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The *C*-terminal domain of glyceraldehyde 3-phosphate dehydrogenase plays an important role in suppression of tRNA^{Lys3} packaging into human immunodeficiency virus type-1 particles

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ABSTRACT

Human immunodeficiency virus type-1 (HIV-1) requires the packaging of human tRNA^{Lys3} as a primer for effective viral reverse transcription. Previously, we reported that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) suppresses the packaging efficiency of tRNA^{Lys3}. Although the binding of GAPDH to Pr55^{gag} is important for the suppression mechanism, it remains unclear which domain of GAPDH is responsible for the interaction with Pr55^{gag}. In this study, we show that Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} of GAPDH are important for the suppression of tRNA^{Lys3} packaging. Yeast two-hybrid analysis demonstrated that the *C*-terminal domain of GAPDH (151–335) interacts with both the matrix region (MA; 1–132) and capsid *N*-terminal domain (CA-NTD; 133–282). The D256R, K263E or E267R mutation of GAPDH led to the loss of the ability to bind to wild-type (WT) MA, and the D256R/K260E double mutation of GAPDH resulted in the loss of detectable binding activity to WT CA-NTD. In contrast, R58E, Q59A or Q63A of MA, and E76R or R82E of CA-NTD abrogated the interaction with the *C*-terminal domain of GAPDH. Multiple-substituted GAPDH mutant (D256R/K260E/K263E/E267R) retained the oligomeric formation with WT GAPDH in HIV-1 producing cells, but the incorporation level of the hetero-oligomer was decreased in viral particles. Furthermore, the viruses produced from cells expressing the D256R/K260E/K263E/E267R mutant restored tRNA^{Lys3} packaging efficiency because the mutant exerted a dominant negative effect by preventing WT GAPDH from binding to MA and CA-NTD and improved the reverse transcription. These findings indicate that the amino acids Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} of GAPDH is essential for the mechanism of tRNA^{Lys3}-packaging suppression and the D256R/K260E/K263E/E267R mutant of GAPDH acts in a dominant negative manner to suppress tRNA^{Lys3} packaging.

1. Introduction

It has recently been shown that cellular proteins regulate HIV-1 replication. Interestingly, several studies of purified HIV-1 virions have shown that, in addition to proteins encoded by the virus, cellular proteins are taken into the virions [1]. Some of these proteins, such as cyclophilin A and lysyl-tRNA synthetase (LysRS), are packaged into virions as a result of their interaction with Pr55^{gag} or p160$^{gag\text{-}pol}$ proteins during assembly [2–5]. These cellular proteins play an important role in viral precursor protein folding and tRNA^{Lys3} packaging. Thus, understanding the packaging mechanism of cellular proteins is one way to elucidate the viral replication capacity.

One of the critical events in HIV-1 replication is reverse transcription. Cellular tRNA^{Lys3} is required for the efficient initiation of reverse transcription and is selectively incorporated into viral particles during its assembly because the 3′ terminal 18 nucleotides must be hybridized to the primer-binding site of HIV-1 genome RNA as a replication primer [6]. Gabor et al. [7] reported that the elevated amount of packaged tRNA^{Lys3} increases viral infectivity. Efficient packaging of tRNA^{Lys3} is facilitated by interaction between Pr55^{gag} or p160$^{gag\text{-}pol}$ and LysRS, which act as carriers of tRNA^{Lys3} [8,9].

Although GAPDH was initially identified as a glycolytic enzyme, it

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIV-1, human immunodeficiency virus type 1; LysRS, lysyl-tRNA synthetase; MA, matrix; CA, capsid

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has been known as a "moonlighting" protein. Several studies have shown that GAPDH is related to apoptosis, the exportation of nuclear RNA, and DNA repair. [10]. Furthermore, GAPDH regulates viral replication by binding to *cis*-acting viral RNAs, such as the hepatitis A virus, hepatitis C virus and human parainfluenza virus [11–13]. Similarly, we previously reported that GAPDH also plays a role in negatively regulating HIV-1 infection [14]. GAPDH is incorporated into virions via its interaction with $Pr55^{gag}$ [14]. Increased GAPDH packaging efficiency decreases reverse transcription efficiency owing to the suppression of LysRS and $tRNA^{Lys3}$ packaging [14]. Thus, the binding of GAPDH to $Pr55^{gag}$ has an important role in the suppression of HIV-1 replication. These findings indicate that GAPDH negatively regulates HIV-1 replication and provide insights into a new host defense mechanism against HIV-1 infection. However, it is remains unclear which amino acid residues of GAPDH are important for the recognition of $Pr55^{gag}$.

In this study, we show that amino acids Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} of GAPDH interact with MA and CA-NTD domain of $Pr55^{gag}$ and the D256R/K260E/K263E/E267R mutant of GAPDH acts as a dominant negative inhibitor of $tRNA^{Lys3}$ packaging. These findings provide a new insight into $tRNA^{Lys3}$ packaging mechanism and indicate a novel regulatory step of HIV-1 replication.

2. Materials and methods

2.1. Cell culture

TZM-bl cells, which were obtained from the NIH AIDS Research and Reference Reagent Program, and HEK293 cells were maintained at 37 °C in DMEM supplemented with 10% fetal calf serum (FCS) containing 100 IU/ml penicillin and 100 μg/ml streptomycin in 5% CO_2.

2.2. Viruses

The infectious molecular clone pNL-CH [15], derived from the pNL4-3 clone of HIV-1, and each mutated GAPDH expression vector (cloned into the pcDNA3.1D/V5-His-TOPO® vector) were cotransfected into HEK293 cells to prepare various GAPDH-mutant-packaging viruses. At 48 h post-cotransfection, the virus-containing supernatant was collected and clarified by filtration using 0.45-μm-pore-size filters [14].

