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Gag Regulates Association of Human Immunodeficiency Virus Type 1 Envelope with Detergent-Resistant Membranes

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Assembly of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein on budding virus particles is important for efficient infection of target cells. In infected cells, lipid rafts have been proposed to form platforms for virus assembly and budding. Gag precursors partly associate with detergent-resistant membranes (DRMs) that are believed to represent lipid rafts. The cytoplasmic domain of the envelope gp41 usually carries palmitate groups that were also reported to confer DRM association. Gag precursors confer budding and carry envelope glycoproteins onto virions via specific Gag-envelope interactions. Thus, specific mutations in both the matrix domain of the Gag precursor and gp41 cytoplasmic domain abrogate envelope incorporation onto virions. Here, we show that HIV-1 envelope association with DRMs is directly influenced by its interaction with Gag. Thus, in the absence of Gag, envelope fails to associate with DRMs. A mutation in the p17 matrix (L30E) domain in Gag (Gag L30E) that abrogates envelope incorporation onto virions also eliminated envelope association with DRMs in 293T cells and in the T-cell line, MOLT 4. These observations are consistent with a requirement for an Env-Gag interaction for raft association and subsequent assembly onto virions. In addition to this observation, we found that mutations in the gp41 cytoplasmic domain that abrogated envelope incorporation onto virions and impaired infectivity of cell-free virus also eliminated envelope association with DRMs. On the basis of these observations, we propose that Gag-envelope interaction is essential for efficient envelope association with DRMs, which in turn is essential for envelope budding and assembly onto virus particles.

Lipid rafts are tightly packed liquid-ordered microdomains enriched in cholesterol, sphingolipids, and glycerophospholipids. The existence of lipid rafts on cell membranes is controversial. Lipid rafts have been defined biochemically as membranes that are resistant to detergent at low temperature (41) and are frequently described as detergent-resistant membranes (DRMs) (5, 6, 40). DRMs can be separated by ultracentrifugation of detergent-lysed cells in sucrose gradients. It is possible that such methods of isolation may affect cell structure and create lipid rafts as an artifactual phenomenon (reviewed in reference 40). Nevertheless, the association of particular membrane proteins with DRMs has frequently been shown to have strong physiological relevance. Thus, lipid rafts have been implicated as platforms for signal transduction and cell activation (39), and it has been suggested that they are involved in the traffic and sorting of membrane proteins at sites throughout the cell, including the endoplasmic reticulum, Golgi complex, cell membrane, and vesicles (24, 30, 31, 39). Rafts may also act as platforms for budding and assembly of enveloped viruses (41, 43). The glycoproteins of several enveloped viruses, such as influenza virus (46), Rous sarcoma virus (32), murine leukemia virus (25), measles virus (27), Ebola virus (2), and human (HIV) and simian (SIV) immunodeficiency virus (4, 38, 45) have been shown to associate with DRMs. As a result, there is now much attention focused on the importance of lipid rafts as a docking site for the assembly of many enveloped viruses.

The assembly and incorporation of human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins onto virions have been shown to be regulated by a stable interaction between the matrix domain of the p55^{gag} precursor and envelope (gp41) cytoplasmic domain (12–16, 21). The matrix domain is myristoylated, and this fatty acid group targets the p55 precursor to membranes (17, 22, 33, 35) and rafts (34). The envelopes of HIV-1, HIV-2, and SIVs carry cysteine residues in the cytoplasmic domains that are palmitoylated and also reported to promote association with rafts and assembly onto budding virions (4, 38, 45). In HIV-1, two relatively conserved cysteine residues occur at the envelope positions 764 and 837. We previously confirmed that these gp41 cytoplasmic-domain cysteines are important for HIV-1 envelope association with lipid rafts. Nevertheless, nearly wild-type (wt) levels of infectivity are retained if C764 and C837 are both substituted by amino acids with bulky hydrophobic side chains (4). The latter envelopes failed to associate with light lipid rafts (DRM-L) but retain association with heavier DRM-H (heavy lipid raft) fractions. In contrast, substitution of C764/C837 by alanine residues eliminated envelope association with rafts and decreased infectivity by over 60%. In contrast, Chan et al. reported that the same gp41 mutations failed to affect envelope association with DRMs (8).

Our previous study was carried out in 293T cells transfected with vectors that encode envelope and all other viral proteins (4). Here, we investigated the roles of other HIV proteins, including Gag in the recruitment of HIV-1 envelope glycopro-

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teins to DRMs and subsequent envelope assembly onto newly synthesized virions. Our results demonstrate that Gag recruits envelope to DRMs, thus facilitating the assembly of envelope glycoproteins onto budding virions.

