Published in final edited form as:
J Immunol. 2013 December 15; 191(12): 6030–6039. doi:10.4049/jimmunol.1302057.

GANP interacts with APOBEC3G and facilitates its encapsidation into the virions to reduce HIV-1 infectivity

Kazuhiko Maeda[#*], **Sarah Ameen Almofty**[#*], **Shailendra Kumar Singh**[*], **Mohammed Mansour Abbas Eid**[*], **Mayuko Shimoda**[*], **Terumasa Ikeda**[†,2], **Atsushi Koito**[†], **Phuong Pham**[‡], **Myron F. Goodman**[‡], and **Nobuo Sakaguchi**[*]

[*] Department of Immunology, Graduate School of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan

[†] Department of Retrovirology and Self-Defense, Graduate School of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan

[‡] Departments of Biological Sciences and Chemistry, University of Southern California, Los Angeles, CA 90089-2910

[#] These authors contributed equally to this work.

Abstract

The single-stranded DNA-dependent deoxycytidine deaminase APOBEC3G (A3G) is a potent restrictive factor against HIV-1 virus lacking viral-encoded infectivity factor (Vif) in $CD4^+$ T cells. A3G antiretroviral activity requires its encapsulation into HIV-1 virions. Here we show that germinal center-associated nuclear protein (GANP) is induced in activated $CD4^+$ T cells and physically interacts with A3G. Overexpression of GANP augments the A3G encapsidation into the virion-like particles and ΔVif HIV-1 virions. GANP is encapsidated in HIV-1 virion and modulates A3G packaging into the cores together with cellular RNAs including *7SL* RNA, and with unspliced *HIV-1* genomic RNA. GANP upregulation leads to a significant increase in A3G-catalyzed G→A hypermutation in the viral genome and suppression of HIV-1 infectivity in a single-round viral infection assay. Conversely, GANP knockdown caused a marked increase in HIV-1 infectivity in a multiple rounds infection assay. The data suggest that GANP is a cellular factor that facilitates A3G encapsidation into HIV-1 virions to inhibit the viral infectivity.

Keywords

GANP; hypermutation; HIV-1; APOBEC3G

To whom correspondence should be addressed: Prof. Nobuo Sakaguchi, Department of Immunology, Graduate School of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan, Tel.: +81-96-373-5134; Fax: +81-96-373-5138; nobusaka@kumamoto-u.ac.jp.
[2]Current address: Department of Biochemistry, Molecular Biology & Biophysics, University of Minnesota

Disclosures
The authors have no conflicting financial interests.

Introduction

Activation-induced cytidine deaminase (AID)/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) protein family is composed of eleven cytidine deaminases that catalyze C→U on ssDNA or RNA to regulate Ab diversification during adaptive immune response and to provide innate resistance against retroviruses and exogenously introduced genetic materials (1-3). AID has been well characterized as a critical initiator of Ig somatic hypermutation (SHM) and class switch recombination (CSR) in germinal center (GC) B cells during the immune responses to T cell-dependent Ags (4-8). AID initiates SHM and CRS by catalyzing C→U conversion at actively transcribed *V-region* and *S-region* of *Ig* genes (4-8). In contrast to AID that acts preferentially at *Ig* loci in B cell genomic DNA, proteins of a subgroup APOBEC3 (A, B, C, DE, F, G, H) primarily target retroviral cDNA in the cytoplasm to cause lethal hypermutation in viral genomes (9). In particular, APOBEC3G (A3G) possesses a potent antiretroviral activity that restricts HIV-1 replication in T cells in the absence of viral infectivity factor, Vif (10). A3G antiviral activity requires its encapsidation into the HIV-1 virions. Upon entry into the secondary infected target cells, A3G catalyzes deamination of C→U, preferentially at many 5'-YCC (Y = C or T) motifs located on the nascent minus-strand cDNA reverse transcribed from *HIV-1* genomic RNA (gRNA) (9). Subsequently, this creates G→A hypermutation in HIV-1 genome that potentially inactivates essential genes required for infectivity in the absence of Vif (11). Although Vif has been shown to inhibit A3G translation and promote A3G degradation through the core binding factor β-mediated proteosomal degradation (12, 13), the action of Vif is not absolute and a few copies of A3G are thought to be encapsidated into virions (14), probably through interaction with *HIV-1* gRNA (15, 16) and/or a number of cellular RNAs, including *7SL* RNA (17-19). A3G-catalyzed G→A hypermutated HIV-1 genomes have been isolated from blood cells of HIV-1 infected patients at different stages of infection (20, 21). A3G encapsidation into HIV-1 virions requires interactions with the nucleocapside (NC) domain of a viral Gag protein, suggesting that incorporation of A3G into HIV virions occurs during viral assembly (18, 22-24). In T cells, A3G is present in RNase-sensitive ribonucleoprotein (RNP) complexes localized in the cytoplasm (25-27) and enriched at mRNA processing bodies (26-31). Cellular proteins interacted with the RNP complex might also be important for A3G encapsidation. However, there is a little knowledge about the molecular mechanism and host cellular proteins responsible for A3G encapsidation into HIV-1 virions (32, 33).

GC-associated nuclear protein (GANP) that was discovered as a protein upregulated in GC B cells during immune responses is physically associated with AID through its carboxyl-terminal side region (34). GANP is a component of transcription and export complex 2 (TREX-2) interacted with RNP complexes and involving in mRNA export in mammals (35, 36). GANP mutant mice studies have shown a strong correlation between the levels of GANP expression and SHM at the rearranged *IgV-loci*. B cell-specific *GANP*-transgenic mice augment the generation of high-affinity Abs against the immunized Ags *in vivo* (37) suggesting that GANP is an important functional AID partner in generation of high-affinity Abs in GC B cells. Recently, we have shown that GANP facilitates AID recruitment from the cytoplasm to the nucleus (38). GANP also augments AID targeting to the rearranged

IgV-region through interaction with many proteins composed of RNP complex and regulation of chromatin modification for nucleosome occupancy at the selective *IgV-region* site (39).

Given the similarity among AID/APOBEC proteins, we have explored a possibility that GANP interacts with A3G to regulate its localization in HIV-1 virions. Here, we showed that GANP is a cellular protein that is upregulated in $CD4^+$ T cells and physically interacts with A3G to facilitate its targeted encapsidation into the HIV-1 virion.

Material and Methods

Antibodies

Following antibodies (Abs) were purchased: β-actin (AC-15), HA-7 and FLAG (M2) from Sigma-Aldrich; HA (ab9110), and RNase A (ab6611) from Abcam; HA (C29F), calnexin (#2433) and β-tubulin (9F3) from Cell Signaling Technology; mouse IgG (sc-2025), rabbit IgG (sc-2027), and GANP (sc-83297) from Santa Cruz Biotechnology; GANP (11054-AP) from ProteinTech. Abs for Gag p24 (VAK4) (40) and Gag p17 (LG20-13-15) (41) were used. Anti-A3G (#9968) serum was provided from the National Institutes of Health AIDS Research and Reference Reagent Program. Allophycocyanin-conjugated anti-human CD4 (Biolegend), FITC-conjugated anti-CD69 (BD Biosciences), and PE-conjugated anti-CD25 (Immunotech) Abs were used for FACS staining.

T cell activation

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers using a protocol approved by the ethics committee of Kumamoto University Faculty of Life Sciences. T cells were purified using a Pan T-cell isolation kit II and an automatic magnetic cell sorter (autoMACS™) (Miltenyi Biotec). $CD4^+$ T cells (1×10^6) purified using CD4 MicroBeads were activated with 40 IU/ml IL-2 (Genzyme) + 5 μg/ml PHA, 50 ng/ml PMA (Sigma-Aldrich), or with CD3/CD28 Dynabeads (4×10^7 beads/ml) (Invitrogen) + IL-2, then stained with anti-CD4, anti-CD69, and anti-CD25 for purification by FACSAria II cell sorter (BD Biosciences).

Cell culture

The 293T cells were maintained in DMEM. RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Invitrogen) was used for culturing H9 T cells and Ramos B cells.

RNA purification, cDNA synthesis, and quantitative real-time PCR analysis

Total RNA was isolated using an RNeasy Mini Kit (Qiagen) from cell lines. To measure the amount of cellular RNAs in cell-free virions, the viral RNA was isolated using QIAamp viral RNA Mini Kit (Qiagen). Gene expression was calculated and normalized to the amount of *gapdh* expression in cells and of HIV-1 *5'-LTR* region of virions. RNA was reverse transcribed using a SuperScript III First-Strand Synthesis System (Invitrogen), and quantitative real-time PCR (qRT-PCR) was performed using MESA BLUE qPCR MasterMix Plus (Eurogentec) on the Applied Biosystems 7500 (Applied Biosystems). Gene-specific primers used in this study are listed in Supplemental Table 1.

Plasmids

FLAG-GANP (38), A3G-HA (42), untagged A3G (43), wild-type (WT) and ΔVif pNL4-3LucE-R- (42), and WT pNL4-3 proviral vectors were used.

Transfection and cell lysate preparation, immunoprecipitation (IP) and western blotting (WB)

Cells were transfected with FuGENE HD transfection reagent (Roche Diagnostics). 48 h after transfection, cells were harvested and lysed using in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% TritonX-100; pH 7.5) with a protease inhibitor cocktail (Nacalai Tesque). IP and WB were carried as described previously (38). In some experiments, cellular and viral RNAs in cell lysates were removed by a treatment with 25 μg/ml RNase A for 30 min at 37°C before IP.