2.3. Plasmid

The coding region of the $HIV\text{-}1_{NL\text{-}CH}$ protein and GAPDH was amplified by PCR using the following primers. $Pr55^{gag}$: $Pr55^{gag}$ UP (5′-AGAATTCATGGGTGCGAGAGCGTCGGTATTA-3′) and $Pr55^{gag}$ DN (5′-TGGATCCTTATTGTGACGAGGGGTCGCTGCC-3′); MA: $Pr55^{gag}$ UP and MA DN (5′-TGGATCCTTAGTAATTTTGGCTGACCTG-3′); CA: CA UP (5′-AGAATTCCCTATAGTGCAGAACCTCCAG-3′) and CA DN (5′-TGGATCCTTACAAAACTCTTGCTTTATGGCC-3′); CA-NTD: CA UP and CA-NTD DN (5′-TGGATCCTTAAATGCTGGTAGGGCTATACAT-3′); CA-CTD: CA-CTD UP (5′-AGAATTCCTGGACATAAGACAAGGACCA-3′) and CA DN; NC: NC UP (5′-AGAATTCATACAGAAAGGCAATTTTAGG-3′) and NC DN (5′-TGGATCCTTAATTAGCCTGTCTCTCAGTACA-3′); p6: p6 UP (5′-AGAATTCCTTCAGAGCAGACCAGAGCCA-3′) and $Pr55^{gag}$ DN; $p160^{gag\text{-}pol}$: $p160^{gag\text{-}pol}$ UP (5′-ACTAGTCATATGGATGAATTCATG-GGTGCGAGAGCGTCGGTATTA-3′) and $p160^{gag\text{-}pol}$ DN (5′-ACCC-GGGGATCCGATGGATCCTTAATCCTCATCCTGTCTACTTGC-3′); Pol: Pol UP (5′-GGAGGCCAGTGAATTCCCTCAGATCACTCTTTGGCAG-3′) and $p160^{gag\text{-}pol}$ DN; GAPDH: GAPDH UP (5′-AGAATTCATGGGGAA-GGTGAAGGTCGGAGTCAAC-3′) and GAPDH DN (5′-TGGATCCTTA-CTCCTTGGAGGCCATGTGGGC-3′); GAPDH-*n*: GAPDH UP and GAP-DH-*n* DN (5′-TGGATCCTTAGCAGGAGGCATTGCTGAT-3′); GAPDH-*c*: GAPDH-*c* UP (5′-AGAATTCTCCTGCACCACCAACTGCTTA-3′) and GAPDH DN. For the yeast-two-hybrid (Y2H) analysis, the HIV-1 protein or GAPDH coding regions were cloned into the *Eco*RI and *Bam*HI sites of pGBKT7 or pGADT7, respectively (Clontech Laboratories, Inc.). The full-length GAPDH coding regions were also cloned into the *Eco*RV and *Bam*HI sites of the pcDNA™ 3.1D/V5-His-TOPO® vector (Thermo Fisher Scientific, Inc.). Each mutated GAPDH construct was obtained by site-directed mutagenesis. All of the mutations were verified by sequencing.

2.4. Yeast two-hybrid analysis

The Matchmaker™ Gold Yeast Two-hybrid System (Clontech Laboratories, Inc.) was used in accordance with manufacturer's recommendations to analyze the interaction between several reconstructed GAPDH and HIV-1 proteins. Briefly, the bait (cloned into pGBKT7) and prey (cloned into pGADT7) constructs were cotransformed into Y2HGold and plated on QDO/X/A plates (without tryptophan leucine, adenine, and histidine and with aureobasidin A and X-α-Gal). As a positive or negative control, pGADT7-T and pGBKT7-53 or pGADT7 AD and pGBKT7 DNA-BD were cotransformed, respectively. To validate transformed protein expression, each yeast strain was lysed and detected using an anti-HA antibody (Wako Pure Chemical Industries, Ltd.) or an anti-c-Myc antibody (Clontech Laboratories, Inc.).

2.5. Coimmunoprecipitation

HEK293 cells transfected with each GAPDH expression vector were lysed and the lysate was used for coimmunoprecipitation, as a previously described [14]. Briefly, the precleaned lysate was incubated with an anti-V5 antibody (Thermo Fisher Scientific Inc.) or an isotype control mouse IgG antibody (R & D SYSTEMS, Inc.), and further incubated with μMACS™ Protein G MicroBeads (Miltenyi Biotec K.K.). The separated proteins were detected by western immunoblot analysis using the anti-GAPDH antibody (Santa Cruz Biotechnology, Inc.). To detect GAPDH interacting $Pr55^{gag}$, pNL-CH and WT or M6 GAPDH expression vector were cotransfected into HEK293 and the resulting lysate was incubated with an anti-GAPDH antibody before incubating with μMACS™ Protein G MicroBeads.

2.6. Measurement of $tRNA^{Lys3}$ packaging levels in virions and reverse transcription products

$tRNA^{Lys3}$ was prepared from various GAPDH mutants packaging viruses, as a previously described [14]. Briefly, $tRNA^{Lys3}$ was collected from each virus and reverse-transcribed using a SuperScript™ III First-strand Synthesis System (Thermo Fisher Scientific, Inc.). The packaging level of $tRNA^{Lys3}$ was normalized by incorporated viral genomic RNA. The reverse transcription products were analyzed using previously described methods [14]. Total DNA from each virus infected TZM-bl cells was subjected to quantitative real-time PCR with primer pair specific for the R/U5 (early) region.