MATERIALS AND METHODS

Reagents and antibodies. β-Methyl cyclodextrin, protease inhibitor cocktail, and EGTA were obtained from Sigma Chemical Co., St. Louis, MO. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was from Fisher Bio-reagents, Inc. Anti-mouse $F(ab')_2$ fragment conjugated to β-galactosidase was from Southern Biotechnology, Inc. Polyclonal anti-rabbit caveolin was obtained from BD Transduction Laboratory, Inc. Monoclonal antibodies (MAbs) to gp41, Chessie 8 (9), and 183-H12-5C (42) to $p55^{gag}$ were provided by the U.S. NIH AIDS Research and Reference Program. Hybridoma supernatant containing p24 MAbs 38:96K and EF7 were provided by the Centralized Facility for AIDS Reagents, Potters Bar, United Kingdom.

Plasmids and molecular constructs. HIV-1 molecular clones pNL4.3 and pHXB2 were described previously (3, 37). pSV2tat72 (11) was obtained from the U.S. NIH Reagents and Reference Program. gp160 envelope fragments were cloned into KpnI sites in the pSVIIIenv vector (36). The LN40 and B33 envelope sequences were amplified from lymph node (LN) and brain autopsy tissue, respectively, of patient NA420 as described previously (36). The L30E substitution in p17 matrix of env^- pNL4.3 molecular clone was prepared by PCR. The pNL4.3 L30E clone was provided by Eric Freed (National Cancer Institute, NCI-Frederick). Point mutations in NA420 LN40 gp41 cytoplasmic domains were introduced by PCR.

Cell culture and transfection. Human embryonic kidney (293T) cells, $CD4^+$ $CXCR4^+$ and $CD4^+$ $CCR5^+$ GHOST and NP2 cells were maintained in Dulbecco's modified Eagle's medium with 4% fetal bovine serum (FBS). pSVIIIenv constructs were cotransfected with env^- pNL4.3 into 293T cells using calcium phosphate precipitation method (Promega, Inc.). Progeny pseudovirions were harvested at 48 h, clarified by centrifugation (1,000 × *g* for 10 min), aliquoted, and stored at −152°C.

Cell-cell fusion. Cell-cell fusion was tested by cocultivating 293T cells cotransfected with env^+ pSVIIIenv and env^- pNL4.3 with target cells that expressed CD4 and appropriate coreceptors. GHOST or NP2 cells were seeded at 2×10^4 cells per well in 48-well tissue culture dishes. Fifteen to 18 h posttransfection, 5×10^5 Env^+ 293T cells were cocultivated with an equal amount of appropriate $CD4^+$, coreceptor-positive target cells. After 6 to 8 h of incubation, cells were washed in phosphate-buffered saline (PBS) and fixed for 10 min with methanol containing 1% methylene blue and 0.25% basic fuchsin. Fixed cells were destained within PBS and examined for syncytium formation by microscopy.

Preparation of VSV-G^+ HIV-1 particles. A vesicular stomatitis virus VSV-G expression vector was cotransfected with either pNL4.3 wt or pNL4.3 L30E into 293T cells using calcium phosphate precipitation method (Promega, Inc.). Progeny pseudovirions were harvested at 48 h, clarified by centrifugation (1,000 × *g* for 10 min), aliquoted, and stored at −152°C.

Infectivity assay, p24 immunostaining, and RT ELISA. $CD4^+$, $CXCR4^+$, or $CCR5^+$ GHOST and NP2 cells were infected with serial dilutions of pseudoviruses in duplicate. VSV-G^+ pseudovirions were titrated onto HeLa cells that lacked CD4. Infected cells were washed 72 h postinfection with PBS and fixed in cold (−20°C) methanol-acetone (1:1) for 10 min. Cells were washed once with PBS and then with PBS containing 1% FBS (PBS–1% FBS) before adding anti-HIV p24 monoclonal antibodies (38:96K and EF7) as hybridoma cell culture fluid (1:40) in PBS–1% FBS. After 1 h, cells were washed with PBS–1% FBS and incubated for 1 hour with anti-mouse β-galactosidase (1:400) in PBS–1% FBS. Cells were washed once with PBS–1% FBS and twice with PBS and stained with the addition of X-Gal substrate (0.5 mg/ml of 5-bromo-4 chloro-3-indolyl-β-D-galactopyranoside in PBS containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mM magnesium chloride). The resultant blue-stained cells were regarded as foci of infection, and the virus infectivity was estimated as focus-forming units (FFU) per ml. The reverse transcriptase (RT) activity of cell-free viruses was estimated by RT–enzyme-linked immunosorbent assay (ELISA) (CavidiTech, Inc., Uppsala, Sweden). Infectivity titers were expressed as FFU/RT activity.

