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ORIGINAL ARTICLE

Identification of cellular factors binding to acetylated HIV-1 integrase

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Abstract The viral protein integrase (IN) catalyzes the integration of the HIV-1 cDNA into the host cellular genome. We have recently demonstrated that IN is acetylated by a cellular histone acetyltransferase, p300, which modifies three lysines located in the C-terminus of the viral factor (Cereseto et al. in EMBO J 24:3070–3081, 2005). This modification enhances IN catalytic activity, as demonstrated by in vitro assays. Consistently, mutations introduced in the targeted lysines greatly decrease the efficiency of HIV-1 integration. Acetylation was proven to regulate protein functions by modulating protein–protein interactions. HIV-1 to efficiently complete its replication steps, including the integration reaction, requires interacting with numerous cellular factors. Therefore, we sought to investigate whether acetylation might modulate the interaction between IN and the cellular factors. To this aim we performed a yeast two-hybrid screening that differs from the screenings so far performed (Rain et al. in Methods 47:291–297, 2009; Studamire and Goff in Retrovirology 5:48, 2008) for using as bait IN constitutively acetylated. From this analysis we have identified thirteen cellular factors involved in transcription, chromatin remodeling, nuclear transport, RNA binding, protein synthesis regulation and microtubule organization. To validate these interactions, binding assays were performed showing that acetylation increases the affinity of IN with specific factors. Nevertheless, few two-hybrid hits bind with the same affinity the acetylated and the unmodified IN. These results further underlie the relevance of IN post-translational modification by acetylation in HIV-1 replication cycle.

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Abbreviations

HIV-1	Human immunodeficiency virus-1
IN	Integrase
PIC	Pre-integration complex
GDBD	Gal4 DNA binding domain
GAD	Gal4 activation domain
HAT	Histone acetyltransferase catalytic domain
wt	Wild type
mut	Mutated
His	Histidine
Ade	Adenine
Ac-Lys	Acetylated lysines
TEV	Tobacco etch virus protease site
HA	Hemagglutinin epitope tag
WB	Western blot analysis
α	Antibodies
NLS	Nuclear localization signal

Introduction

An essential step in the retroviral life cycle is the integration of the viral DNA into the host cellular genome; a reaction catalyzed by the viral protein integrase (IN). Following virus entry and uncoating in the cytoplasm, the viral double strand cDNA is synthesized through reverse transcription starting from the RNA genome (Coffin et al. 1997). The viral DNA molecules then translocate into the nuclei of infected cells as part of a large nucleoprotein

complex, the pre-integration complex (PIC), which is formed by IN and other viral/cellular proteins (Suzuki and Craigie 2007). Within PICs both ends of the linear DNA molecule are processed by IN to form recessed 3′ OH termini. At the integration site, IN uses the recessed 3′OH groups to cut opposing strands of chromosomal DNA in a staggered fashion, thus concomitantly connecting the viral DNA 3′ ends to the generated 5′ overhangs. The resultant DNA recombination intermediate harbors single strands discontinuities that must be repaired by the host cellular repair system to complete provirus formation (Vandegraaff and Engelman 2007). Purified HIV-1 IN displays 3′ processing and DNA strand transfer activities that are sufficient to catalyze the cDNA integration reaction in vitro. However, in vivo numerous cellular proteins are required for efficient integration. The host factors regulate IN enzymatic functions by modulating its stability and by mediating nuclear import and perhaps access to specific regions of the chromatin (Goff 2007; Van Maele et al. 2006). We have recently demonstrated that p300, a histone acetyltransferase, binds IN and acetylates three lysines (K264, K266, K273) located in its C-terminus leading to enhanced IN activity. Acetylable lysines are necessary for virus integration and thus for optimal replication, as demonstrated by the inefficient infectivity observed following their mutations into arginine residues (K264, 266, 273R) (Cereseto et al. 2005). Since it has been demonstrated that acetylation modulates the activities of cellular and viral proteins by affecting protein–protein interactions (Sterner and Berger 2000), in this study we investigated whether this protein modification could affect the interaction of IN with cellular factors. To this aim we have employed the tethered catalysis two-hybrid system, a method previously reported to efficiently identify factors binding specifically to acetylated proteins (p53, histones H3 and H4) (Guo et al. 2004). From this screening we identified thirteen new factors binding IN with variable affinities based on the acetylation level of the viral factor.

Results

Constitutive acetylation of IN fused to the HAT domain of p300

To produce constitutively acetylated IN, a cDNA cassette was constructed containing a codon-optimized sequence for IN (IN-CO) fused to the acetyltransferase catalytic domain of p300 (IN-HATwt). As control, IN was also fused to a sequence coding for a catalytically inactive HAT containing a D1395Y mutation (IN-HATmut). The IN-HAT fusion proteins produced by these constructs contain at the N-terminus a histidine tag (6xHis) which allows affinity purification of the protein product by means of cobalt based resin columns (see 'Materials and methods'). In addition, the C-terminus of the chimera is in frame with a hemagglutinin (HA) epitope tag used to analyze the fusion protein by immunoblot with anti-HA antibodies. Finally, between the IN and the HAT domains a tobacco etch virus (TEV) proteolytic cleavage site was introduced allowing IN separation from the HAT domain (Fig. 1a).