3. Results

3.1. C-terminal domain of GAPDH interacts with HIV-1 MA and CA

We previously demonstrated that GAPDH, which is expressed in HIV-1 producer cells, is incorporated into viral particles via its interaction with viral precursor proteins [14]. To further investigate which domain is required for the interactions, we prepared viral proteins- or GAPDH-expression vectors, and performed Y2H analysis. The Y2HGold yeast strain was cotransformed with the constructed bait (Fig. 1A) and prey (Fig. 1B) vectors and grown on the QDO/X/A plates. As shown in Fig. 1C, the growth of blue colonies on the QDO/X/A plates signifies the positive interaction between the *C*-terminal domain of GAPDH (GAPDH-*c*) and $Pr55^{gag}$. Furthermore, Y2H analysis using

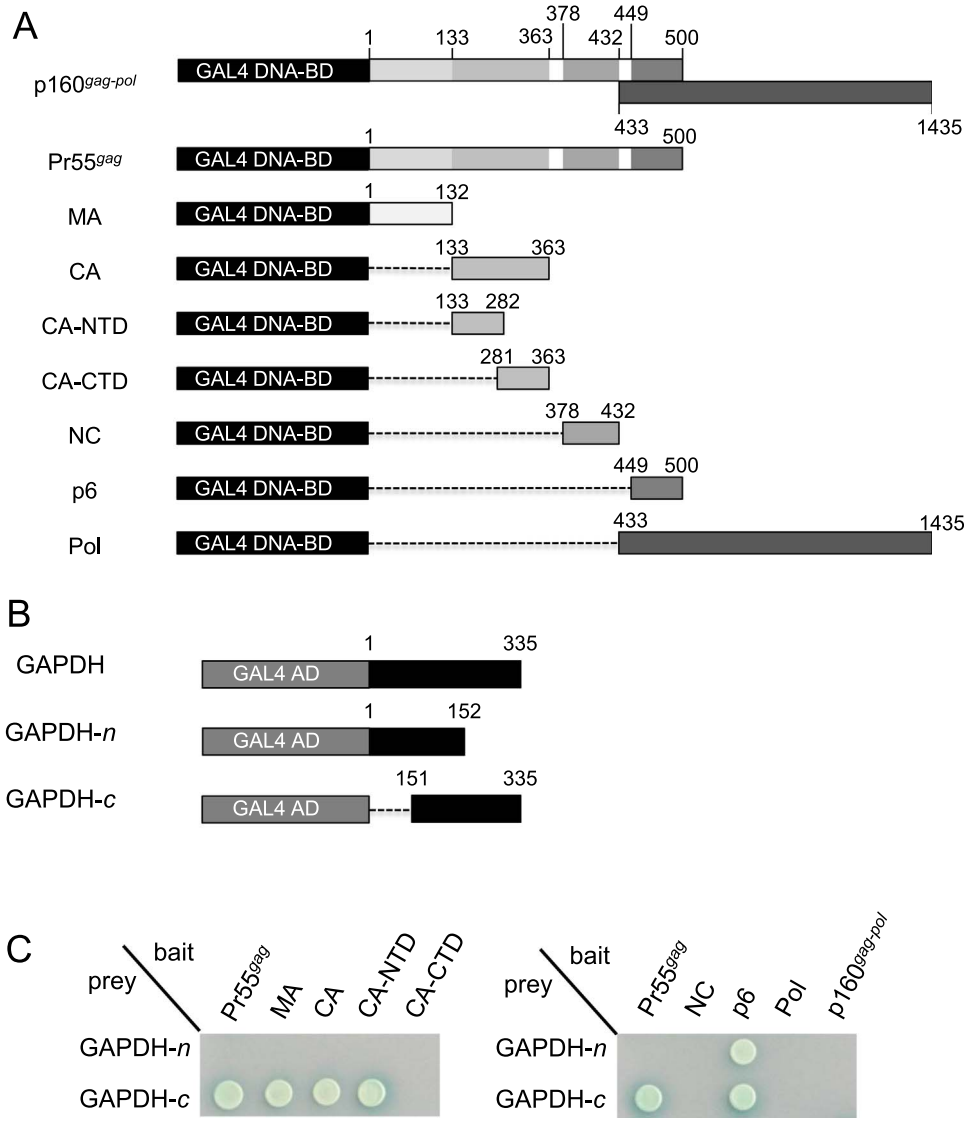

Fig. 1. *Y2H analysis of interaction between GAPDH and HIV-1 precursor proteins.* (A) Bait constructs obtained from pNL-CH and (B) prey constructs obtained from human GAPDH are illustrated. (C) Y2H analysis of *N*-terminal domain of GAPDH (GAPDH-*n*) or *C*-terminal domain of GAPDH (GAPDH-*c*) with $p160^{gag\text{-}pol}$, $Pr55^{gag}$, MA, CA, CA-NTD, CA-CTD, NC, p6 or Pol. The Y2HGold strain was cotransformed with the constructs-expressing bait (as indicated in Fig. 1A) and prey proteins (as indicated in Fig. 1B). Growth on QDO/X/A plates indicates the positive interaction.

processing forms of $Pr55^{gag}$ indicated that MA and CA-NTD interact with GAPDH-*c*. In contrast, the Y2HGold yeast strain cotransformed with GAPDH-*c* and $p160^{gag\text{-}pol}$ did not grow on the QDO/X/A plates, despite sufficient protein expression levels, because the GAL4-activation domain (GAL4 AD) fused to $p160^{gag\text{-}pol}$ could not translocate to the nucleus [16]. Although the p6 bait protein also indicated positive interaction with GAPDH-*c*, this result reflected its autoactivity, which was confirmed by transforming only the bait vector into Y2HGold in the absence of the prey vector (Supplementary Fig. 1). These results suggest that MA and CA-NTD are essential for specific binding via the multiple-site binding of GAPDH to $Pr55^{gag}$.