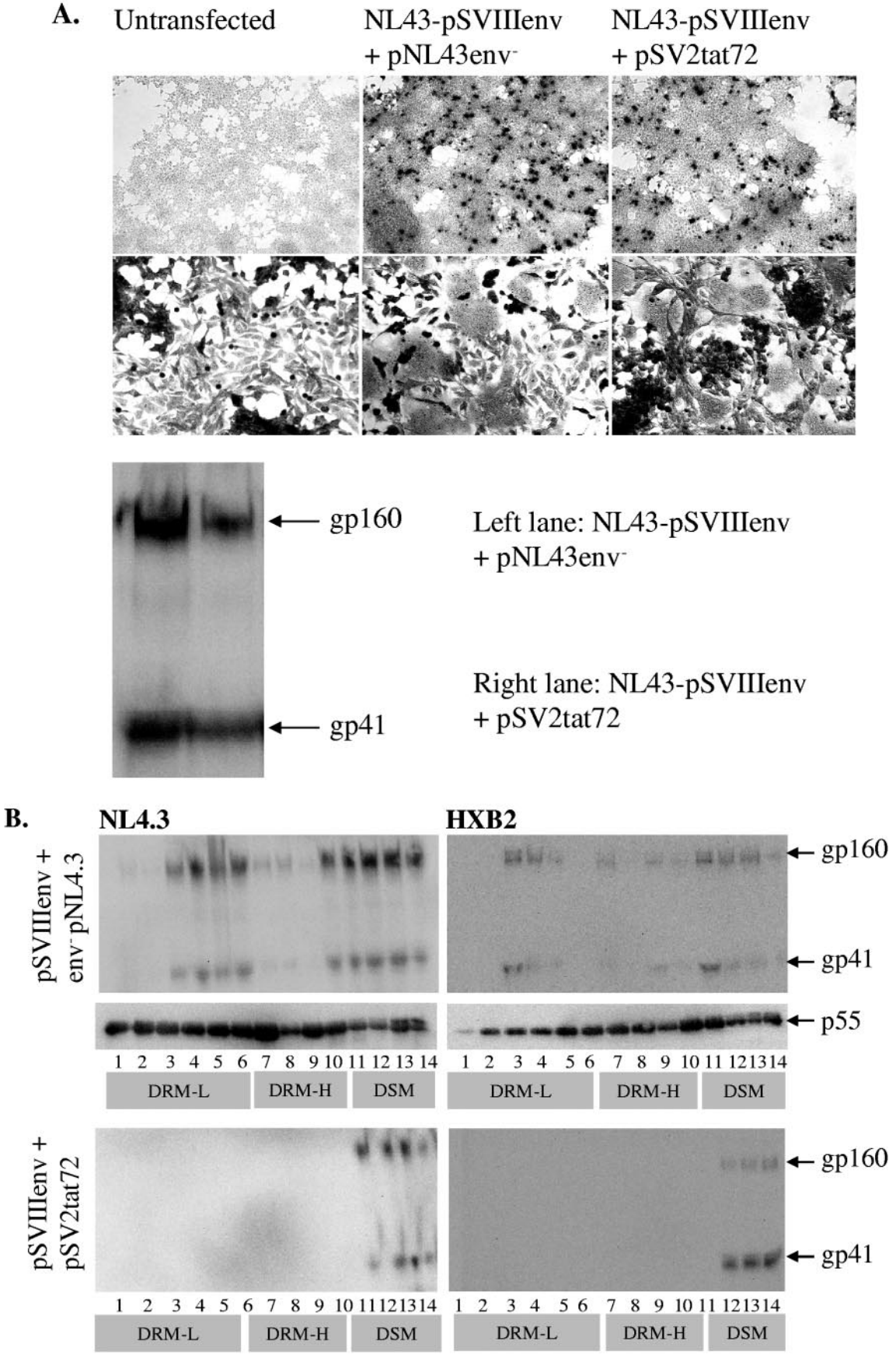
Isolation of detergent-resistant membranes and sucrose density centrifugation. gp160 expressing 293T or MOLT 4 cells were lysed with Triton X-100 at a final concentration of 0.5% in TNE buffer (10 mM Tris [pH 7.5], 100 mM sodium chloride, and 10 mM EGTA) supplemented with protease inhibitor cocktail for 30 min on ice. Cellular lysates were homogenized and centrifuged at low speed to remove nuclei. Supernatants were adjusted to 60% sucrose in 1-ml volume and loaded on top of 250 μl of 80% sucrose. Subsequent sucrose gradients of 50%, 40%, 35%, 10%, and 5%, respectively, were layered on top to a final volume of 5 ml. These steps were carried out on ice. Sucrose gradients were centrifuged at 100,000 × *g* for 18 h at 4°C in a SW 50.1 Ti rotor (Beckman, Inc.). Fourteen fractions were collected from the top, and each fraction was immunoprecipitated with HIV-positive human serum (1:1,000). Immunoprecipitated samples were analyzed for envelope (gp160 and gp41) and p55 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting, using monoclonal antibodies Chessie 8 and 183-H12-5C to probe envelope and $p55^{gag}$, respectively. The density of each fraction was quantified using a refractometer.