Viral preparation

Virus-like particles (VLPs) were produced from HIV-1 Gag mutant constructs (44). Vesicular stomatitis virus (VSV)-G pseudotyped HIV-1 and ΔVif HIV-1 virions with a luciferase reporter were produced from WT pNL4-3 and ΔVif pNL4-3 in 293T. For the multiple rounds infection assay, Vif proficient env-intact pNL4-3 vector was used. VLPs were produced from HIV-1 Gag mutant constructs. Virus particles or VLPs were harvested from the culture supernatant and purified by centrifugation on 20% sucrose cushion at 35,000 × g for 1 h. Relative amounts of HIV-1 virions in viral preparations were calculated based on p24 titer by ELISA (HIV-1 p24 antigen ELISA kit, ZeptoMetrix).

Fractionation of HIV-1 virion cores

HIV-1 virions were fractionated as reported by Soros et al. (45) with some modification. The virus pellet was treated with the hypotonic buffer containing 0.25% Triton X-100 (TX-100 buffer) for 2 min at 25°C and spun at 14,000 rpm for 8 min at 4°C to collect the supernatant (first sup). The insoluble pellet was incubated with the TX-100 buffer for 5 min, centrifuged at 14,000 rpm for 8 min at 4°C to separate into the second supernatant (second sup) and the final pellet that was enriched for virion cores.

Virion immunoprecipitation (Virion-IP)

Detection of the protein-bound RNA in virion was performed either with anti-HA (for A3G) or anti-FLAG (for GANP) by modification of the RNA chromatin IP assay (38, 46). The virion amount was adjusted with p24 and fixed with 1% of formaldehyde and cross-linked with 0.25 M glycine (pH 7.0) to preserve the RNA-protein interaction, then washed and centrifuged at 16,000 rpm. Virions were cross-linked and lysed for the IP of RNA-protein complex by the method described previously (46). A3G-bound or GANP-bound RNAs were detected by qRT-PCR using primers designed for various regions of *HIV-1* gRNA listed in Supplemental Table 1.

Mutation analysis in HIV-1 proviral DNA

Genomic DNA was isolated from cells at 48 h post-infection. A 408 bp region of HIV-1 *pol* was amplified using the high-fidelity PrimeSTAR HS DNA polymerase (Takara Bio Inc.)

and subsequently digested with *Dpn*I to remove the plasmid DNA used for transfection. PCR products were purified by agarose gel electrophoresis and ligated to the pCR4 Zero Blunt TOPO sequence vector (Invitrogen) to be sequenced using ABI3130 Genetic Analyzer with BigDye Terminator (Applied Biosystems).

siRNA treatment

293T cells (2.5×10^5 cells/well) in OPTI-MEM GlutaMAX medium were transfected with 10 nM of siRNA using lipofectamine RNAiMAX (Invitrogen) transfection reagent. H9 cells (5×10^5 cells) were transfected with 2 μM of siRNA using the Amaxa Nucleofection Kit V (program A-30) according to the manufacturer's protocol. All siRNA against GANP and negative control SIC were obtained from Sigma. The duplex sense sequence of siGANP-A and siGANP-B was CCUGUAUCAUCUGCGGUUUTT and GCAUUGAACAGUAAUAAUUTT, respectively.

Infection assays

In single-round infectivity assay, 293T cells (1.5×10^5), activated $CD4^+$ primary T cells (5×10^5), and H9 cells (5×10^5) were infected with similar amount (2 ng of p24-equivalent) of ΔVif HIV-1 viruses and cultured for 48 h. Cells were lysed and assayed for luciferase activity as described previously (42). For multiple rounds infections assay, H9 targeted cells (5×10^5) were infected with WT HIV-1 viruses (20 ng of p24-equivalent) in the presence of polybrene (8 μg per ml). After 3 h, cells were washed and cultured in RPMI medium. Multiple rounds infection of HIV-1 was monitored as p24 production in the culture supernatant at 5, 7, 10 and 12 d after infection.

Statistical analysis

Differences were analyzed statistically by the unpaired two-tailed Student's *t*-test for significant values of $P < 0.05$.

Results

A3G interacts with GANP in T cells

We examined the expression of GANP and A3G in $CD4^+$ T cells from PBMC after stimulation with PMA, PHA + IL-2, or anti-CD3/CD28 beads + IL-2 for 3 days *in vitro* (Fig. 1A). A3G mRNA expression is increased in stimulated T cells (47). *A3G* and *ganp* mRNAs were increased by stimulation with anti-CD3/CD28 beads + IL-2; a 6.4-fold increase for *A3G* and 19.2-fold increase for *ganp* after stimulation (Fig. 1A). Expression of A3G and GANP was examined by WB in cell lines (293T, Ramos, and H9) (Fig. 1B). A3G and GANP were expressed at a higher level in H9 T cell and Ramos B cell lines (Fig. 1B, 1C) in whole cell lysates (WCLs). Next, we examined whether GANP interacts with A3G as a complex for efficient targeting. FLAG-tagged GANP (FLAG-GANP) and A3G tagged-HA (A3G-HA) expression vectors were used to transfect 293T cells. WCLs were further IP with anti-FLAG Ab and analyzed using anti-HA Ab. A3G-HA was present in the IP precipitate with FLAG-GANP (Fig. 1D). A similar IP experiment using anti-GANP and anti-A3G Abs showed co-IP of the endogenous A3G and GANP in H9 cells and $CD4^+$ T cells from PBMC (Fig. 1E). These results show that A3G interacts with GANP in T cells.

GANP enhances A3G encapsidation in VLPs

The Gag protein alone is sufficient for A3G packaging into VLPs, thus, we examined GANP incorporation into VLPs with WT and a series of myristoylated Gag mutants (42) (Fig. 2A). As previously shown, A3G is encapsidated into WT Gag, δ10-110 and δ10-277, but not into Zwt-p6 (Fig. 2B, left panel; A3G-HA). We found that GANP alone is encapsidated in WT Gag, mutant Gag δ10-110 or Zwt-p6 although with less efficiency, but not in δ10-277 (Fig. 2B, left panel; FLAG-GANP). These data indicate that different regions of Gag are required for A3G and GANP encapsidation in VLPs. Encapsidation of GANP requires the capsid region, whereas A3G encapsidation requires the NC region. No A3G was observed in the Zwt-p6 VLPs without GANP (Fig. 2B, A3G-HA). Notably, A3G can be encapsidated into the Zwt-p6 VLPs with co-expressed GANP (Fig. 2B, right panel). These data suggest that GANP may actively mediate a NC-independent A3G encapsidation into Zwt-p6 VLPs in the absence of *HIV-1* gRNA.

GANP is encapsidated in the HIV-1 virion core fraction accompanying A3G

Next we examined whether GANP is assembled and encapsidated into virions. Viruses were produced from 293T cells using HIV-1 proviral vector pNL4-3LucE-R- (WT pNL4-3) and Vif-deficient variant (ΔVif pNL4-3) pseudotyped with VSV-G envelope (42). Virions from 293T cells transfected with pNL4-3 and FLAG-GANP, or with Mock (empty vector), were subjected to WB analysis to determine the amount of GANP incorporation after normalization to p24 protein content. FLAG-GANP was detected in both WT and ΔVif HIV-1 virions, but the level of incorporated FLAG-GANP in WT virions was slightly less compared with the level of FLAG-GANP in ΔVif HIV-1 virions (Fig. 3A). The absence of cellular organelles in virion preparations was confirmed by WB against calnexin, an endoplasmic reticulum membrane marker protein. The data clearly indicated that GANP is encapsidated into HIV-1 virions and suggest that Vif protein inhibits not only A3G but also GANP encapsidation. The influence of A3G and GANP on their mutual encapsidation into ΔVif HIV-1 virions was examined. Encapsidated A3G and GANP were quantified by WB from purified ΔVif HIV-1 virions produced in 293T cells transfected with ΔVif pNL4-3, FLAG-GANP, and A3G (0, 2, 10 or 50 ng). FLAG-GANP was detected in all virion preparations and was not affected by the expression of A3G (Fig. 3B), indicating that A3G does not affect GANP encapsidation in the absence of Vif.

We next examined whether GANP affects the incorporation of A3G into HIV-1 virion compartments. First, ΔVif HIV-1 virions were produced from the 293T cells transfected with ΔVif pNL4-3, A3G-HA, and FLAG-GANP or a Mock (empty vector) control. Virions were subjected to fractionation to determine whether A3G and GANP are located in the same virion compartments. FLAG-GANP was detected solely in the virion pellet fraction, presumably composed of virion cores (Fig. 3C). Thus, GANP is incorporated exclusively into the virion cores, but not in any other virion compartment (Fig. 3C). In contrast, A3G was present in all three virion fractions, that is consistent with earlier studies (16, 45). In the absence of FLAG-GANP, the majority of encapsidated A3G was present in supernatant fractions, whereas approximately 10% of the A3G was present in the pellet. FLAG-GANP expression markedly increased (2.5-fold) the amount of A3G in the pellet core fraction (Fig. 3D). In contrast, A3G levels in non-core matrix fractions (1st sup and 2nd sup) decreased 3-

and 10-fold, respectively (Fig. 3D). GANP overexpression led to a significant increase in A3G encapsidation into the virion pellet fraction while reducing A3G incorporation in the HIV-1 virion outside core region (Fig. 3D).

