, 2001).

DNA-binding assays

His-IN (1 μg) was incubated with the GST-HAT domain of p300 in binding buffer (25 mM Hepes, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 2% glycerol, 0.5% Tween 20, 0.1 mM 2-mercaptoethanol, 2 mM sodium butyrate) with or without the addition of 20 μM of cold acetyl-CoA. The amounts indicated in each experiment of His-IN mut or His-IN wt following acetylation in the presence or absence of Lys-CoA, or nonacetylated, were incubated in binding buffer for 30 min at 4°C with 20 mM ^{32}P-labeled U5-LTR oligonucleotide (5′-GTCAGTGTGGAAAATCTCTAGCAGT-3′) annealed with the complementary strand. Samples were irradiated with UV (0.8 J for 5 min in ice) and loaded on a 10% SDS–PAGE gel that was then analyzed by phosphoimager (Cyclone).

Viral assays

Viral stocks were prepared by transfection of the viral clones HIV-1_{BRU} (Petit *et al*, 1999) and HIV-$1_{BRU(K264,266,273R)}$ in 293T cells. The same amounts of virus were used for the subsequent infections

Table I Conservation of the lysines that are substrates of acetylation (positions 264, 266, 273 of HIV-1 IN, in red) in the IN proteins of other retroviruses

	264 266 273	
Lentivirus	VVPRRK-AKIIRDYGK	HIV-1 (subtypes A,B,H,F,G,O)
	VVPRRK-AKIIKDYGK	HIV-1 (subtype C)
	VVPRRK-VKIIRDYGK	HIV-1 (subtype D)
	VVPRRK-AKIIRHYGK	HIV-1 (subtype O2)
	VVPRRK-AKIIRDYGG	HIV-2
	VVPRRK-AKIIKDYGE	SIV
	LIPRRHIRRVPEPCA	FIV
	LYRHRHMRFIPPPDS	BIV
	VIANKDVKFIPPPKE	VISNA
	VIPYKDAKFIPPPTK	CAEV
	VIAKKDVKFIPQPKE	OLV
	AVPLTR-TKLLIKPN	EIAV
Nonlentivirus	WVPSRKVKPDITQKDE	RSV
	WVPWRLLKAFKCPKND	BLV
	WIPWRFLKKAACPRPD	HTLV-II
	WIPWRLLKRAACPRPV	HTLV-I
	WIHYSRLKKAPDNQEE	REV-A
	WIHASHVKAAGPTTNQ	FeLV
	WIHAAHVKAADPGGGP	MoMLV
	WIHAAHVKAATTPPIK	AKVMLV

The region 220–270 of HIV-1 IN corresponds to the minimal DNA-binding domain of the protein (modified from Cannon *et al*, 1996).

using both viral preparations, as monitored by assessing both the p24 levels (p24 ELISA kit, Perkin-Elmer) and the RT activity in the supernatants, following standard procedures. The integrated proviral DNA in CEM cells was evaluated by Alu-PCR as previously described (Chun *et al*, 1997), with minor modifications. The sequences of the primers used are as follows. First amplification: Alu-LTR 5′ (5′-TCCCAGCTACTCGGGAGGCTGAGG-3′) and Alu LTR 3′ (5′-AGGCAAGCTTTATTGAGGCTTAAGC-3′) (Chun *et al*, 1997); nested amplification: H1F (5′-GAAGGGCTAATTTGGTCCCA-3′) and H1R (5′-GATGCAGCTCTCGGGCCATG-3′) (Lusic *et al*, 2003). The sequences of the primers used to analyze the 2-LTR circles are: 477 (5′-GTGACTCTGGTAACTAGAGA-3′) and 515 (5′-GTGTGTAGTTCTGC TAATCAGGGAA-3′) (Cara *et al*, 1995). The PCR products were resolved by gel electrophoresis and quantified by densitometric scanning; all quantification experiments were performed at least three times.

Immunoprecipitation and in vivo acetylation assay

The following antibodies were used: anti-acetylated lysine (Cell Signaling), anti-p300 (Pharmingen), anti-Flag (M2 Stratagene), either free or bound to agarose beads. For immunoprecipitation, cell pellets were lysed in a RIPA buffer containing 10 mM of sodium butyrate (Sigma) and protease inhibitors (Roche). The protein concentration of the extracts was determined by the Bradford assay (BioRad). Anti-FLAG M2-Agarose beads (Sigma) were incubated overnight at 4°C with cell extracts (2.5–4.5 mg). After incubation, the immunocomplexes were analyzed by Western blotting using the appropriate antibodies.

In vitro binding assays

Binding of GST-IN and its truncated variants to ^{35}S-p300 was performed as described previously (Marzio *et al*, 1998, 2000). Briefly, 1 μg of recombinant proteins, after pretreatment in a solution containing DNase I 0.25 U/μl and RNase 0.2 μg/μl to remove contaminant bacterial nucleic acids, were incubated with 600 c.p.m. of *in vitro* translated p300 in a solution containing 0.2 mg/ml ethidium bromide. Following extensive washes, the reaction mixture was resolved by SDS–PAGE electrophoresis and analyzed by PhosphoImager.

Recombinant proteins

GST, GST-IN, and GST-IN fragments and mutants were prepared as already described (Marzio *et al*, 1998, 2000). The pcDNA3-p300 was used as a template to produce the *in vitro* ^{35}S p300 for *in vitro* binding by using the TNT Reticulocyte Lysate System (Promega). His-IN wt and mut were purified as in Bushman *et al* (1993).

FRET analysis

U2OS cells (5×10^5) were seeded into four-well glass chamber slides (LabTek II-Nalge Nunc) and transfected with the Effectene transfection kit (Qiagen) for 24 h with 400 ng of pEYFP-IN and pECFP-p300 at a 1 to 5 molar ratio, or with 400 ng control plasmids pEYFP-CDK9 and pECFP-Rb. Fluorescent images of samples fixed with 2% paraformaldehyde were acquired using a TCS-SL Leica confocal microscopy. The quantitative FRET analysis was performed using the Leica Confocal software that elaborates the FRET efficiency by applying the formula $1–F_{da}/F_d$, where F_{da} stands for the fluorescence emitted by the donor fluorophore before bleaching and F_d for the emission of the donor fluorophore after bleaching.

Plasmids

pGEX-IN was constructed using the recombinant PCR technology from the HIV-1_{HXB2} viral clone. pINSD-His was obtained from the NIH AIDS Research & Reference Reagent Program. The pGEX-IN and pINSD-His mutated in K264, K266, and K273 were constructed using recombinant PCR starting from each original vector. pFlag-IN-codon optimized was kindly provided by Dr A Engelman; pEYFP-IN was prepared by subcloning the IN-codon optimized cDNA from the pFLAG-IN into pEYFP vector (Clontech) by the PCR method. The pECFP-p300 was prepared by PCR-subcloning the p300 cDNA from the pcDNA3-p300 into the pECFP vector (Clontech). pcDNA3-p300 and pCMVβ-p300 have been described previously (Marzio *et al*, 1998, 2000). The pGEX-p300 HAT (aa 1195–1810) was a kind gift of Dr E Verdin. The HIV mut viral clone was prepared by subcloning IN from the pGEX-IN mutated vector. All constructs were verified by DNA sequencing.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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