Both IN-HATwt and IN-HATmut chimera were produced and purified in bacteria (*Escherichia coli*, BL21 strain). The purified products were immunoprecipitated with anti His-tag antibodies and analyzed by Western blot using anti-acetylated lysine (Ac-Lys) antibodies to assess the acetylation status of the recombinant chimera. As shown in Fig. 1b, upper panel, high levels of acetylation were detected with the IN-HATwt chimera at the two quantities tested (1 and 5 μg), while no signal was reported with the same amounts of IN-HATmut chimera. In order to verify appropriate protein loading the same filter was also incubated with antibodies anti-IN (Fig. 1b, lower panel).

Since p300 HAT domain contains 44 lysine residues that are substrate of auto-acetylase catalytic activity, we sought to verify whether the acetylation reported with the IN-HAT chimera (Fig. 1b) included modification of the individual IN domain. To this end IN was separated from the fusion protein through TEV digestion; the cleavage product was purified with nickel affinity chromatography and checked by Western blot analysis using anti-IN antibodies (Fig. 1c, left panel). As shown in Fig. 1c, right panel, IN derived from the IN-HATwt chimera gave a strong acetylation signal with anti-Ac-Lys antibodies, while no acetylation was detected with IN derived from the HATmut. Thus, these results clearly demonstrate that IN is specifically acetylated as a fusion product of HATwt but not of HATmut.

The IN-HAT cDNA cassette was then expressed in yeast cells in frame with the Gal4 DNA-binding domain (GDBD) under the control of a yeast promoter (GDBD-IN-HATwt/mut). The expression of the IN-HATwt/mut chimeras in yeast cells (AH109 strain) showed that the IN-HATwt chimera expressed high levels of acetylated IN, while the IN-HATmut chimera was negative for acetylation (A. Allouch et al., manuscript in preparation).

In conclusion, these results demonstrate that IN expressed as a fusion product with the HAT domain of p300 is highly acetylated. In addition to bacteria, acetylated IN can be expressed in eukaryotic (yeast) cells.

Two-hybrid screening analysis using the IN-HATwt fusion protein

To identify the cellular factors interacting with acetylated IN, we used the GDBD-IN-HATwt as bait to screen a