GANP binds to HIV-1 gRNA

A modified RNA chromatin IP assay, designated virion-IP, was carried out to examine whether encapsidated GANP and A3G associate with *HIV-1* gRNA. GANP and A3G were found to associate with *HIV-1* gRNA within virions (Fig. 4A). The virion-IP assay suggested that both A3G and GANP associated with *HIV-1* gRNA inside the virion core (Fig. 4B). Using the region-specific primers for qRT-PCR for GANP-bound fragments, we probed GANP binding to different regions of *HIV-1* gRNA (Fig. 4C). The data showed that GANP can associate with all regions spanning the entire *HIV-1* gRNA (H1 to H6), but has a significantly higher binding to the H5 region where the Rev-responsive element is located (Fig. 4D). In virions encapsidated with both GANP and A3G, the A3G binding preference was at H1 (SL1; stem-loop 1) and H5 regions of *HIV-1* gRNA (Fig. 4E).

GANP modulates cellular RNA incorporation with A3G

A3G encapsidation can either increase or decrease the encapsidation of cellular RNAs (15, 17, 48, 49). Thus, we investigated whether GANP, A3G or GANP + A3G are able to mediate the packaging of various cellular RNA molecules into virions. A3G alone reduced the encapsidation of U4, U5, U6, and hY5, but did not cause any change in *7SL* RNA encapsidation (Fig. 5A, gray bars). NC-independent GANP packaging in virions was consistent with observed changes in encapsidation of cellular RNAs in FLAG-GANP (Fig. 5B). GANP alone had small, but significant effects on the encapsidation of U2, U4, and U6 cellular RNAs, but not on encapsidation of cellular RNAs, hY1, hY3, hY4 and hY5 (Fig. 5B). A3G and GANP co-expression significantly reduced U2, U4, U6, and hY4, but still maintained *7SL* RNA in virions (Fig. 5B, white bars), implying that A3G and GANP cooperate in the assembly of *HIV-1* gRNA together with the preferential cellular RNAs, such as *7SL* RNA, for their efficient encapsidation (17, 48). A cleaved form of *7SL* RNA, known as S-domain fragment with several cleavage sites, was found in Zwt-p6 VLPs (Fig. 5C) (50). Virion-IP analysis showed that A3G and GANP have a significant binding to *7SL* RNA in virions (Fig. 5D). In the presence of A3G, GANP reduced the level of cleaved S-domain of *7SL* RNA in Zwt-p6 VLPs (Fig. 5E), suggesting that GANP-mediated packaging of A3G is initiated through GANP binding and protection of *HIV-1* gRNA and cellular *7SL* RNA.

GANP enhances A3G-mediated anti-HIV-1 activity

The ΔVif HIV-1 virions were first generated from 293T cells in the presence of A3G and FLAG-GANP or A3G and Mock (empty vector) control, and then added for secondary infection against 293T cells. Virions subsequently produced from the secondary infected 293T cells were subjected to mutational analysis of the HIV-1 *pol* gene. Consistent with previous reports (42), a high frequency of G→A hypermutation (2.08×10^{-3}) was observed at the *pol* gene when viruses were produced with A3G alone (A3G + Mock) (Fig. 6A). In the presence of FLAG-GANP, A3G-catalyzed G→A mutations were significantly increased

(89%) in the same region (Fig. 6A). The G→A mutations occurred preferentially at A3G hotspot motifs GG→AG (Fig. 6B; flags). Thus, GANP overexpression that increases A3G encapsidation into virion cores enhances C→U deamination, resulting in accumulation of G→A mutations in the genome of ΔVif HIV-1.

The effect of GANP overexpression on A3G-mediated anti-HIV-1 activity was examined using a single-round infectivity assay (42). The ΔVif HIV-1 virions were isolated from culture supernatant of 293T cells transfected with ΔVif pNL4-3, A3G, and FLAG-GANP (Fig. 6C, left panel). The effect of GANP on viral infectivity was comparable to 293T cells with the Mock (empty vector) control in the absence of A3G (Fig. 6C, right graphs). The presence of A3G significantly decreased the viral infectivity in a dose-dependent manner, up to an approximate 10-fold reduction (50 ng A3G). We observed the similar effect of GANP overexpression upon viral infectivity in primary $CD4^+$ T cells. Overexpression of GANP resulted in further reduction of viral infectivity at every A3G transfection dosage, even when the amount of A3G was minimal.

GANP is ubiquitously expressed and an essential protein for cell survival and normal development (34). To confirm GANP effect on A3G encapsidation and anti-HIV-1 activity, we carried out the single round infection assay for ΔVif HIV-1 produced from endogenous GANP-knockdown cells. Two different siRNAs targeting distinct regions of the *ganp* gene severely reduced endogenous GANP expression in 293T cells respectively (Fig. 6D). The ΔVif HIV-1 virions were generated from the 293T siControl and GANP-knockdown cells after co-transfection with ΔVif pNL4-3 and A3G-HA (Fig. 6D, left panel). As expected, downregulation of endogenous GANP markedly suppressed A3G-encapsidation in ΔVif HIV-1 virions (Fig. 6D, blot data). The reduction in A3G-encapsidation into the virion core coincided with a higher infectivity of ΔVif HIV-1 virions prepared from GANP-knockdown cells compared with siControl. The results showed the similar increase of HIV-1 infectivity against H9 cells and primary $CD4^+$ T cells in a single-round infection assay with VSV-G envelope (Fig. 6D, graphs).

GANP is important for restriction of WT Vif-proficient HIV-virus

The potential role of GANP in restriction of HIV-1 infectivity was further studied using a multiple rounds infection assay in H9 cell line that expresses endogenous GANP and A3G. The level of endogenous GANP from 2 to 4 days post-transfection in siGANP-treated H9 cells was approximately 2-fold lower compared to the siControl H9 cells (Fig. 7A). WT Vif-proficient HIV-1 viruses were produced in siGANP-treated or siControl-treated 293T cells. Multiple rounds infection was performed in GANP-knockdown H9 cells as the target cells to examine infectivity of the WT HIV-1 virions with the intact envelope (Fig. 7B). Knockdown of GANP in both virus-producing and target cells showed a dramatic increase (50-fold) in WT HIV-1 production as monitored by the p24 protein levels at day 12 in GANP-knockdown compared to the control cells (Fig. 7B). These data suggest that HIV-1 virions produced in cells with a limited amount of GANP exhibited a significantly higher infectivity compared to HIV-1 virions produced in cells expressing a high level of GANP.

Discussion

HIV-1 infected patients are now treated with a highly active antiretroviral therapy. HIV-1 viral load and the disease progression are controlled by the immune responsiveness of the infected individuals and balance with the production of serum Abs and generation of $CD8^+$ CTLs. Innate and adaptive immune responses to HIV-1 infection are complex and involve multiple viral and host factors (51). A3G is a host cellular factor that has been shown to inactivate HIV-1 virus lacking Vif (10). For the WT HIV-1 virus, the antiviral activity of A3G might not be sufficient to shut down the viral infection at the initial infection stage because the Vif protein inhibits expression and causes degradation of A3G in the infected cells. Vif-mediated inhibition of A3G in HIV-1 infected cells, however, is not complete. A3G action on HIV-1 genomes is readily observed in patients at different stages of HIV-1 infection (20, 21). In addition to direct action on HIV-1 replication, A3G has been shown to enhance the ability of the immune system to recognize HIV-1 infected cells and to activate of HIV-1 specific $CD8^+$ CTLs (52). A3G mutator activity produces defective HIV-1 proviruses expressing truncated or misfolded viral proteins, which can be served as the pool of MHC-I restricted HIV-1 Ags (52). Thus, A3G-mediated C→U conversions on HIV-1 cDNA play an important role to evoke the immunogenicity of HIV-infected cells and CTL activation.

A3G encapsidation in HIV-1 virions is required for its antiviral activity. When expressed at a low level, active A3G is packaged primarily in HIV-1 virion cores (45). Only those A3G molecules packaged into the virion cores and that associate with viral gRNA are “biologically active”, i. e. having an ability to restrict HIV-1 infectivity (14, 16, 45). GANP is upregulated in activated $CD4^+$ T cells (Fig. 1) and GANP overexpression caused a higher level of encapsidated active A3G in virions (Fig. 3C, 3D) leading to a marked elevation of G→A hypermutation (Fig. 6A, 6B). Consistent with active A3G encapsidation in virions, ΔVif HIV-1 virions produced in 293T with overexpressed GANP exhibited significantly lower infectivity compared with the control cells (Fig. 6C). Conversely, HIV-1 virions produced from GANP downregulated 293T or H9 appeared to have considerably higher infectivity, compared with the virions produced in control cells, in either single-round or multiple rounds infection assay (Fig. 6D and Fig. 7B). A significant increase in infectivity of WT HIV-1 virions propagated in GANP-knockdown H9 T-cells in multiple rounds infection assay (Fig. 7) suggests that GANP has an effect on the packaging of a very low level of A3G present in T cells infected with Vif-proficient HIV-1. Overall, our data showing that GANP promotes A3G packaging into virion (Fig. 3D) and enhances A3G antiviral activity indicate a key role of GANP in recruiting A3G to its physiological targets, thereby leading to observed G→A hypermutation of *HIV-1* genome.

GANP alters the location of encapsidated A3G inside the ΔVif HIV-1 virions. Although A3G is packaged both in the virion core and non-core fractions (45), we have determined that GANP promotes A3G-encapsidation into the virion core compartment (Fig. 3C, 3D), presumably through the stable association with *HIV-1* gRNA (Fig. 4A, 4B). Co-expression of GANP and A3G depressed encapsidation of cellular RNAs, except *7SL* RNA (Fig. 5A), suggesting that cellular RNA-mediated packaging of A3G into virions appears to be non-specific and is inhibited by GANP. GANP-mediated localization of A3G into the virion

cores is likely to occur during maturation of the virus particles, when the appearance of Gag and Gag-Pol cleaved proteins leads to structural rearrangements and formation of the virion particles. Since GANP itself is incorporated into the virion, it may recruit A3G into the virion through interaction and with the RNP complex containing *HIV-1* gRNA. Thus, GANP-facilitated A3G encapsidation into virion represents an additional pathway of packaging A3G into the virion, which was supported by a previous report showing that A3G incorporation into the virion core can be mediated through its interaction with *HIV-1* gRNA or *7SL* RNA (50).