3.2. Asp^{256}, Lys^{263} and Glu^{267} of GAPDH interact with HIV-1 MA and Asp^{256} and Lys^{260} of GAPDH are essential for the interaction between GAPDH and CA-NTD

To explore the MA- or CA-NTD-interacting domain of GAPDH, we performed docking simulation of interaction between GAPDH (PDB ID: 1ZNQ) [17] and MA (PDB ID: 2H3I) [18] or CA (PDB ID: 1E6J) [19] with a software system molecular operating environment (MOE)-Dock (Docking software). The docking simulation proposed one possible model that the GAPDH helix 10 (255–267), which is located at the surface of GAPDH, plays a role in the interaction between GAPDH-*c* and MA or CA-NTD (Fig. 2A). Because GAPDH actually exists as a stable tetramer, which is in equilibrium with a metastable dimer, it is possible that GAPDH tetramer interacts with more than two proteins using same region. Therefore, on the basis of these deduced interaction domains, 5 single-point and 5 multiple-point mutants of the helix 10 domain of GAPDH were prepared to perform mutagenesis study (Fig. 2B).

In Y2H analysis using GAPDH mutants and WT MA, as shown in the Fig. 3A, the K260E and Q264A of GAPDH mutants retained their ability to interact with WT MA. In contrast, the D256R, K263E or E267R of GAPDH mutants showed the loss of the ability to bind to WT MA. In addition, combined mutations except for D256R/K260E (M1) caused the loss of interaction. Western immunoblot analysis indicated that these effects were apparently not due to the low expression levels of the bait and prey proteins (Fig. 3B). On the other hand, the docking simulation predicted that residues Arg^{58}, Gln^{59} and Gln^{63} of MA formed an ion or a hydrogen bond network with Asp^{256}, Lys^{263} and Glu^{267} of GAPDH. As shown in the Fig. 3C, the R58E, Q59A and Q63A of MA mutants did not interact with GAPDH-*c*. These effects were also