Western blotting and cell lysis. The efficiency of envelope expression was assessed by Western blotting. Env^+ cells were lysed in 1% Triton X-100 supplemented with protease inhibitor cocktail and homogenized with a 30-gauge syringe needle (three times). Debris was removed by microcentrifugation. Lysate supernatants were resolved by SDS-PAGE before blotting and probing for gp160/gp41 with MAb Chessie 8 and for p24 with MAb 183-H12-5C.

Envelope incorporation onto virus particles. To investigate envelope incorporation onto virus particles, 293T cells were cotransfected with env^+ pSVIIIenv constructs together with env^- pNL4.3. Env^+ pseudotype viruses were harvested at 48 h, centrifuged at low speed, and passed through 0.45-μm-pore-size syringe filters to remove cell debris. Virus particles were pelleted at 100, 000 × *g* for 2 h at 4°C, and pellets were reconstituted in PBS. The RT activity of each pellet was measured by RT-ELISA, and equal amounts of RT activity were then resolved by SDS-PAGE before blotting and probing for gp160/gp41 with MAb Chessie 8 and for p24 with MAb 183-H12-5C.

RESULTS

Envelope association with lipid rafts is eliminated in the absence of the Gag precursor. We first investigated whether HIV-1 envelope required other viral proteins to associate with DRMs. Envelope was expressed from pSVIIIenv, which encodes envelope and Rev but also requires Tat for efficient envelope expression. 293T cells were therefore cotransfected with pSVIIIenv (NL4.3 envelope) with either env^- pNL4.3 (encodes all HIV-1 proteins except envelope) or the Tat expression vector pSV2tat72. We examined envelope expression in the transfected 293T cells by immunostaining with Chessie 8, an anti-gp41 MAb. As shown in Fig. 1A (top row of micrographs), envelope expression was detected for both cotransfections. Envelope expression via each cotransfection also conferred equivalent levels of cell-cell fusion GHOST/CXCR4 cells (Fig. 1A, bottom row of micrographs). Envelope expression was also evaluated by Western blotting of cell lysates. Slightly larger amounts of envelope were detected for cotransfections of pSVIIIenv and Env^- pNL4.3 compared to cotransfections with pSVIIIenv and pSV2tat72 (Fig. 1A, blot). We next tested envelope association with DRMs using flotation gradient centrifugation as described in Materials and Methods. Thus, transfected env^+ 293T cells were lysed with Triton X-100 to a final concentration of 0.5% in TNE buffer at 4°C. Subsequently, postnuclear supernatant contents were fractionated by sucrose density gradient centrifugation, and each fraction was analyzed for envelope content. As shown in Fig. 1B, in the absence of viral proteins other than Tat and Rev, envelope was excluded from the classical raft fractions (DRM) with most of envelope present in the detergent-sensitive membrane (DSM) fraction. When all other viral proteins were present, envelope was clearly detected in DRM-L and to some extent in DRM-H fractions. Densitometry measurements revealed that >96% of gp160 was present in the DSM fractions when envelope was expressed with just Tat and Rev. However, 19.4% of gp160 was present in DRM-L and 12% in DRM-H when all other viral



proteins were present. These observations are similar to those of our previous report (4). Most HIV-1 strains have two cysteine residues in the gp41 cytoplasmic domain at positions 764 and 837 that are targets for palmitoylation (45). However, NL4.3 contains only one cysteine residue at residue 764. We therefore also tested the HXB2 envelope that has both cysteine residues. Figure 1B shows that in the absence of viral proteins other than Tat and Rev, HXB2 envelope also failed to associate with DRM with >96% of gp160 present in the DSM fractions. This result confirmed that envelope was unable to associate with lipid rafts without the cooperation of one or more viral proteins.