GANP interacts with A3G (Fig. 1) and with AID, another member of APOBEC family of cytidine deaminases (38). In B cells, AID localizes primarily in the cytoplasm, but acts on genomic DNA in the nucleus. GANP facilitated recruitment of AID into the nucleus and targeting AID to the *IgV-region* to initiates SHM (38, 39). A3G, however, appears exclusively in the cytoplasm and is recruited to HIV-1 virions during the assembly and maturation of the viral particles. Our study here reveals an important regulatory role for GANP as a key cellular factor that regulates A3G packaging into the HIV-1 virion. Thus, GANP interacts with both cytidine deaminases, AID and A3G, and helps to facilitate their targeting the intended physiological targets *in vivo*.

GANP's effect on A3G encapsidation into the virion consequently leading to G→A hypermutation of the viral genome might have a potential use in HIV-1 vaccine development. Co-expression of GANP and A3G in HIV-1 infected cells causes an increase in production of defective HIV-1 mutant proviruses expressing truncated, hypermutated or misfolded viral proteins. These hypermutated proviruses do not produce infectious particles but generate a sustained pool of “natural” occurring inactive HIV-1 variants. With a good selection strategy, A3G-generated HIV-1 inactive variants can be selected to use as “natural occurring” multiple-type HIV-1 vaccines that are effective to broad-spectrum of viral clades in many areas including North and South American, Eurasian, African, and Asian countries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Yoshimi Fukushima, Kazuko Fukuda, and Hiromi Terasawa for technical and secretarial assistance. We thank Prof. Shinji Harada and Dr. Yosuke Maeda (Department of Medical Virology) for their support.

This study was supported in part by a Global-COE program (Global Education and Research Center Aiming at the control of AIDS, Kumamoto University), a MEXT KAKENHI Grant Number 22021036, a Contract Research Fund from the MEXT for Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, JSPS KAKENHI Grant Numbers 23390122, 24659224, 22590436, and 23590546) a Fund from Higo Bank, and in part by NIH Grants GM21422 and ES013192. S.A.A. is supported by the King Abdullah Scholarship Program (Ministry of Higher Education in Saudi Arabia). S.A.A and M.M.A.E. are supported by the Advanced Education Program for Integrated Clinical, Basic and Social Medicine (Graduate School of Medical Sciences, Kumamoto University).

Abbreviations used in this article

AID	activation-induced cytidine deaminase
A3G	APOBEC3G
APOBEC3	apolipoprotein B mRNA–editing, enzyme-catalytic, polypeptide-like 3
GC	germinal center
gRNA	genomic RNA
IP	immunoprecipitation
NC	nucleocapsid
PBMC	peripheral blood mononuclear cell
qRT-PCR	quantitative real-time PCR
RNP	ribonucleoprotein
TREX-2	transcription and export complex 2
Vif	viral infectivity factor
VLP	virus like particle
VSV-G	vesicular stomatitis virus G protein
WB	Western blot
WCL	whole cell lysate
WT	wild-type

References

1. Conticello SG, Thomas CJ, Petersen-Mahrt SK, Neuberger MS. Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. Mol. Biol. Evol. 2005; 22:367–377. [PubMed: 15496550]
2. Conticello SG, Langlois MA, Yang Z, Neuberger MS. DNA deamination in immunity: AID in the context of its APOBEC relatives. Adv. Immunol. 2007; 94:37–73. [PubMed: 17560271]
3. Schmitz KM, Petersen-Mahrt SK. AIDing the immune system-DIAbolic in cancer. Semin. Immunol. 2012; 24:241–245. [PubMed: 22841422]
4. Odegard VH, Schatz DG. Targeting of somatic hypermutation. Nat. Rev. Immunol. 2006; 6:573–583. [PubMed: 16868548]
5. Di Noia JM, Neuberger MS. Molecular mechanisms of antibody somatic hypermutation. Annu. Rev. Biochem. 2007; 76:1–22. [PubMed: 17328676]
6. Peled JU, Kuang FL, Iglesias-Ussel MD, Roa S, Kalis SL, Goodman MF, Scharff MD. The biochemistry of somatic hypermutation. Annu. Rev. Immunol. 2008; 26:481–511. [PubMed: 18304001]
7. Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. Annu. Rev. Immunol. 2008; 26:261–292. [PubMed: 18370922]
8. Maul RW, Gearhart PJ. AID and somatic hypermutation. Adv. Immunol. 2010; 105:159–191. [PubMed: 20510733]
9. Chiu YL, Greene WC. The APOBEC3 cytidine deaminases: an innate defensive network opposing exogenous retroviruses and endogenous retroelements. Annu. Rev. Immunol. 2008; 26:317–353. [PubMed: 18304004]

10. Sheehy AM, Gaddis NC, Choi JD, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature. 2002; 418:646–650. [PubMed: 12167863]
11. Lecossier D, Bouchonnet F, Clavel F, Hance AJ. Hypermutation of HIV-1 DNA in the absence of the Vif protein. Science. 2003; 300:1112. [PubMed: 12750511]
12. Jager S, Kim DY, Hultquist JF, Shindo K, LaRue RS, Kwon E, Li M, Anderson BD, Yen L, Stanley D, Mahon C, Kane J, Franks-Skiba K, Cimermancic P, Burlingame A, Sali A, Craik CS, Harris RS, Gross JD, Krogan NJ. Vif hijacks CBF-beta to degrade APOBEC3G and promote HIV-1 infection. Nature. 2012; 481:371–375. [PubMed: 22190037]
13. Zhang W, Du J, Evans SL, Yu Y, Yu XF. T-cell differentiation factor CBF-beta regulates HIV-1 Vif-mediated evasion of host restriction. Nature. 2012; 481:376–379. [PubMed: 22190036]
14. Browne EP, Allers C, Landau NR. Restriction of HIV-1 by APOBEC3G is cytidine deaminase-dependent. Virology. 2009; 387:313–321. [PubMed: 19304304]
15. Svarovskaia ES, Xu H, Mbisa JL, Barr R, Gorelick RJ, Ono A, Freed EO, Hu WS, Pathak VK. Human apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is incorporated into HIV-1 virions through interactions with viral and nonviral RNAs. J. Biol. Chem. 2004; 279:35822–35828. [PubMed: 15210704]
16. Khan MA, Kao S, Miyagi E, Takeuchi H, Goila-Gaur R, Opi S, Gipson CL, Parslow TG, Ly H, Strebel K. Viral RNA is required for the association of APOBEC3G with human immunodeficiency virus type 1 nucleoprotein complexes. J. Virol. 2005; 79:5870–5874. [PubMed: 15827203]
17. Wang T, Tian C, Zhang W, Luo K, Sarkis PT, Yu L, Liu B, Yu Y, Yu XF. 7SL RNA mediates virion packaging of the antiviral cytidine deaminase APOBEC3G. J. Virol. 2007; 81:13112–13124. [PubMed: 17881443]
18. Huthoff H, Autore F, Gallois-Montbrun S, Fraternali F, Malim MH. RNA-dependent oligomerization of APOBEC3G is required for restriction of HIV-1. PLoS Pathog. 2009; 5:e1000330. [PubMed: 19266078]
19. Didierlaurent L, Racine PJ, Houzet L, Chamontin C, Berkhout B, Mougel M. Role of HIV-1 RNA and protein determinants for the selective packaging of spliced and unspliced viral RNA and host U6 and 7SL RNA in virus particles. Nucleic Acids Res. 2011; 39:8915–8927. [PubMed: 21791531]
20. Kieffer TL, Kwon P, Nettles RE, Han Y, Ray SC, Siliciano RF. G-->A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting CD4+ T cells in vivo. J. Virol. 2005; 79:1975–1980. [PubMed: 15650227]
21. Kijak GH, Janini LM, Tovanabutra S, Sanders-Buell E, Arroyo MA, Robb ML, Michael NL, Birx DL, McCutchan FE. Variable contexts and levels of hypermutation in HIV-1 proviral genomes recovered from primary peripheral blood mononuclear cells. Virology. 2008; 376:101–111. [PubMed: 18436274]
22. Alce TM, Popik W. APOBEC3G is incorporated into virus-like particles by a direct interaction with HIV-1 Gag nucleocapsid protein. J. Biol. Chem. 2004; 279:34083–34086. [PubMed: 15215254]
23. Cen S, Guo F, Niu M, Saadatmand J, Deflassieux J, Kleiman L. The interaction between HIV-1 Gag and APOBEC3G. J. Biol. Chem. 2004; 279:33177–33184. [PubMed: 15159405]
24. Bogerd HP, Cullen BR. Single-stranded RNA facilitates nucleocapsid: APOBEC3G complex formation. RNA. 2008; 14:1228–1236. [PubMed: 18456846]
25. Chiu YL, Witkowska HE, Hall SC, Santiago M, Soros VB, Esnault C, Heidmann T, Greene WC. High-molecular-mass APOBEC3G complexes restrict Alu retrotransposition. Proc. Natl. Acad. Sci. USA. 2006; 103:15588–15593. [PubMed: 17030807]
26. Kozak SL, Marin M, Rose KM, Bystrom C, Kabat D. The anti-HIV-1 editing enzyme APOBEC3G binds HIV-1 RNA and messenger RNAs that shuttle between polysomes and stress granules. J. Biol. Chem. 2006; 281:29105–29119. [PubMed: 16887808]
27. Gallois-Montbrun S, Kramer B, Swanson CM, Byers H, Lynham S, Ward M, Malim MH. Antiviral protein APOBEC3G localizes to ribonucleoprotein complexes found in P bodies and stress granules. J. Virol. 2007; 81:2165–2178. [PubMed: 17166910]