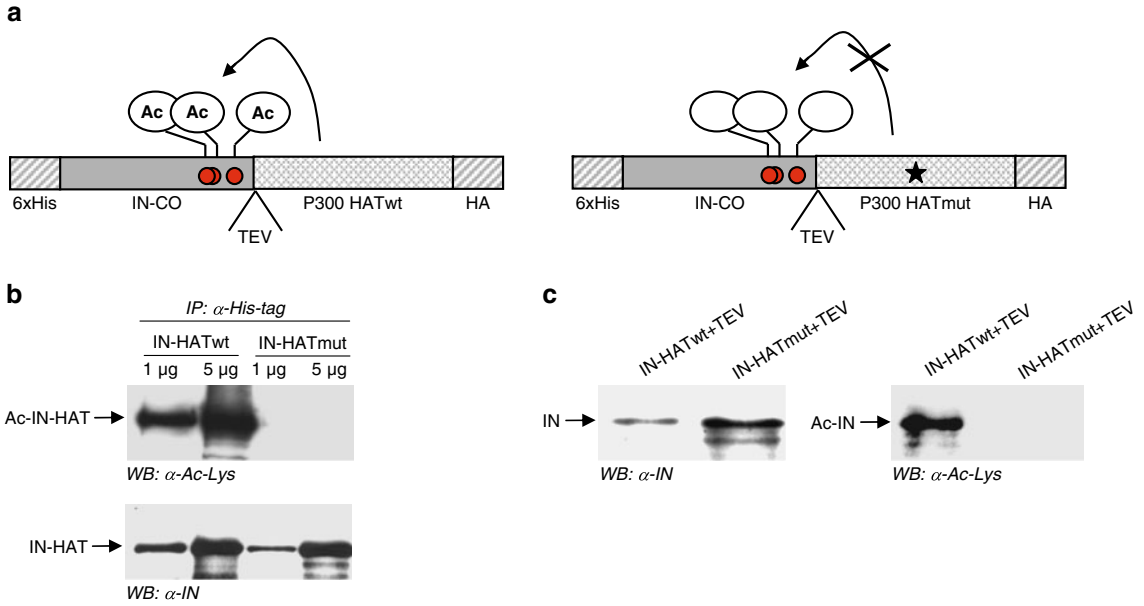

Fig. 1 Generation of in vitro and in vivo of highly acetylated HIV-1 IN. **a** Schematic representation of the constructs engineered to produce constitutively acetylated IN (IN-HATwt) or control unmodified IN (IN-HATmut). IN codon optimized (*CO*) was fused to HATwt or HATmut (D1395Y). The fusion proteins are tagged at N terminus with 6xHis and at C-terminus with HA. A TEV proteolytic cleavage site is introduced between IN and the HAT domains. **b** Two amounts (1 and 5 µg) of IN-HATwt and IN-HATmut recombinant proteins were immunoprecipitated (*IP*) by anti His-tag antibodies (*α-His*). Immunoprecipitates were analyzed by Western blot (*WB*) using anti-acetylated lysine antibodies (α-Ac-Lys) (*upper panel*) and the same filter was incubated with anti-IN antibodies (*α-IN*) (*lower panel*). **c** 6xHisIN separated from either IN-HATwt or IN-HATmut fusion proteins by TEV digestion was purified by affinity chromatography (see 'Materials and methods') and analyzed by WB using α IN (*left panel*) and αAc-Lys (*right panel*)

human lymphocytes cDNA library fused to the Gal4 activation domain (GAD). The two-hybrid screening was performed in the AH109 yeast strain. This strain contains *Ade* and *His* genes as reporters which allows the selection of positive clones using the selective medium (-Ade and -His). From almost 10.6×10^6-screened transformants, 754 were positive clones encoding thirteen cellular proteins which are listed in Table 1. According to their proprieties and proposed functions, these cellular factors could be divided in three categories:

1. Transcription regulatory and chromatin remodeling factors: LEDGF/p75, KAP1, BTF3b, THRAP3 and HMGN2.
2. Translation regulatory and RNA binding proteins: eIF3h, eEF1A-1 and hnRNPA2.
3. Nuclear import-export proteins: Exp2 and RanBP9.

In addition to factors grouped in categories, we also identified RPL23, a structural ribosomal subunit, STMN1, a factor involved in microtubule organization and finally CCDC32, a protein with still unknown functions.

These newly identified factors, were subsequently tested by two-hybrid analysis with the IN-HATwt chimera, as well as with each single domain (IN and HATwt), to verify their association properties with the acetylated or unmodified IN and also with the HAT domain contained in the chimera. In addition, the analysis was performed with the GDBD to check the bait specificity. Results shown in Table 2 indicate that all identified factors associate with IN as a separate domain and that the majority do not bind the HAT domain except for THRAP3, RanBP9, eEF1A-1, STMN1 and CCDC32.

In conclusion, thirteen new factors binding to acetylated IN have been identified. These factors positively interact also with IN separated from the HAT domain, suggesting that acetylation modulates but is not absolute requirement for virus cell interaction.

Binding of two-hybrid factors with acetylated and un-modified IN

To verify the interaction between IN and the cellular factors identified by two-hybrid screening (Table 1), pull down assays with the same factors expressed in human cells have been performed. Experiments were carried out with few selected factors based on their possible involvement in HIV-1 replication: transcription related proteins (BTF3b, THRAP3 and HMGN2) potentially involved in tethering viral integration in transcription units; a nuclear transport factor (Exp2) possibly involved in nuclear–cytoplasmic translocation. In addition, eIF3h, a factor involved in protein synthesis, was tested due to its high frequency of

Table 1 Cellular factors identified by yeast two-hybrid screening using constitutively acetylated IN (IN-HATwt) as bait