A matrix mutation that abrogates envelope incorporation onto virions disrupts envelope assembly in lipid rafts. Since it has been reported that the envelope interacts directly with the matrix domain of the $p55^{gag}$ precursor, we next investigated the role of Gag in envelope association with DRMs. A single specific amino acid substitution in the matrix (p17) domain of the Gag protein was previously shown to eliminate envelope incorporation onto virus particles (16). The L30E substitution was therefore introduced into the matrix domain of the *env*$^-$ pNL4.3 construct to create pNL4.3 (L30E) env. pSVIIIenv (NL4.3 envelope) was cotransfected with *env*$^-$ pNL4.3 (L30E) in 293T cells, and pseudotype viruses were harvested. Immunostaining of the transfected 293T cells with MAb Chessie 8 (Fig. 2A, left panels) and cell-cell fusion assays (Fig. 2A, right panels) confirmed that the envelope protein was expressed and functional for fusion. Similar levels of envelope expression after cotransfection with either Env$^-$ pNL4.3 wt or L30E, were also indicated by Western blotting of transfected cell lysates (data not shown). Harvested viruses were titrated on CD4$^+$ CXCR4$^+$ GHOST and NP2 cells to evaluate their infectivity and also subjected to Western blot analysis to assess the amount of envelope incorporated onto virions. As expected, the matrix mutation resulted in over 95-fold reduction in infectivity (Fig. 2B) and dramatic reduction in the amount of envelope incorporation onto virions (Fig. 2C).

We next evaluated the effect of the L30E matrix mutation on envelope association with DRMs. 293T cells were cotransfected with pSVIIIenv with either the *env*$^-$ pNL4.3 or *env*$^-$ pNL4.3 (L30E) construct. Env$^+$ 293T cells were washed, lysed, and processed for sucrose density gradient centrifugation as described above. When expressed in conjunction with a Gag L30E protein, envelope proteins were absent from DRM fractions, with >96% of gp160 in the DSM fractions and only trace amounts in DRM-H fractions (Fig. 3). In contrast, when wt Gag was present, substantial amounts of envelope (37% of gp160) were found in DRM-L. However, the L30E mutation had no effect on Gag (p55) association with raft fractions. In a final confirmatory experiment, pSVIIIenv and pSV2tat72 were cotransfected with and without pCMV-p55 that expresses only Gag. Again, envelope associated with DRMs only if Gag was expressed (data not shown). Together, these results show that envelope association with DRM-L is dependent on Gag.

Gag is required for envelope to associate with DRMs in MOLT 4 T-cells. The experiments described above were all based on the analysis of the human embryonic kidney 293T cell line. To evaluate whether the observations made were relevant for HIV replication in T cells, the role of Gag in envelope association with DRMs was investigated in the MOLT 4 T-cell line. Pseudovirions carrying the VSV-G glycoprotein were first prepared by cotransfecting 293T cells with pVSV-G and either pNL4.3 or pNL4.3 L30E full-length clone. MOLT 4 cells were then infected with VSV-G$^+$ NL4.3 or VSV-G$^+$ NL4.3 L30E virus at a multiplicity of infection of 1. After 72 h, cells were lysed with Triton X-100 at 4°C and analyzed by sucrose density gradient centrifugation as described in Materials and Methods. Envelope was associated with DRMs only in the presence of wt Gag (28% in DRM-L and 19% in DRM-H) and located only in the soluble fraction (>90%) when expressed with the mutated Gag L30E (Fig. 4). Thus, envelope association with DRMs required a functional Gag precursor in T cells as well as in 293T cells.

Do buoyant Gag particles affect envelope location in sucrose gradients? Gag particles are themselves buoyant (20, 26), and it was possible that such particles float up in sucrose gradients carrying envelope with them independently of DRM association. HIV virions have a buoyant density of about 1.15 g/ml in sucrose gradients (7). This density is at the highest end of the densities of DRM-L fractions (1.09 to 1.15 g/ml). The majority of buoyant envelope detected in DRM-L here conferred a range of densities generally lower than 1.15 g/ml. Treatment of 293T cells with 10 mM of β-methyl cyclodextrin to disrupt DRMs eliminated Gag from the DRM-L fractions but did not affect Gag association within the fractions that represent heavier rafts (DRM-H at 1.16 to 1.2 g/ml). The Gag in these fractions may thus contain buoyant Gag complexes as described by Lindwasser and Resh (26) and Holm et al. (20). Despite the presence of Gag in fractions associated with DRM-H, envelope was completely removed from all DRM-L