28. Wichroski MJ, Robb GB, Rana TM. Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. PLoS Pathog. 2006; 2:e41. [PubMed: 16699599]
29. Nathans R, Chu CY, Serquina AK, Lu CC, Cao H, Rana TM. Cellular microRNA and P bodies modulate host-HIV-1 interactions. Mol. Cell. 2009; 34:696–709. [PubMed: 19560422]
30. Burdick R, Smith JL, Chaipan C, Friew Y, Chen J, Venkatachari NJ, Delviks-Frankenberry KA, Hu WS, Pathak VK. P body-associated protein Mov10 inhibits HIV-1 replication at multiple stages. J. Virol. 2010; 84:10241–10253. [PubMed: 20668078]
31. Phalora PK, Sherer NM, Wolinsky SM, Swanson CM, Malim MH. HIV-1 replication and APOBEC3 antiviral activity are not regulated by P bodies. J. Virol. 2012; 86:11712–11724. [PubMed: 22915799]
32. Strebel K, Khan MA. APOBEC3G encapsidation into HIV-1 virions: which RNA is it? Retrovirology. 2008; 5:55. [PubMed: 18597677]
33. Smith HC. APOBEC3G: a double agent in defense. Trends Biochem. Sci. 2011; 36:239–244. [PubMed: 21239176]
34. Sakaguchi N, Maeda K, Kuwahara K. Molecular Mechanism of Immunoglobulin V-Region Diversification Regulated by Transcription and RNA Metabolism in Antigen-Driven B Cells. Scand. J. Immunol. 2011; 73:520–526. [PubMed: 21388430]
35. Wickramasinghe VO, McMurtrie PI, Mills AD, Takei Y, Penrhyn-Lowe S, Amagase Y, Main S, Marr J, Stewart M, Laskey RA. mRNA export from mammalian cell nuclei is dependent on GANP. Curr. Biol. 2010; 20:25–31. [PubMed: 20005110]
36. Jani D, Lutz S, Hurt E, Laskey RA, Stewart M, Wickramasinghe VO. Functional and structural characterization of the mammalian TREX-2 complex that links transcription with nuclear messenger RNA export. Nucleic Acids Res. 2012; 40:4562–4573. [PubMed: 22307388]
37. Sakaguchi N, Kimura T, Matsushita S, Fujimura S, Shibata J, Araki M, Sakamoto T, Minoda C, Kuwahara K. Generation of high-affinity antibody against T cell-dependent antigen in the Ganp gene-transgenic mouse. J. Immunol. 2005; 174:4485–4494. [PubMed: 15814669]
38. Maeda K, Singh SK, Eda K, Kitabatake M, Pham P, Goodman MF, Sakaguchi N. GANP-mediated recruitment of activation-induced cytidine deaminase to cell nuclei and to immunoglobulin variable region DNA. J. Biol. Chem. 2010; 285:23945–23953. [PubMed: 20507984]
39. Singh SK, Maeda K, Eid MM, Almofty SA, Ono M, Pham P, Goodman MF, Sakaguchi N. GANP regulates recruitment of AID to immunoglobulin variable regions by modulating transcription and nucleosome occupancy. Nat. Commun. 2013; 4:1830. [PubMed: 23652018]
40. Koito A, Hattori T, Matsushita S, Maeda Y, Nozaki C, Sagawa K, Takatsuki K. Conserved immunogenic region of a major core protein (p24) of human and simian immunodeficiency viruses. AIDS Res. Hum. Retroviruses. 1988; 4:409–417. [PubMed: 2464360]
41. Koito A, Kameyama Y, Cheng-Mayer C, Matsushita S. Susceptibility of mink (Mustera vision)-derived cells to replication by human immunodeficiency virus type 1. J. Virol. 2003; 77:5109–5117. [PubMed: 12692213]
42. Ikeda T, Ohsugi T, Kimura T, Matsushita S, Maeda Y, Harada S, Koito A. The antiretroviral potency of APOBEC1 deaminase from small animal species. Nucleic Acids Res. 2008; 36:6859–6871. [PubMed: 18971252]
43. Chelico L, Prochnow C, Erie DA, Chen XS, Goodman MF. Structural model for deoxycytidine deamination mechanisms of the HIV-1 inactivation enzyme APOBEC3G. J. Biol. Chem. 2010; 285:16195–16205. [PubMed: 20212048]
44. Zennou V, Perez-Caballero D, Gottlinger H, Bieniasz PD. APOBEC3G incorporation into human immunodeficiency virus type 1 particles. J. Virol. 2004; 78:12058–12061. [PubMed: 15479846]
45. Soros VB, Yonemoto W, Greene WC. Newly synthesized APOBEC3G is incorporated into HIV virions, inhibited by HIV RNA, and subsequently activated by RNase H. PLoS Pathog. 2007; 3:e15. [PubMed: 17291161]
46. Ikeda T, Abd El Galil KH, Tokunaga K, Maeda K, Sata T, Sakaguchi N, Heidmann T, Koito A. Intrinsic restriction activity by apolipoprotein B mRNA editing enzyme APOBEC1 against the mobility of autonomous retrotransposons. Nucleic Acids Res. 2011; 39:5538–5554. [PubMed: 21398638]

47. Refsland EW, Stenglein MD, Shindo K, Albin JS, Brown WL, Harris RS. Quantitative profiling of the full APOBEC3 mRNA repertoire in lymphocytes and tissues: implications for HIV-1 restriction. Nucleic Acids Res. 2010; 38:4274–4284. [PubMed: 20308164]

48. Khan MA, Goila-Gaur R, Opi S, Miyagi E, Takeuchi H, Kao S, Strebel K. Analysis of the contribution of cellular and viral RNA to the packaging of APOBEC3G into HIV-1 virions. Retrovirology. 2007; 4:48. [PubMed: 17631688]

49. Bach D, Peddi S, Mangeat B, Lakkaraju A, Strub K, Trono D. Characterization of APOBEC3G binding to 7SL RNA. Retrovirology. 2008; 5:54. [PubMed: 18597676]

50. Keene SE, King SR, Telesnitsky A. 7SL RNA is retained in HIV-1 minimal virus-like particles as an S-domain fragment. J. Virol. 2010; 84:9070–9077. [PubMed: 20610725]

51. Deeks SG, Walker BD. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. Immunity. 2007; 27:406–416. [PubMed: 17892849]

52. Casartelli N, Guivel-Benhassine F, Bouziat R, Brandler S, Schwartz O, Moris A. The antiviral factor APOBEC3G improves CTL recognition of cultured HIV-infected T cells. J. Exp. Med. 2010; 207:39–49. [PubMed: 20038599]

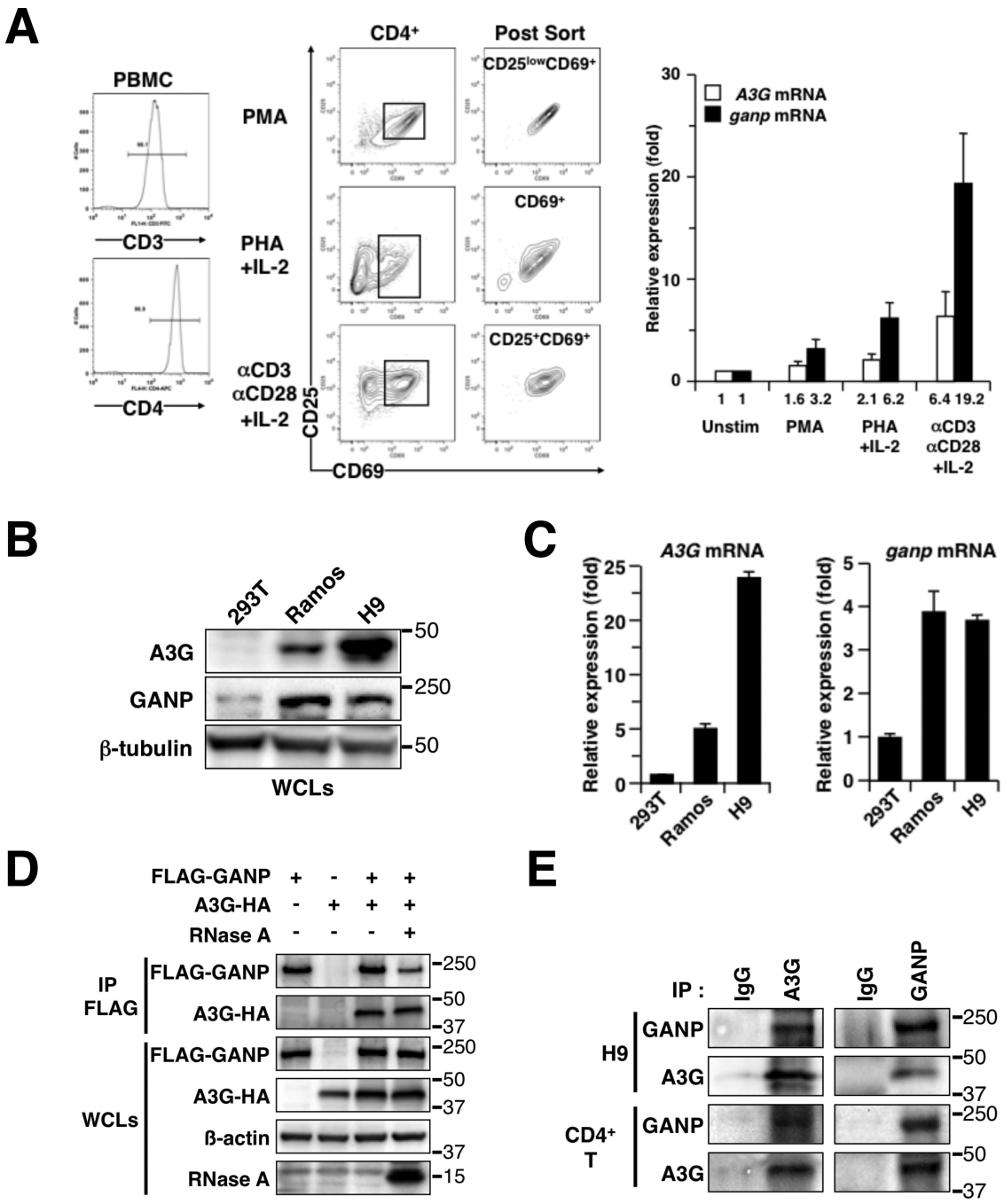

FIGURE 1.