Protein names (number of isolated clones)	Proposed function	Complete residues: peptides retrieved	GenBank accession number	Reference
Lens epithelium-derived growth factor: LEDGF/p75 (6 clones)	Transcription coactivator. Factor interacting with lentiviral INs determining IN association to chromatin. Putative tethering factor for HIV-1 integration	530: 344–530	AF063020.1	Ge et al. (1998), Cherepanov et al. (2003)
Krüppel-associated protein 1: KAP1 (3 clones)	Transcription corepressor and DNA damage response factor. Factor inhibiting infectivity of MoMLV in embryonic cells	835: 304–835	HSU78773	Sripathy et al. (2006), Wolf and Goff (2007)
Basic transcription factor 3 isoform b: BTF3b (1 clone)	Component of the RNA polymerase II complex required for transcription initiation	162: 1–162	NM_001207.4	Zheng et al. (1990)
Thyroid hormone receptor protein 3: THRAP3 (1 clone)	Subunit of the large transcription mediator TRAP complex; positive regulator of RNA polymerase II promoters transcription	373: 1–361	BC054046.1	Rachez and Freedman (2001)
High-mobility group nucleosomal binding domain 2: HMGN2 (1 clone)	Component of the HMG non histone chromatin remodeling family of proteins. Inducer of chromatin decondensation and transcription activity	90: 1–90	BC014644.1	West (2004)
Ran-binding protein 9: RanBP9 (8 clones)	Ran binding protein involved nuclear transport pathway NLS mediated	729: 149–729	BC063849.1	Gorlich (1998)
Exportin 2 (synonyme: CAS): Exp2 (1 clone)	Importin α binding protein. Mediator of importin α nuclear export after NLS cargo release into the nucleus.	971: 244–971	BC108309.1	Kutay et al. (1997), Solsbacher et al. (1998)
Eukaryotic translation initiation factor 3 subunit H: eIF3h (726 clones)	Component of the eIF3 complex: promotes translation preinitiation complex formation, mRNA recruitment and scanning for AUG recognition in the ribosomes	352: 12–352; 172–352	BC000386.2	Hinnebusch (2006)
Elongation factor 1 alpha 1: eEF1A-1 (1 clone)	Component of the alpha subunit of EF1 complex: promotes protein biosynthesis by delivering aminoacylated tRNA to ribosomes. Binds HIV-1 matrix and nucleocapsid and stimulates HIV-1 transcription. Its yeast homologue binds HIV-1 IN	46: 268–462	BC082268.1	Calado et al. (2002)
Heterogenous nuclear ribonucleoprotein A2: hnRNPA2 (1 clone)	RNA binding protein containing two RNA recognition motifs (RRM): involved in mRNA regulation (splicing and trafficking). Regulator of HIV-1 RNA trafficking	341: 1–180	NM_002137.2	Shyu et al. (2000)
Stathmin 1: STMN1 (2 clones)	Tubulin binding protein: involved in microtubule depolymerization and signal transduction cascade	149: 1–149	BC082228.1	Cassimeris (2002)
Ribosomal protein L23: RPL23 (1 clone)	Structural component of 60S subunit of ribosomes. Activates p53 by inhibiting MDM2	140: 18–140	NM_000978.3	Berchtold and Berger (1991)
Coiled-coil domain containing 32 isoform1: CCDC32 (2 clones)	Unknown functions	185: 12–185	BC001673.2	Ota et al. (2004)

identification (726 clones) in the two-hybrid screening. These factors were fused to a Flag tag and expressed in HEK293T cells. The derived cell lysates were incubated with either IN-HATwt or IN-HATmut recombinant proteins. Subsequently, the immunocomplexes were recovered with anti-Flag antibodies and analyzed by Western blot with anti-HA and anti-Flag antibodies. As an experimental control for binding specificity the same analysis was performed using HEK293T cells expressing an unrelated control protein, luciferase, fused to the Flag tag (Flag-Luciferase). As shown in Fig. 2a and b higher amounts of IN-HATwt than IN-HATmut were found associated with Flag-Exp2 and Flag-elF3h. Conversely, similar amounts of IN-HATwt and IN-HATmut bound Flag-BTF3b, Flag-THRAP3 and Flag-HMGN2 (Fig. 2c, d, e). Finally, no specific binding was observed with the unrelated control protein, Flag-Luc (Fig. 2f). As shown in lower panels of Fig. 2a–f, incubation of the same filters with anti-Flag antibodies proved that similar amounts of Flag proteins were immunoprecipitated with either IN-HATwt or IN-HATmut. These results suggest that Exp2 and eIF3h bind with higher affinity the acetylated form of IN, whereas BTF3b, THRAP3 and HMGN2 show no preferential binding for either forms of the viral protein. In a separate

Table 2 Interactions in yeast between GAD prey proteins and GDBD hybrid baits (IN-HATwt, IN and HATwt)

	IN-HATwt	IN	HATwt	GDBD
LEDGF/p75	+	+	−	−
KAP1	+	+	−	−
BTF3b	+	+	−	−
THRAP3	+	+	+	−
HMGN2	+	+	−	−
Exp2	+	+	−	−
RanBP9	+	+	+	−
EIF3h	+	+	−	−
EEF1A-1	+	+	+	−
HnRNPA2	+	+	−	−
STMN1	+	+	+	−
RPL23	+	+	−	−
CCDC32	+	+	+	−

+ Indicates interaction, − indicates no interaction

report we show that another factor, KAP1, also identified by the two-hybrid screening (Table 1), preferentially binds acetylated IN (A. Allouch et al., manuscript in preparation).

Next, the interaction between the two-hybrid hits and the single HAT and IN domains was verified using lysates of HEK293T cells expressing the Flag-tagged factors. As shown in Fig. 3a, upper panel, no binding was observed with the HAT domain, even though high expression of two-hybrid Flag-tagged factors could be detected (lower panel). Thus, these data demonstrate that the interaction between IN and the two-hybrid hits is specific and not mediated by the HAT domain. Moreover, this result suggests that the positive interactions observed by two-hybrid analysis with the HAT domain (Table 2), were likely a result of the synergistic transactivation properties of these factors (THRAP3, RanBP9, eEF1A-1, STMN1 and CCDC32) together with the HAT domain over the yeast promoter.