FIG. 1. Roles of viral proteins on HIV-1 envelope association with rafts. (A) Envelope expression and fusigenicity in the presence and absence of other viral proteins. 293T cells were cotransfected with pSVIIIenv (NL4.3 env) with either *env*$^-$ pNL4.3 or pSV2tat72 and were immunostained for envelope. Cells were fixed and permeabilized with methanol-acetone (1:1) 48 h after transfection before immunostaining with anti-gp41 MAb Chessie 8 (top row of micrographs). Cell-cell fusion and syncytium induction were detected by cocultivation of 293T cells expressing NL4.3 envelopes in the presence and absence of *env*$^-$ pNL4.3 with GHOST/CXCR4 cells. Cells were fixed and stained with 1% methylene blue and 0.25% basic fuchsin in methanol (bottom row of micrographs). Western blot of 293T cell lysate taken 48 h after transfection with pSVIIIenv (NL4.3 env) with either *env*$^-$ pNL4.3 or pSV2tat72 is shown below the micrographs. (B) HIV-1 envelope association with DRMs in the presence and absence of viral proteins other than Tat and Rev. 293T cells were cotransfected with pSVIIIenv which expressed Rev and contained either NL4.3 and HXB2 envelopes together with *env*$^-$ pNL4.3, which expresses all viral proteins except for envelope, or pSV2Tat72, which expresses Tat. Cells were lysed with cold 0.5% Triton X-100 in TNE buffer on ice. Lysates were homogenized, cleared of nuclei, adjusted to 60% sucrose, and applied to the bottom of a sucrose gradient (as described in Materials and Methods). Gradient fractions were Western blotted and probed for envelope (gp160 and gp41) and Gag (p55) with mouse MAbs. Note that both NL4.3 and HXB2 envelopes associate with DRM fractions in the presence of *env*$^-$ pNL4.3 but were excluded from rafts when expressed in the absence of *env*$^-$ pNL4.3 (bottom blots).