GANP is upregulated in activated T cells and interacts with A3G. (**A**) A3G and GANP in 293T, Ramos B cell and H9 T cell lines. WCLs were subjected to WB. (**B**) *A3G* and *ganp* mRNAs. *A3G* and *ganp* mRNA levels were normalized to those found in 293T cells. (**C**) *A3G* and *ganp* mRNA in activated $CD4^+$ T cells. $CD4^+$ T cells were stimulated with PMA, PHA + IL-2, or anti-CD3/CD28 beads + IL-2 for 3 days, and sorted using CD25 and CD69. *A3G* and *ganp* mRNAs were measured by qRT-PCR and normalized to those found in unstimulated cells. (**D**) A3G co-IP with GANP in 293T cells. FLAG-GANP and A3G-HA

were transfected into 293T cells. WCLs were subjected to anti-FLAG IP/WB with anti-HA. RNase A, if present, was pre-incubated with the WCL to digest RNA before the IP. (**E**) Interaction between GANP and A3G in H9 cells and $CD4^+$ primary T cells. WCLs were IP with anti-A3G, anti-GANP or IgG and the endogenous GANP and A3G were detected by WB. The error bar indicates standard error.

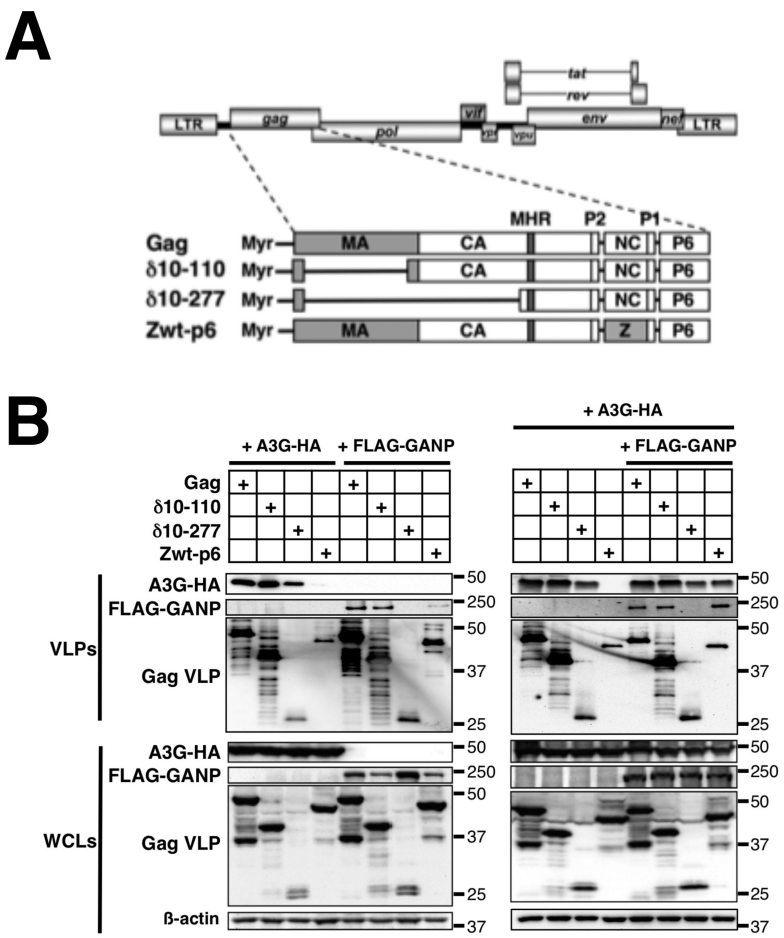

FIGURE 2.

GANP enhances A3G encapsidation in VLPs. (**A**) Domain representation of myristoylated Gag deletion mutants (Myr - myristoylated; MA - matrix; CA - capsid; MHR - major homology region; NC - nucleocapsid; Z - a leucine zipper). (**B**) NC-dependent incorporation of A3G and CA-dependent incorporation of GANP in VLPs. VLPs were produced in 293T cells transfected with WT or mutant Gag constructs. VLPs and WCLs from infected 293T cells were subjected to WB to detect the presence of A3G and GANP.

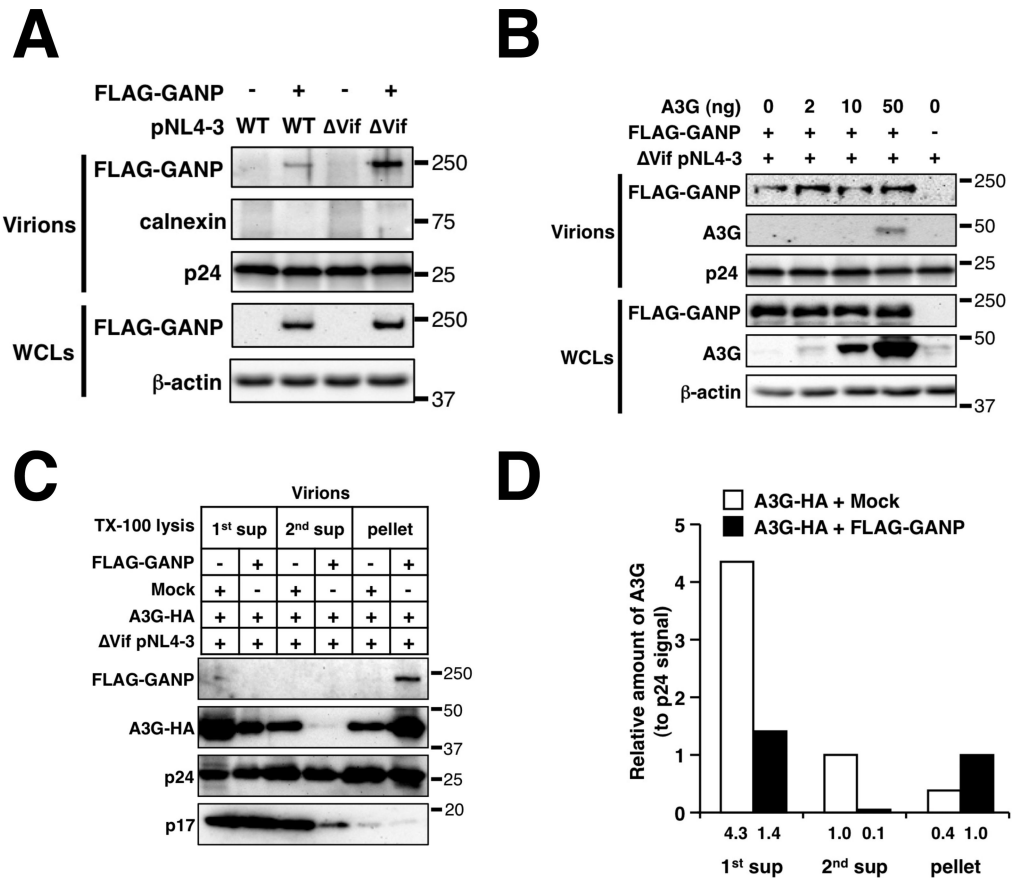

FIGURE 3.

GANP is encapsidated in virions and associated with *HIV-1* gRNA. (**A**) WT and ΔVif HIV-1 virions were purified from culture supernatants of virus producing 293T cells transfected with FLAG-GANP and WT or ΔVif pNL4-3. FLAG-GANP was detected in the virions by WB. (**B**) A3G does not affect on GANP encapsidation. A3G does not influence GANP-encapsidation into ΔVif HIV-1 virions. Viruses were produced in the presence of FLAG-GANP alone or together with A3G (transfection dosage of 2, 10 or 50 ng of DNA). (**C**) GANP localizes exclusively in the virion core and promotes A3G packaging into virion cores. The ΔVif HIV-1 virions were fractionated into supernatant fractions (first and second sup) and a pellet fraction enriched with virion cores. (**D**) The relative amount of A3G in each fraction was calculated after normalization with p24.