Finally, the unmodified form of IN (6xHis-IN) was verified by pull down assays with Flag-tagged two-hybrid hits expressed in HEK293T cells. High levels of IN was found associated with BTF3b, HMGN2 and THRAP3 (Fig. 3b), while much lower amounts could be detected in complex with Exp2, eIF3h and KAP1.

Therefore, these data are in agreement with results in Fig. 2, showing that BTF3b, HMGN2 and THRAP3 bind efficiently unmodified IN and do not require IN acetylation. Conversely, the low amounts of Exp2, eIF3h and KAP1

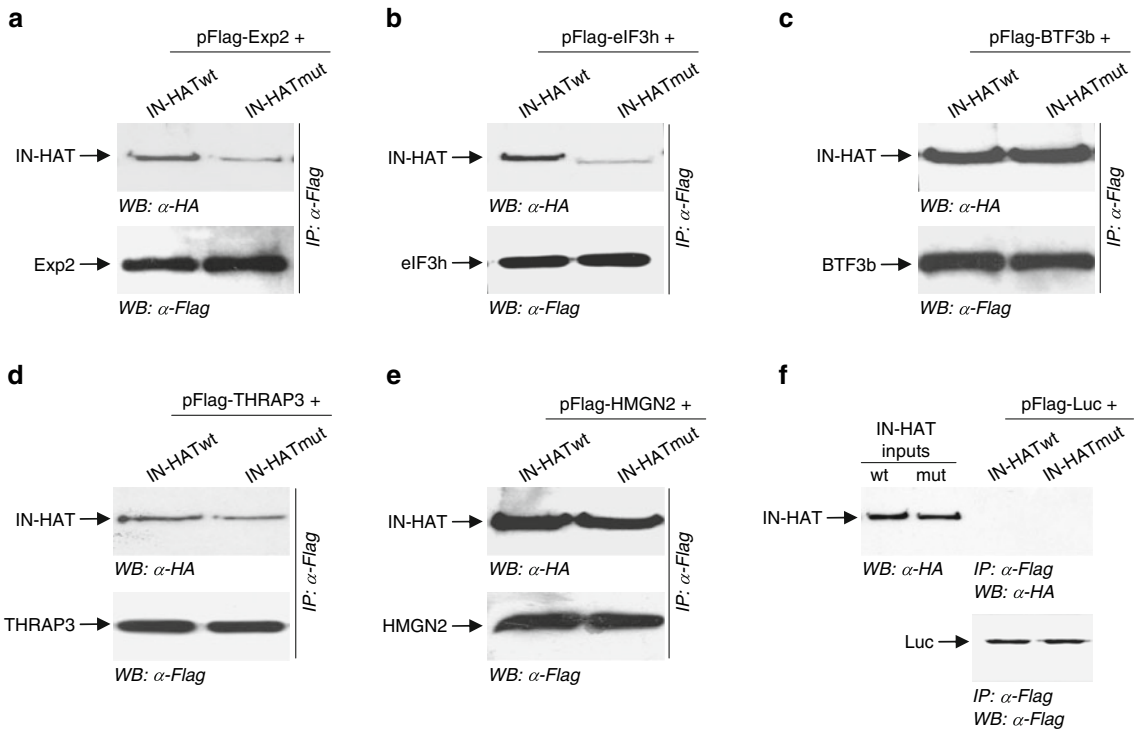

Fig. 2 Binding analysis between acetylated IN and proteins identified by the two-hybrid screening. **a** Lysates from HEK293T cells expressing Flag-Exp2 (**a**), Flag-eIF3h (**b**), Flag-BTF3b (**c**), Flag-THRAP3 (**d**), Flag-HMGN2 (**e**) and FLAG-Luc (**f**) were incubated with recombinant IN-HATwt or IN-HATmut-HA and immunoprecipitated (*IP*) with monoclonal anti-Flag antibodies. Immunoprecipitates were then analyzed by Western blot (*WB*) with anti-HA antibodies (*α-HA*) (*upper panels*) and with polyclonal anti-Flag antibodies (*α-Flag*) (*lower panels*)