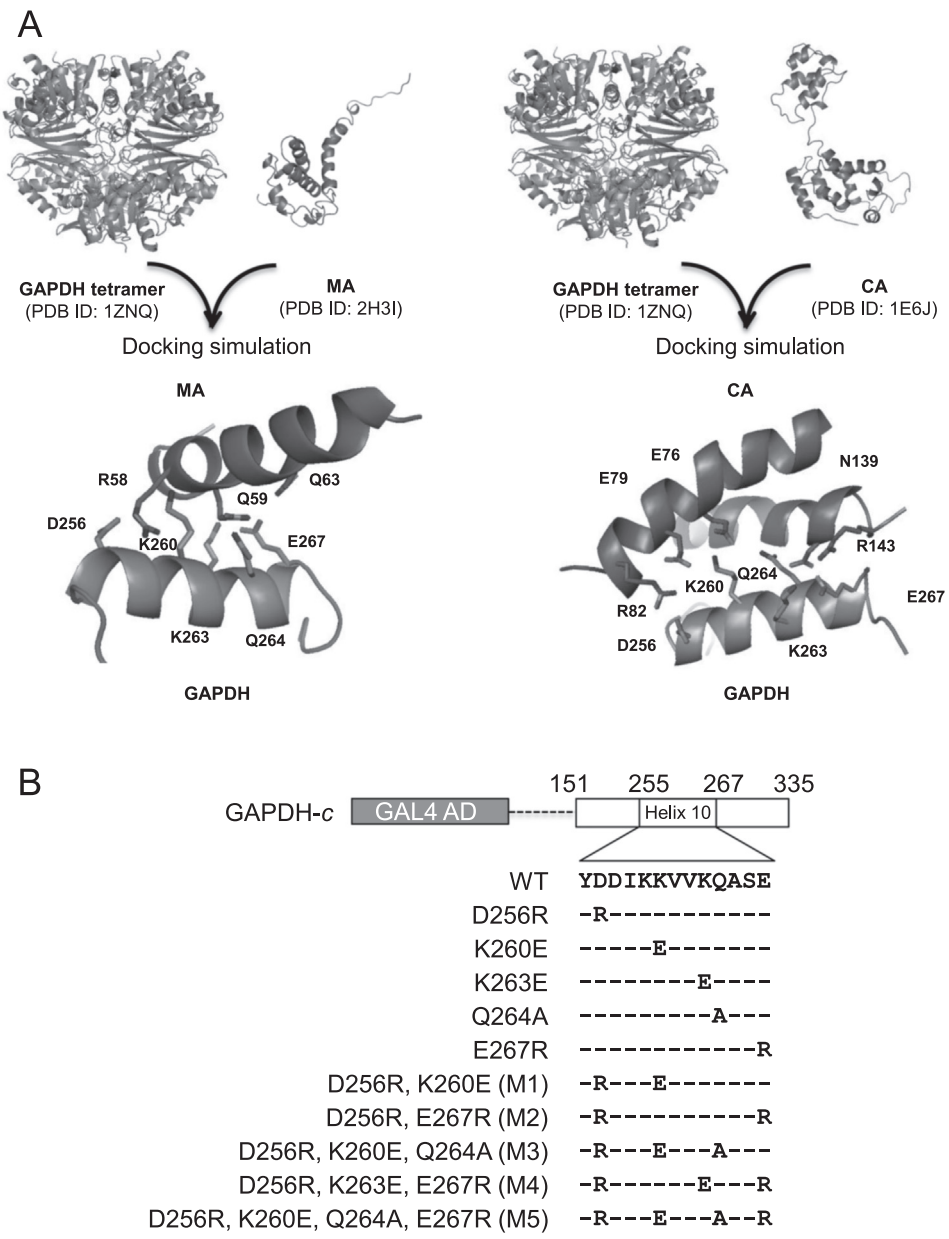

Fig. 2. *Deduced interaction between GAPDH and MA or CA.* (A) Proposed models for the interaction of GAPDH with MA (left) or CA (right). (B) GAPDH constructs mutated in GAPDH-*c* in Y2H analysis.

not due to the low protein expression levels of the bait and prey proteins (Fig. 3D). These findings suggest that Asp^{256}, Lys^{263} and Glu^{267} of GAPDH are crucial for the interaction between GAPDH and MA.

Since the docking simulation proposed that GAPDH helix 10 is also required for the interaction with CA-NTD, Y2H analysis focusing on the interaction GAPDH and CA-NTD was carried out. The D256R, K260E, K263E, Q264A and E267R of GAPDH single-point mutants maintained the interaction between GAPDH and CA-NTD (Fig. 3E). In contrast, the multiple-point mutations of GAPDH, D256R/K260E (M1), D256R/K260E/Q264A (M3) and D256R/K260E/Q264A/E267R (M5) lacked the binding ability to the WT CA-NTD. These results suggest that both of Asp^{256} and Lys^{260} of GAPDH play an important role in GAPDH interaction with CA-NTD. Furthermore, the docking simulation predicted that Asp^{256} and Lys^{260} of GAPDH interact with Arg^{82}, Glu^{79} and Glu^{76} of CA-NTD. Therefore, we prepared the E76R, E79R and R82E of CA-NTD mutants and coexpressed them with GAPDH-*c*. As shown in the Fig. 3G, the E79R of CA-NTD mutant retained its ability to interact, but the E76R and R82E mutants lost their ability to interact with GAPDH-*c*. These effects were apparently not due to the low expression levels of the bait and prey proteins (Fig. 3F and H). These results suggest that GAPDH-*c* requires Asp^{256} and Lys^{260} to bind to CA-NTD.

3.3. Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} of GAPDH are critical residues for tRNA^{Lys3}-packaging suppression effect

The Y2H analysis demonstrated that GAPDH helix 10 contributes to the interaction of GAPDH with both MA and CA-NTD. Because MA and CA exist as the precursor protein Pr55^{gag} in HIV-1 producer cells, we next prepared the D256R/K260E/K263E/E267R mutant (M6) of GAPDH (Fig. 4A). To examine whether M6 GAPDH interacts with WT Pr55^{gag}, M6 GAPDH and Pr55^{gag} were coexpressed in the Y2HGold strain. M6 GAPDH did not show interaction with WT Pr55^{gag}, MA or CA-NTD (Fig. 4B, left panel), despite the expression levels being sufficient for examining protein-protein interaction (Fig. 4B, right