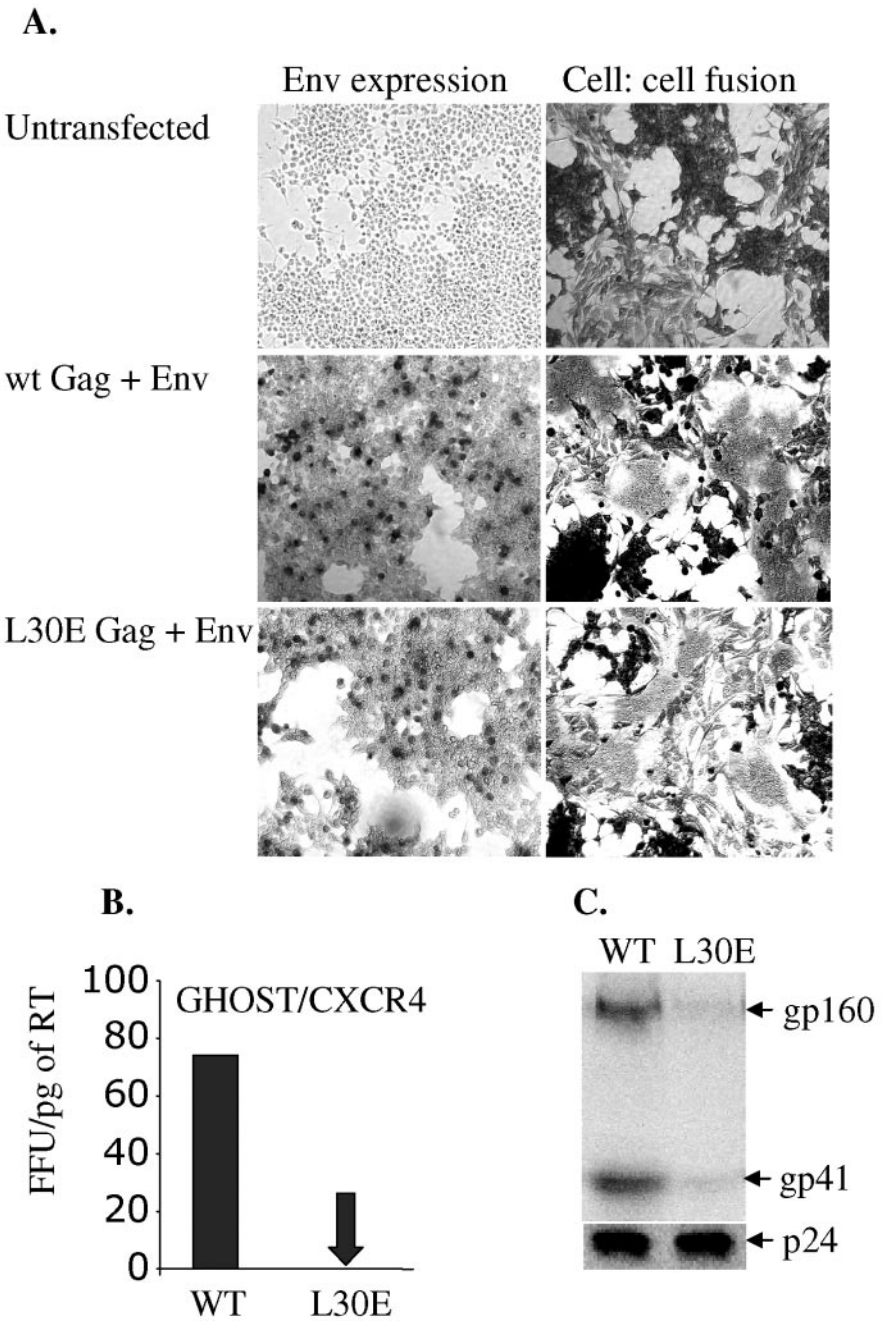


FIG. 2. Effect of a matrix mutation (L30E) on envelope function. (A) 293T cells were transfected with pSVIIIenv (NL4.3 env) with either *env*$^-$ pNL4.3 or *env*$^-$ pNL4.3 (L30E) and assessed for envelope expression (left panels) and cell-cell fusion (right panels). (B) Impact of L30E mutation in p17^{gag} on virus infectivity tested on GHOST/CXCR4 cells and represented as infectivity (FFU) to particle (RT) ratio. (C) Envelope incorporation into virus particles was assessed by measuring the amounts of envelope in transfected 293T cell supernatants. Virus samples were precipitated and resuspended in PBS, and equivalent amounts of RT activity were then resolved on a sodium dodecyl sulfate–8% polyacrylamide gel, followed by Western blotting. Envelope and p24 were probed with Chessie 8 and 183-H12-5C monoclonal antibodies, respectively.

and DRM-H fractions and transferred entirely to the DSM fractions, following β-methyl cyclodextrin treatment (Fig. 5). These observations are consistent with a specific association of envelope and DRM-L and do not support a role for buoyant Gag particles conveying envelope into the lighter fractions of the sucrose gradient.

A tissue-derived HIV-1 envelope, NA420 LN40, that fails to incorporate onto virions. An envelope gene that was PCR amplified from lymph node tissue of an AIDS patient (NA420 LN40) (36) encoded an envelope that was defective for infectivity. LN40 envelope contrasted with a related envelope amplified from brain tissue of the same patient (NA420 B33) that

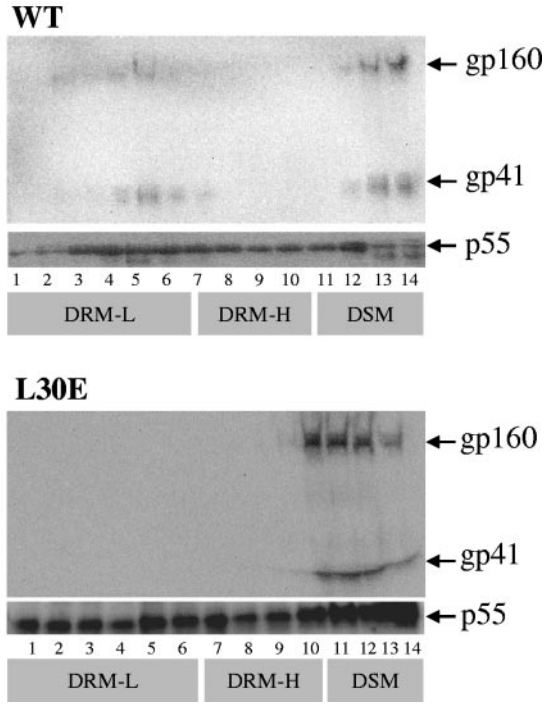


FIG. 3. Effect of matrix mutation on envelope association with lipid rafts. 293T cells were cotransfected with pSVIIIenv (NL4.3 env) construct and either *env*$^-$ pNL4.3 or *env*$^-$ pNL4.3 (L30E). Cells were lysed with cold 0.5% Triton X-100 and homogenized, and postnuclear supernatants were subjected to sucrose density gradient centrifugation as described in Materials and Methods. Gradient fractions were subsequently probed for envelope (gp160 and gp41) and Gag (p55) with mouse MAbs by Western blotting. Note that envelope proteins coexpressed with the L30E Gag mutation failed to associate with DRM-L (bottom panel) compared with that with wild-type Gag (top panel).