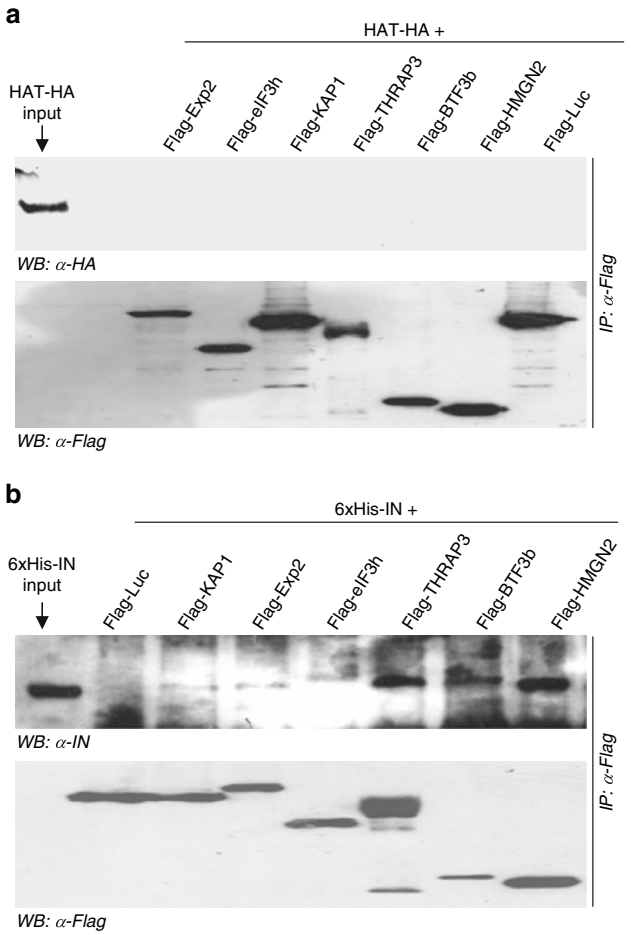

Fig. 3 Binding between proteins identified by the two-hybrid screening and the HATwt or IN domains. **a** HEK293T cell lysates expressing Flag-eIF3h, Flag-BTF3b, Flag-THRAP3, Flag-HMGN2 or FLAG-Luc were incubated with either HATwt (**a**) or 6xHis-IN (**b**) recombinant proteins and immunoprecipitated (*IP*) with monoclonal anti-Flag antibodies. Immunoprecipitates were then analyzed by Western blot (*WB*) with anti-HA antibodies (*α-HA*) (*upper panels*) and with polyclonal anti-Flag antibodies (*α-Flag*) (*lower panels*)

bound to IN is indicative that IN acetylation enhances binding affinity.

Discussion

This study exploits the tethered catalysis system (Guo et al. 2004) to produce an HIV-1 viral protein, IN, constitutively acetylated by p300. The construct verified to produce acetylated IN in bacteria, was used to screen by two-hybrid a human lymphocytes cDNA library. From this screening we have identified 13 cellular factors, 12 of which have never been reported to interact with HIV-1 IN. The binding analysis performed by two-hybrid and pull down assays revealed that a basal association of all factors was reported with the unmodified IN while the acetylation of the viral factor variably affect the affinity with the two-hybrid hits.

The newly identified factors interacting with acetylated or un-modified IN showed no obvious simple sequence similarity. Nevertheless, it is plausible that the IN recognizes common elements present in these proteins. In fact, these factors can be grouped in three categories based on their functional properties: (a) transcription regulatory and chromatin remodeling factors; (b) translation regulatory and RNA binding proteins; (c) nuclear import–export proteins.

Interestingly, LEDGF/p75, one of the factors identified in this screening, is one of the most described IN interactor required for efficient HIV-1 integration (Cherepanov et al. 2003; Engelman and Cherepanov 2008). This result validates the system of analysis employed in this study and proves that fusion of IN to the HAT domain does not significantly alter the IN structure. LEDGF/p75 binds the core catalytic domain of HIV-1 IN. Nevertheless, the N-terminal domain of IN was proven to enhance the binding affinity with this factor (Maertens et al. 2003). Therefore, the identification of LEDGF/p75 in our screening might suggest that also the C terminus of IN and its acetylation could affect IN/LEDGF-p75 interaction.

HIV-1 integration preferentially occurs in regions of the chromatin rich in genes transcriptionally active (Bushman et al. 2005; Mitchell et al. 2004; Schroder et al. 2002). Recent studies performed by sequence analysis using the ENCODE annotation (Wang et al. 2007) and by a visualization analysis (Albanese et al. 2008) demonstrated that HIV-1 targets decondensed regions of the chromatin. It has been hypothesized that cellular factors interacting with IN may tether the virus to appropriate sites for integration. Indeed, LEDGF/p75 knockdown and knockout cells show a significant reduction of integration frequency in transcription units (Ciuffi et al. 2005; Shun et al. 2007). Nevertheless, since in the absence of LEDGF/p75 the virus still does not integrate randomly in the genome, additional factors may be required for integration specificity. The screening reported in this study, uncovered factors involved in transcription and chromatin structure regulation, thus good candidate proteins to tether HIV-1 integration. BTF3b and THRAP3, found in the two-hybrid screening, are positive regulators of gene transcription that act by associating with RNA polymerase II (Rachez and Freedman 2001; Zheng et al. 1990). The HMNG2 protein, another two-hybrid hit, is involved in chromatin structure regulation by binding to nucleosomes in a DNA sequence independent manner. This factor induces chromatin decompaction, which in turn facilitates DNA transcription and replication (West 2004). In fact, HMNG2 was found to localize in active transcription chromatin regions (Bustin 2001; Hock et al. 1998). Finally, HMGA1, another HMG family member, was previously reported to stimulate HIV-1 integration by promoting the formation of IN/cDNA complexes (Hindmarsh et al. 1999; Li et al. 2000).