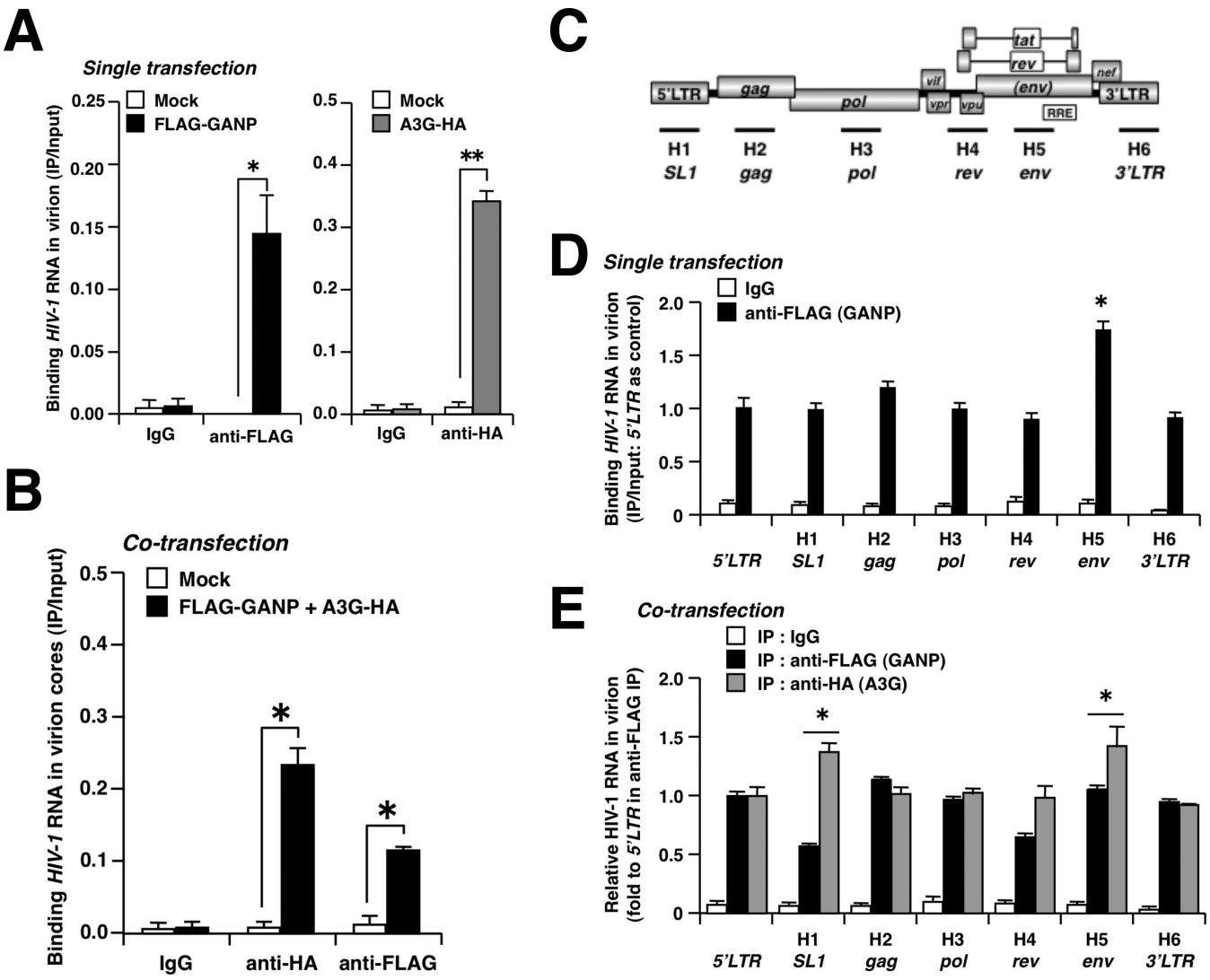

FIGURE 4.

GANP is associated with *HIV-1* gRNA. (**A**) Association of GANP and A3G with viral gRNA. The ΔVif HIV-1 virions produced in the presence of FLAG-GANP or A3G-HA were subjected to virion-IP assay using anti-FLAG or anti-HA. qRT-PCR was used to quantify the fraction (%) of *HIV-1* gRNA. IgG was used as a control. (**B**) GANP and A3G association with *HIV-1* gRNA in virion cores. The ΔVif HIV-1 virion core pellet prepared in (Fig. 3C) was subjected to the virion-IP assay. (**C**) Schematic representation of HIV-1 regions examined for association with GANP. (**D**) GANP binds to different *HIV-1* gRNA regions within the virion. Binding regions were quantified by qRT-PCR and normalized to the GANP binding to the *5'-LTR*. (**E**) Comparison of FLAG-GANP and A3G-HA binding regions of *HIV-1* gRNA in ΔVif HIV-1 virions produced in the presence of both FLAG-GANP and A3G-HA. Data represent the means ± standard deviations calculated from three experiments. **P* < 0.05, ***P* < 0.01.

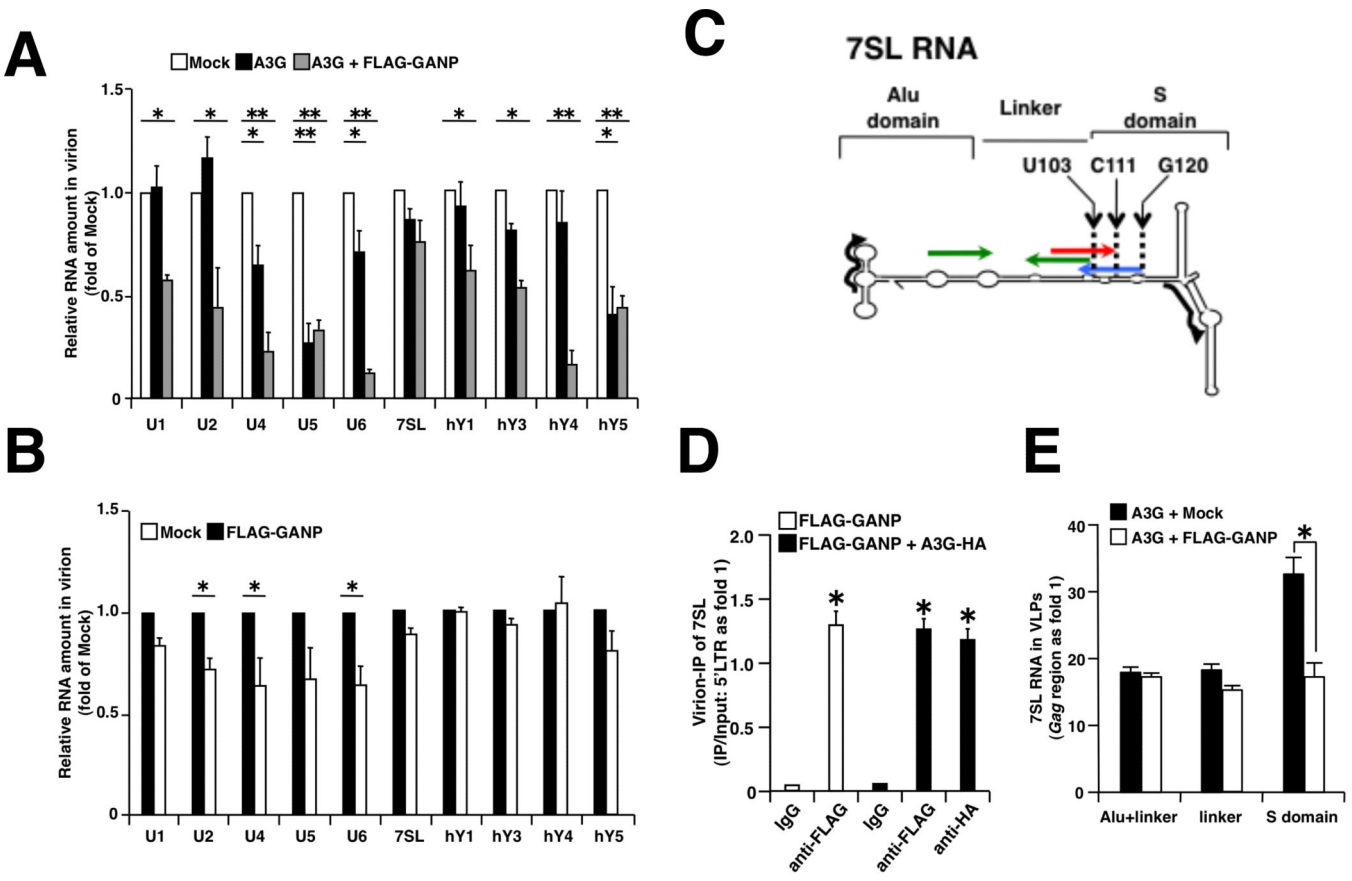

FIGURE 5.

GANP modulates cellular RNA incorporation with A3G. (**A**) Encapsidation of cellular RNAs into ΔVif HIV-1 virions. Virions were produced from ΔVif pNL4-3 in 293T cells in the presence of A3G-HA, FLAG-GANP, and A3G-HA or Mock. Cellular RNA encapsidation was normalized to the Mock (empty vector) control. (**B**) Encapsidation of cellular RNAs into ΔVif HIV-1 virions in the presence of FLAG-GANP alone. (**C**) The *7SL* RNA consists of Alu domain, linker region, and S-domain. Primers (arrows) for *7SL* RNA cleavage sites and the three frequent cleavage sites U104, C111, and G120 are indicated (29). (**D**) Virion-IP assay was used to detect the association of *7SL* RNA with FLAG-GANP and A3G-HA in ΔVif HIV-1 virions. (**E**) Cleavage of *7SL* RNA in Zwt-p6 VLPs is detected by qRT-PCR using primer pairs for Alu+linker, linker or S-domain. Quantified *7SL* RNA fragments were normalized based on the Gag region measurement. (**F**) Full-length *7SL* RNA was detected by straddling over the cleavage sites as in (C) using the specific primers. The data were normalized with VLPs from Mock (empty vector) cells. Data represent the means ± standard deviations calculated from three experiments. **P* < 0.05, ***P* < 0.01.