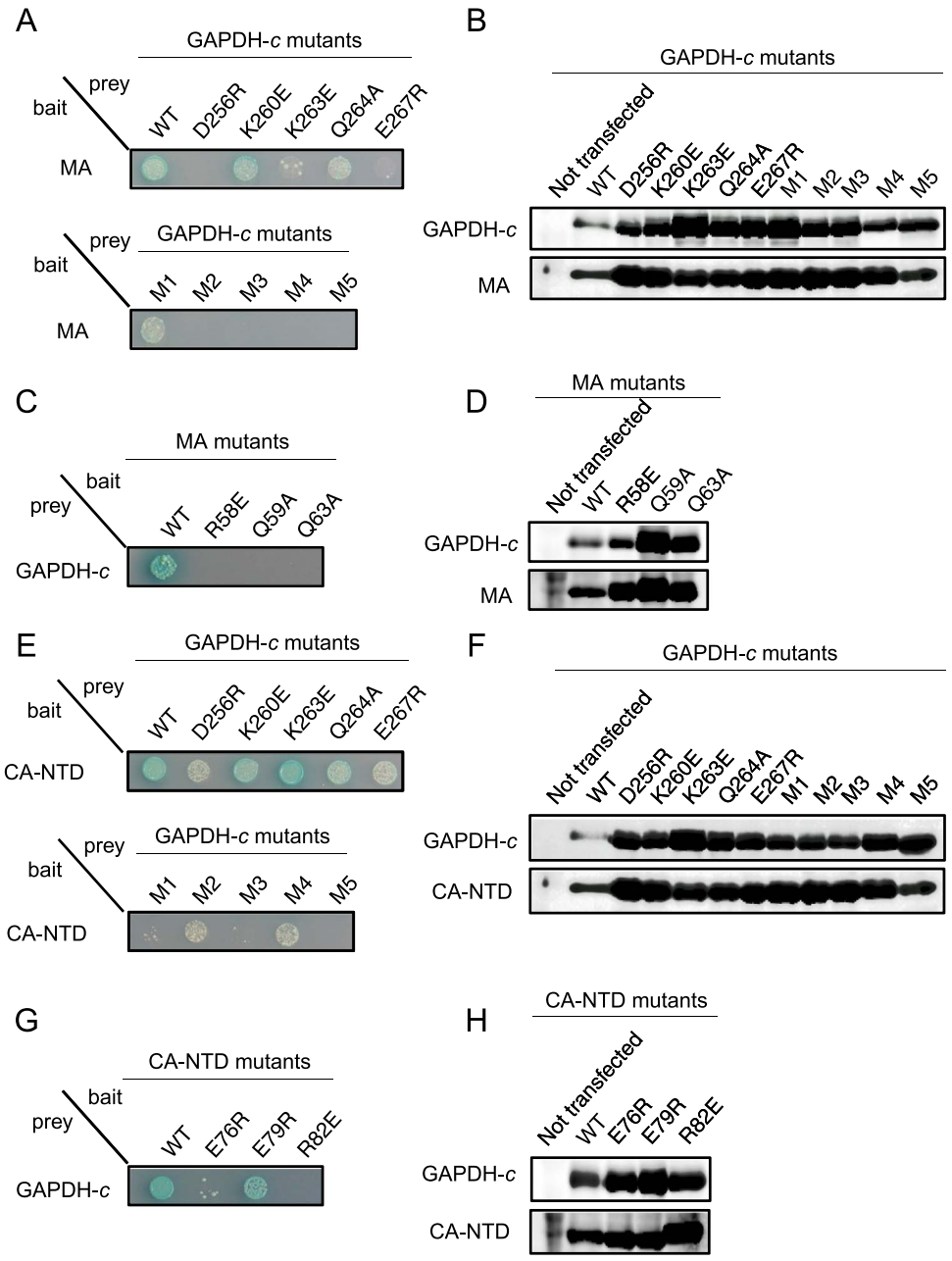

Fig. 3. *Y2H analysis of the interaction between C-terminal domain of GAPDH (GAPDH-c) and MA or CA-NTD.* (A) The *C*-terminal domain of WT or GAPDH mutants was used as prey proteins, and WT MA was used as the bait protein. (B) Bait and prey proteins expression level in Y2HGold strain using Fig. 3A. (C) WT or MA mutants were used as bait proteins, and WT GAPDH-*c* was used as the prey protein. (D) Bait and prey proteins expression level in Y2HGold strain using Fig. 3C. (E) The *C*-terminal domain of WT or GAPDH mutants was used as prey proteins, and WT CA-NTD was used as the bait protein. (F) Bait and prey proteins expression level in Y2HGold strain using Fig. 3E. (G) WT or CA-NTD mutants were used as bait proteins, and WT GAPDH-*c* was used as the prey protein. (H) Bait and prey proteins expression level in Y2HGold strain using Fig. 3G. Western immunoblot analysis of total protein extracts from each transformed Y2HGold strain was performed using the anti-HA antibody (against prey proteins) and the anti-c-Myc antibody (against bait proteins), respectively.

panel), suggesting that Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} of GAPDH are important for the interaction between GAPDH and $Pr55^{gag}$.

We previously reported that viruses produced from GAPDH-overexpressing cells enhanced GAPDH packaging and suppressed $tRNA^{Lys3}$ packaging [14]. Therefore, V5-tagged WT or M6 GAPDH expression vectors were prepared and cotransfected into HEK293 cells with pNL-CH to investigate whether the viruses produced from M6-GAPDH-expressing cells showed suppressed GAPDH packaging and restored $tRNA^{Lys3}$ packaging efficiency. We first performed coimmunoprecipitation assay to validate whether endogenous GAPDH and exogenous V5-tagged GAPDH retained oligomatic formation, because GAPDH exists primarily as a homotetramer in the cytoplasm [17]. The assay showed that both V5-tagged WT and M6 GAPDH retained oligomatic formation with endogenous GAPDH in HIV-1 producer cells (Fig. 4C), suggesting that M6 did not affect the oligomatic formation of GAPDH. Furthermore, overexpression of V5-tagged WT GAPDH in HIV-1 producer cells (Fig. 4D, WT lane of producer cells) increased the endogenous and V5-tagged WT GAPDH incorporation levels in viruses (Fig. 4D, WT lane of viral particles) as previously described [14]. However, as expected, a similar level of expression of M6 GAPDH (Fig. 4D, M6 lane of producer cells) decreased the incorporation levels of both endogenous and V5-tagged M6 GAPDH (Fig. 4D, M6 lane of viral particles) in comparison with that of WT GAPDH. To address whether the decreased incorporation level of V5-tagged M6 GAPDH depended on the poorer interaction of GAPDH with $Pr55^{gag}$ than V5-tagged WT GAPDH, we examined the interaction level in the virus