HIV-1 nuclear import occurs through still incompletely understood mechanisms. One of the viral factor hypothesized to be involved in nuclear translocation is the IN protein which contains several putative nuclear localization signals (NLSs) (Bouyac-Bertoia et al. 2001; Gallay et al. 1997; Hearps and Jans 2006). In our study, we identified two proteins that regulate the nuclear importin pathway Exp2 and RanBP9. These factors may trigger the nuclear import of IN by importin α and β complex. However, the implications of both importin factors have been explored for HIV-1 infectivity leading to contradictory results. Indeed, recent reports suggested that IN may lack a functional NLS and attributed its karyophilic proprieties to the LEDGF/p75 interacting factor (Devroe et al. 2003; Llano et al. 2004). More recently IN has been demonstrated to interact with a cellular factor, transportin SR2 (TNPO3) which mediates HIV-1 transport into the nucleus (Christ et al. 2008).

A recent two-hybrid screening performed with IN of another retrovirus, the Moloney Murine Leukemia Virus (MoMLV), identified the murine eIFs2, a subunit part of the translation initiation factor 3 (eIF3) complex (Studamire and Goff 2008). Moreover, in this study it was demonstrated that HIV-1 IN does not interact with eIFs2. The human eIF3f, another component of eIF3 complex, was reported to inhibit HIV-1 replication at post-integration step by interfering with the 3′ end processing of HIV-1 mRNAs (Valente et al. 2009). From our screening we identified eIF3h, another subunit belonging to the eIF3 complex, as a factor interacting with acetylated HIV-1 IN. Thus, since numerous studies report the association of retroviruses with the eIF3, this protein complex presumably plays an important function in the viral replication cycle, even though the detailed molecular mechanism has not yet been unraveled.

One of the two-hybrid hits was eEF1A-1 factor which has been previously reported to be involved in HIV-1 biology. Most interestingly, in agreement with our results, a former study using a yeast expression experimental system suggested that IN interacts with eEF1A-1 (Parissi et al. 2001). Moreover, eEF1A-1 was identified to be involved in HIV-1 replication by binding with the viral gag polyproteins (matrix and nucleocapsid) (Cimarelli and Luban 1999) and also by activating the viral promoter (Wu-Baer et al. 1996). All these reports implicate multi-roles of eEF1A-1 in HIV-1 replication cycle.

STMN1, identified in our two-hybrid screening, regulates microtubule organization by binding tightly tubulin and inducing microtubule destabilization (Cassimeris 2002; Howell et al. 1999). This observation is in line with a previous report showing that HIV-1 IN bind microtubule-associated proteins such as the yeast STU2p, a centrosomal protein, and Dyn2p (dynein light chain protein). It has been hypothesized that IN interaction with these factors may be responsible for IN nuclear import (de Soultrait et al. 2002; Desfarges et al. 2009).

In conclusion, here we report a list of cellular proteins that interact with acetylated HIV-1 IN. These are new potential factors involved in HIV-1 replication by either inhibiting or favoring the virus at specific steps involving IN activity. Further analyses are required to establish their role in HIV-1 biology.

Materials and methods

Vectors and constructs

pASK-IN-HATwt and pASK-IN-HATmut to express and purify IN-HATwt and IN-HATmut in bacteria were constructed by cloning IN codon optimized (CO) in frame with the HAT domain of p300 (a.a. 1195–1673) wild-type or mutated (D1395Y) in the pASK-IBA37 plus vector (IBA, Gottingen, Germany) containing at 5′ of the MCS a 6xHis tag. During the cloning procedure a 3′ HA tag and a TEV protease cleavage site between IN and HAT were introduced by PCR. HATwt-HA was cloned in pASK-IBA37 plus by PCR. PINSD-IN encoding for 6xHis-IN was described in (Cereseto et al. 2005). From the pASK-IBA37-IN-HATwt/mut vectors the IN-HATwt/mut, HATwt and IN were PCR amplified and cloned in frame with the GDBD in the pBD-Gal4 vector (Stratagene, La Jolla, CA, USA) for expression in yeast cells. KAP1, BTF3b, THRAP3, HMGN2, Exp2, eIF3h cDNAs were cloned by PCR in pFlag-CMV2 vectors starting from their truncated cDNAs isolated in the two-hybrid screening.