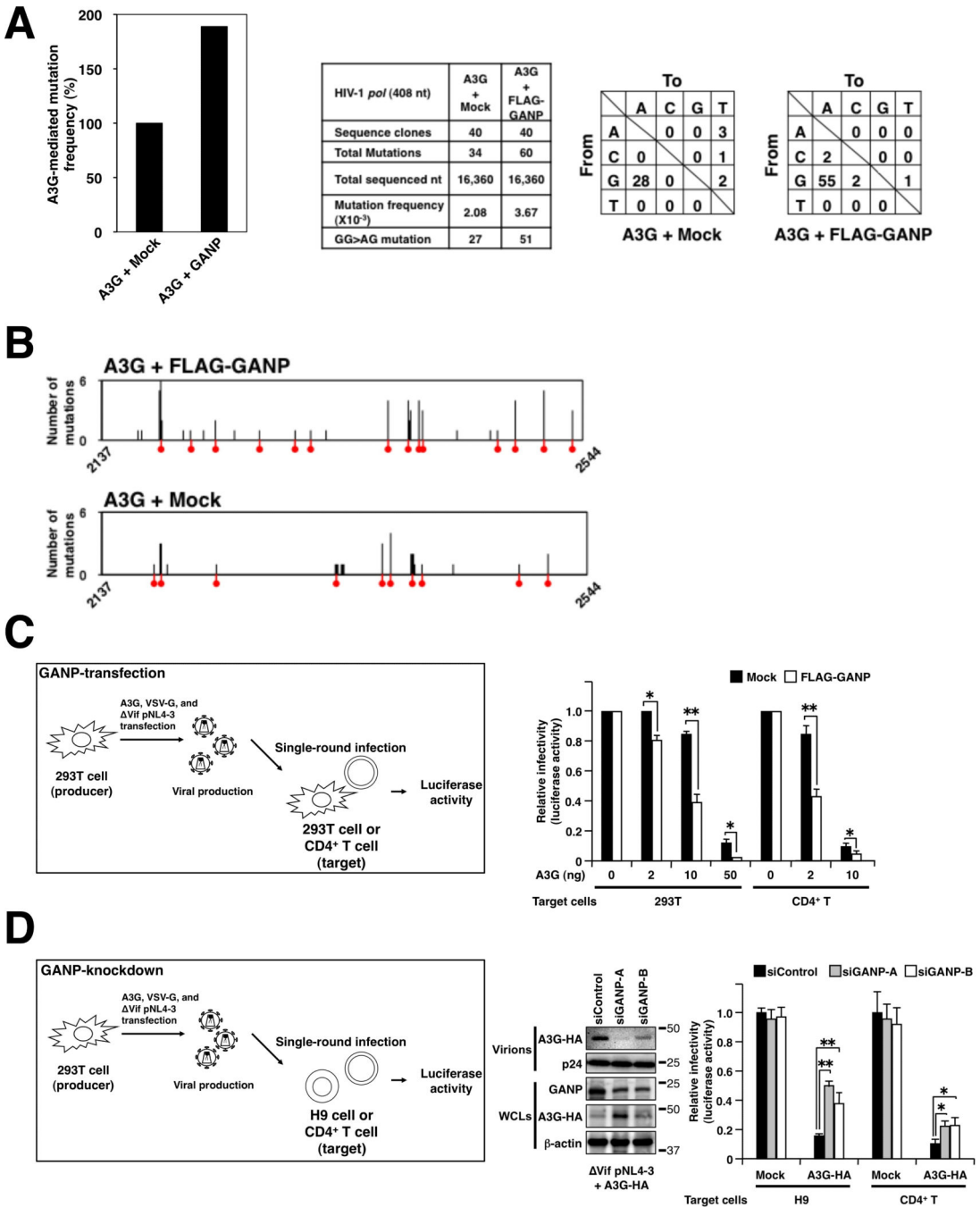

FIGURE 6.

GANP enhances A3G-catalyzed G→A hypermutation and promotes A3G anti-HIV-1 activity. (**A**) Mutation analysis of 408 nt *pol* region of HIV-1 proviral DNA. Virions produced in 293T cells with a reduced amount of A3G (2 ng) in combination with or without FLAG-GANP were infected to the target cells and subjected to sequence analysis. (**B**) A3G-catalyzed mutation spectra in the HIV-1 *pol* region (nt positions: 2,137-2,544). Flags indicate mutations at an A3G hotspot motif (GG→AG). (**C**) GANP overexpression in 293T virus-producing cells enhances A3G-mediated inhibition of ΔVif HIV-1 infectivity in

a single-round infection assay. The ΔVif HIV-1 virions were produced with a low dose (0, 2, 10 or 50 ng) of A3G ± FLAG-GANP and a similar amount of virions from each preparation (2 ng of p24-equivalent) was used to infect 293T cells and $CD4^+$ primary T cells. Relative viral infectivity was calculated by the luciferase activity in WCLs of infected cells at 48 h post-infection. (**D**) Single-round infection assay in H9 cells. The ΔVif HIV-1 virions containing A3G were produced in 293T cells treated with either siControl or siGANP. A3G was examined by WB in both WCLs and virion core fractions. Relative viral infectivity was calculated based on the luciferase activity in WCLs of infected H9 cells and $CD4^+$ primary T cells. Data represent the means ± standard deviations calculated from three experiments. $*P < 0.05$, $**P < 0.01$.

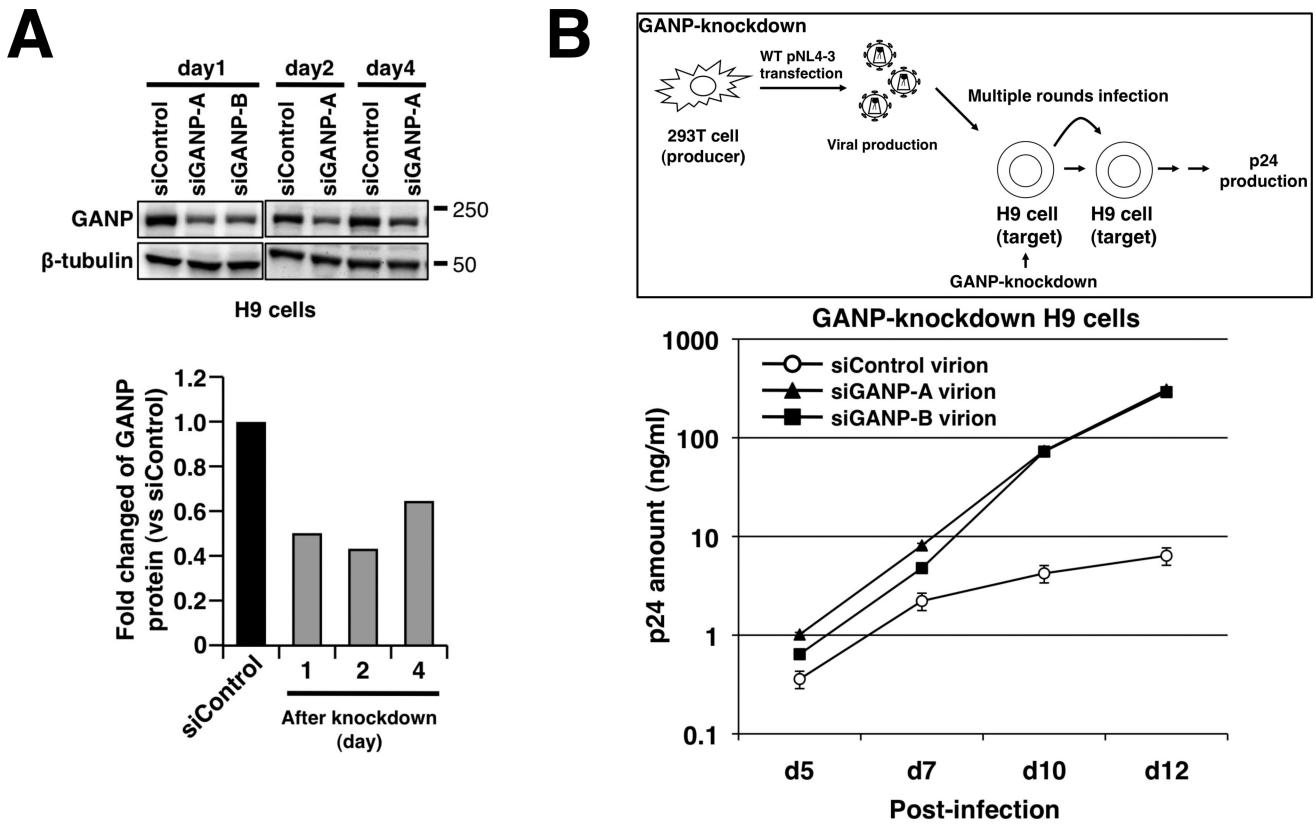

FIGURE 7.

Effect of GANP-knockdown on WT Vif-proficient HIV-1 infectivity. (**A**) Downregulation of GANP in H9 cells at days 1, 2, and 4 after treatment with siGANPs. (**B**) Multiple rounds infection assay in GANP-knockdown H9 cells. Vif- proficient intact env HIV-1 virions produced in siControl- or siGANP-treated 293T cells were used to infect GANP-knockdown H9 cells. Infectivity was analyzed by measuring p24 production in the culture supernatant by ELISA. Data represent the means ± standard deviations calculated from three experiments.