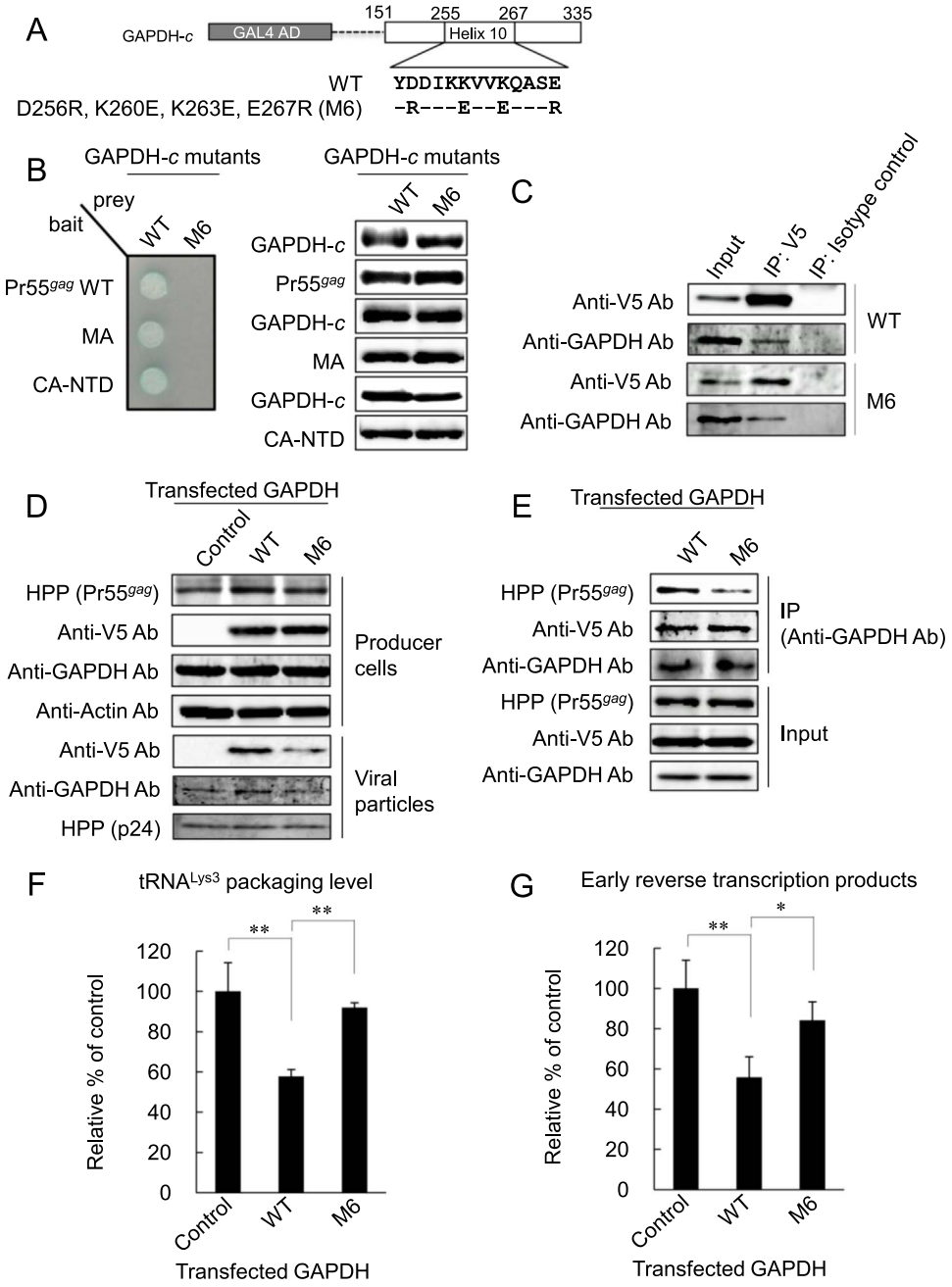

Fig. 4. *Effect of mutations of Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} in GAPDH on HIV-1 replication.* (A) To validate the critical residues of GAPDH, D256R/K260E/K263E/E267R mutation (M6) was introduced in GAPDH. (B) Y2H analysis of interaction between M6 GAPDH and MA or CA-NTD. The M6 GAPDH prey and MA or CA-NTD bait vector were cotransfected into Y2HGold. M6 GAPDH abrogated the GAPDH interaction with MA and CA-NTD (left panel), although each protein was expressed (right panel). (C) Coimmunoprecipitation assay of endogenous and exogenous GAPDH (V5-tagged WT or M6 GAPDH). (D) GAPDH expression in HIV-1 producer cells and incorporation level of GAPDH in viral particles. V5-tagged WT or M6 GAPDH was used to distinguish between endogenous and exogenous GAPDH. HEK293 cells were cotransfected with pNL-CH and V5-tagged WT, M6 GAPDH or empty (indicated as control) expression vector. Pr55^{gag} and p24 were detected by HIV-1-positive plasma (HPP). (E) Coimmunoprecipitation assay of GAPDH and Pr55^{gag}. (F) Packaging level of tRNA^{Lys3}. The amount of tRNA^{Lys3} in the control virus was set as 100%. (G) Effects of each virus that is produced from cells transfected with WT or M6 GAPDH vector on early reverse transcription products in TZM-bl cells. The value in the control experiment was set as a 100%. The significance of difference (Nonrepeated measures ANOVA and Dunnett's test versus WT) is indicated as follows: **, $p < 0.01$; *, $p < 0.05$. The error bars denote the standard deviation. The mean values of at least three independent experiments are shown.

producer cells by coimmunoprecipitation assay. As a result, the interaction between oligomatic GAPDH, which was composed of endogenous GAPDH and V5-tagged WT or M6 GAPDH, and Pr55^{gag} was found to be weaker in V5-tagged M6 GAPDH-expressing cells (Fig. 4E, M6 lane of IP) than in V5-tagged WT GAPDH-expressing cells (Fig. 4E, WT lane of IP). This finding suggests that the amino acid residues Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} of GAPDH play critical roles in the interaction of GAPDH with Pr55^{gag} and GAPDH packaging into virions. Finally, we examined the effects of M6 GAPDH on the viral replication by measuring tRNA^{Lys3} packaging level and reverse transcription products. Although the expression of WT GAPDH suppressed packaging of tRNA^{Lys3} as previously described, the expression of M6 GAPDH rescued packaging of tRNA^{Lys3} (Fig. 4F). Furthermore, the levels of early reverse transcription products were also recovered by M6 GAPDH expression in virus producer cells (Fig. 4G). These findings indicate that the Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} residues within GAPDH are critical for the mechanism of tRNA^{Lys3}-packaging suppression and that M6 GAPDH acts as a dominant negative regulator of

HIV-1 replication.