Yeast two-hybrid screening

A human T-lymphocytes cDNA library fused to Gal4 Activating Domain in a pACT vector (BD biosciences Clontech, Palo Alto, CA, USA) was expressed in AH109 yeast cells and screened with the pGDBD-IN-HATwt (bait) expression vector. Library transformation and screening were performed following manufacturer's instructions (Matchmaker GAL4 two-hybrid system 3). GAD identified prey proteins (Table 1) were co-expressed in AH109 yeast cells with GDBD fused to IN-HATwt, HATwt and IN to check for interactions.

IN-HATwt and IN-HATmut, IN and HATwt purifications

pASK-IN-HATwt/mut encoding for 6xHis-IN-HATwt/mut-HA, pASK-HATwt encoding for 6xHis-HATwt-HA and pINSD-IN encoding for 6xHis-IN were transformed in

E. coli BL21 competent cells (Stratagene, La Jolla, CA, USA). Induction of protein expression was performed using 43 mM anhydrotetracycline hydrochloride (AHT) (for pASK plasmids) or 0.5 mM IPTG (for pINSD-IN) for 4 h at 30°C. Bacteria culture was lysed in binding buffer (1 M NaCl, 20 mM Tris HCl pH 7.9 and 0.5% Triton X-100) containing 1 mM PMSF and protease inhibitor cocktail (Roche, Mannheim, Germany). TALON Metal Affinity Resin (BD Biosciences, Palo Alto, CA, USA) incubated for 2 h at 4°C was used to recover the 6xHis recombinant proteins. Following two washes in binding buffer containing 5 mM imidazole, proteins were eluted using binding buffer containing 200 mM imidazole and dialysed in buffer containing 150 mM NaCl, 50 mM Tris HCl pH 8, 10% glycerol and 0.5 mM EDTA. 6xHis-IN was dialyzed in 1 M NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT and 10% glycerol. For TEV digestion 20 μg of 6xHis-IN-HATwt/mut were incubated with 30 units of AcTEV protease (Invitrogen, Paisley, UK) in 50 mM Tris–HCl pH 8, 0.5 mM EDTA and 1 mM of DTT in 250 μl total volume. To recover His-IN from the digested product the TEV treated samples were adjusted to 1 M NaCl and incubated with Ni-NTA agarose resin (Qiagen, Hilden, Germany) for 2 h at 4°C. Following washes in binding buffer containing 5 mM imidazole, His-IN was eluted using the binding buffer containing 250 mM imidazole and dialysed in 1 M NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT and 10% glycerol. 6xHis-IN was dialysed in 1 M NaCl, 20 mM Tris–HCl pH 7.5, 1 mM DTT and 10% glycerol.

Antibodies

Primary antibodies for Western blot analyses were: monoclonal HIV-1 IN antibody (8G4) from NIH AIDS Research and Reference Reagent Program (Germantown, MD), polyclonal anti-acetylated lysines (Cell Signaling Technology, Beverly, MA, USA), polyclonal anti-HA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), polyclonal anti-Flag (Sigma-Aldrich, St Louis, MO, USA) and monoclonal anti-His (Qiagen, Hilden, Germany). Secondary antibodies HRP conjugated anti-mouse or anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, YSA).

Immunoprecipitations and anti-Flag beads pull down

A total of 1 and 5 μg of either purified and dialyzed 6xHis-IN-HATwt-HA or 6x His-IN-HATmut-HA proteins were incubated with 1 mg of monoclonal anti-His antibody (Qiagen, Hilden, Germany) for 1 h and then 20 μl of Protein G UltraLink resin (Pierce Biotechnology, Rockford, IL, USA) were added for additional 1 h. Immunocomplexes were washed three times in buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 8, 10% glycerol and 0.5 mM EDTA and analyzed by Western blot. For Flag pull down experiments HEK293T expressing Flag proteins (KAP1, BTF3b, THRAP3, HMGN2, Exp2 and eIF3h) were lysed in 50 mM Hepes pH 7.4, 150 mM NaCl and 0.5% NP-40. A total of 250 μg lysate was mixed with 250 ng of recombinant and purified proteins (6xHis-IN-HATwt-HA or 6xHis-IN-HATmutHA, 6xHis-HATwt-HA or 6xHis-IN) together with 5 μM of Lys-CoA (synthesized at the ICGEB Peptide Synthesis Core Faculty, Trieste, Italy). Following 1 h incubation at 4°C, 20 μl of monoclonal anti-Flag M2 antibodies immobilized on agarose beads (Sigma, St Louis, MO, USA) were added and incubated for 1 h. Following washes in lysis buffer samples were analyzed by Western blot using polyclonal anti-HA or monoclonal anti-IN and polyclonal anti-Flag antibodies.

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