4. Discussion

During HIV-1 assembly, the selective cellular $tRNA^{Lys3}$ packaging is required for the effective reverse transcription [6]. Thus, the disruption of the interaction between the $Pr55^{gag}$/$p160^{gag\text{-}pol}$/viral genome RNA complex and the $tRNA^{Lys3}$/LysRS complex likely provides a novel therapeutic strategy. Interestingly, we previously identified cellular GAPDH inside virions as a $tRNA^{Lys3}$ packaging inhibitor and demonstrated that the inhibitory mechanism is dependent on the interaction between cellular GAPDH and HIV-1 precursor proteins ($Pr55^{gag}$ and $p160^{gag\text{-}pol}$) [14]. However, it remained unclear how GAPDH interacts with these proteins. Our findings indicate that $tRNA^{Lys3}$ packaging is interrupted by the interaction of GAPDH with MA and CA-NTD translated as part of viral precursor proteins. The GAPDH mutagenesis assay indicated that Asp^{256}, Lys^{260}, Lys^{263} and Glu^{267} of GAPDH are important residues in the interaction of GAPDH with MA and CA-NTD. Importantly, the crystal structure of GAPDH tetramer (PDB ID: 1ZNQ) [17] shows that all of these amino acids in helix 10 are exposed on four each of monomer GAPDH, suggesting that two subunits of the GAPDH tetramer simultaneously interact with MA and CA-NTD, respectively (Supplementary Fig. 2A and B). On the other hand, Y2H analysis demonstrated that the R58E, Q59A or Q63A of MA, and E76R or R82E of CA-NTD mutants abrogated their interaction with the *C*-terminal domain of GAPDH. MA (Arg^{58}, Gln^{59} and Gln^{63}) or CA (Glu^{76} and Arg^{82}) residues, which contribute to ionic or hydrogen bond interaction with Asp^{256}, Lys^{260}, $Lys^{263}$and Glu^{267} of GAPDH, are also exposed on the surface of MA or CA and located on the same side of MA helix 3 or CA helix 4 (Supplementary Fig. 2A). Interestingly, a MOE candidate model (Fig. 2A) conferred a somewhat different helix orientation between GAPDH-MA or GAPDH-CA-NTD (Supplementary Fig. 2C) and was supported by Y2H analysis and data regarding oligomatic formation, $tRNA^{Lys3}$ packaging level and reverse transcription using M6 GAPDH. Furthermore, we tried to prepare mutant pNL-CH proviral clones encoding R58E/Q59A/Q63A in MA and E76R/R82E in CA to clarify the effects of these mutations on $tRNA^{Lys3}$ incorporation. The mutated pNL-CH expressed a similar level of the virus precursor protein in HIV-1 producer cells to WT pNL-CH. However, we did not examine whether the $tRNA^{Lys3}$ incorporation level inside virions was increased because the mutational introduction within the capsid-coding gene (E76R, R82E) impaired HIV-1 budding (data not shown).

Many studies have denoted how $tRNA^{Lys3}$ is incorporated into virions. The findings of such studies demonstrated that the $tRNA^{Lys3}$/LysRS complex interacts with the $Pr55^{gag}$/$p160^{gag\text{-}pol}$/viral genome RNA complex and is efficiently packaged into virions [4,5,9,20,21]. Javanbakht et al. [5] demonstrated that the domains critical for the $Pr55^{gag}$-LysRS interaction are mapped to include the dimerization domains of both LysRS and CA. Kovaleski et al. [20] more specifically reported that the interaction between LysRS and $Pr55^{gag}$ is dependent on the helix 7 of LysRS and the helix 4 of CA-CTD of $Pr55^{gag}$. In addition, Khorchid et al. [21] reported that the interaction between $tRNA^{Lys3}$ and $p160^{gag\text{-}pol}$ is involved in the thumb domain sequence of reverse transcriptase (RT). However, the amounts of all $tRNA^{Lys}$s ($tRNA^{Lys1,2}$ and $tRNA^{Lys3}$) and non-$tRNA^{Lys}$s incorporated into virions are significantly increased when $p160^{gag\text{-}pol}$ is present with $Pr55^{gag}$ [22]. Thus, Kleiman et al. [23] reviewed that $p160^{gag\text{-}pol}$ probably increases the incorporation of all tRNAs into $Pr55^{gag}$ virus-like particles through the nonspecific binding of tRNAs to RT sequences within $p160^{gag\text{-}pol}$. Since there is no evidence that RT sequences in $p160^{gag\text{-}pol}$ show a preference for interacting with $tRNA^{Lys3}$ and not with other tRNAs, $Pr55^{gag}$ does specifically interact with LysRS to play an important role in concentrating $tRNA^{Lys3}$ in the virions. These findings suggest that by sterically inhibiting the interaction between the $Pr55^{gag}$ and $tRNA^{Lys3}$/LysRS complex, the GAPDH tetramer efficiently suppresses the incorporation of $tRNA^{Lys3}$ into the virions. Taken together, these findings indicate that increasing the stability of the GAPDH tetramer or shifting the equilibrium toward the tetramer by increasing the expression level of GAPDH in HIV-1-infected cells might provide an effective approach to interrupt the $tRNA^{Lys3}$ packaging into virions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2016.09.015.

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