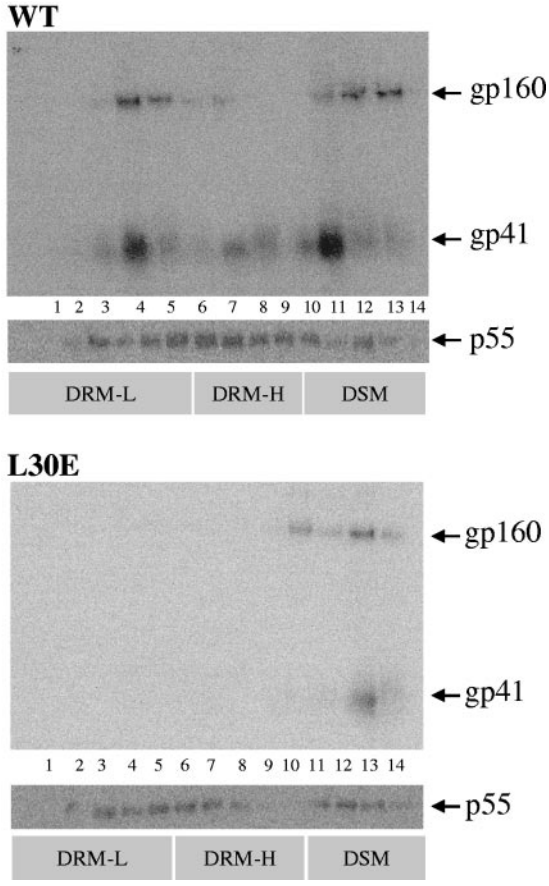


FIG. 4. Envelope association with lipid rafts in MOLT 4 T cells. MOLT 4 cells were infected with VSV-G$^+$ pseudotype virions prepared with either pNL4.3 wt or pNL4.3 (L30E). After 48 h, cells were lysed with cold 0.5% Triton X-100 and homogenized, and postnuclear supernatants were subjected to sucrose density gradient centrifugation as described in Materials and Methods. Gradient fractions were subsequently probed for envelope (gp160 and gp41) and Gag (p55) with mouse MAbs by Western blotting. Note that envelope proteins coexpressed with the L30E Gag mutation failed to associate with DRM-L (bottom panel) compared with that with wild-type Gag (top panel).

assembled efficiently onto virions and conferred high infectivity (36). Nevertheless, NA420 LN40 was efficiently expressed in 293T cells and functional for CCR5-dependent cell-cell fusion (Fig. 6B). In contrast to NA420 B33, LN40 envelope does not contain gp41 cytoplasmic-domain cysteine residues that are targets for palmitoylation and implicated for raft association. Nevertheless, LN40 envelope carried residues with bulky hydrophobic side chains (F764 and Y847) that we previously showed were sufficient to confer envelope association with heavy rafts and for nearly wild-type levels of envelope assembly and infectivity (4). In addition, LN40 envelope had further changes in sites reported to confer envelope-Gag interactions (12, 23, 28) (see Discussion). As shown in Fig. 6C and D, replacement of LN40 gp41 sequences with residues 758 to 856 from B33 sequences (B33$^{758\text{-}856}$) fully restored envelope incorporation onto virions and infectivity. This region encompasses the envelope cytoplasmic region that contains the cysteine residues and sites implicated in Gag interactions (28, 38, 45).

Envelope incorporation onto virions correlates with lipid raft association. We next tested whether the LN40 wt or LN40 (B33$^{758\text{-}856}$) envelopes could associate with rafts. 293T cells were transfected with pSVIIIenv encoding each of LN40 wt, B33 wt, and LN40 (B33$^{758\text{-}856}$) envelopes and pNL4.3 env$^-$. Env$^+$ 293T cells were lysed in cold Triton X-100 and processed for sucrose density centrifugation as described above. Fractions were collected and analyzed for the presence of envelope and Gag. As shown in Fig. 7, p55^{gag} was consistently detected in all fractions irrespective of envelope mutant constructs. However, NA420 LN40 wt envelope was found to be excluded from fractions representing DRMs and detected only in DSM fractions. Densitometry measurement showed that >96% of gp160 was present in DSMs. In contrast, B33 and LN40 (B33$^{758\text{-}856}$) both showed abundant envelope association with DRM-L, with 22.8% and 25.9% of gp160 associated with DRM-L. These results show a clear correlation between envelope association with DRMs and incorporation onto virions.

DISCUSSION

The existence of lipid rafts on cell membranes has been controversial. Lipid rafts are usually characterized by ultracentrifugation of detergent-lysed cells in sucrose density gradients. It is possible that this method may affect the structure of cell membranes in unknown ways and create the phenomenon of

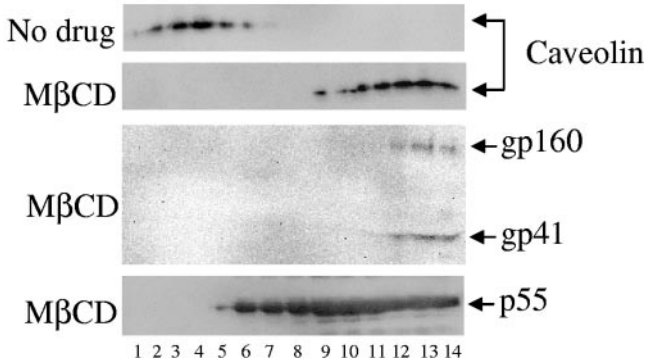


FIG. 5. Effect of β-methyl cyclodextrin on envelope and Gag association with lipid rafts. Untransfected 293T cells were treated with and without 10 mM β-methyl cyclodextrin (MβCD) and subsequently lysed with cold Triton X-100. Postnuclear supernatants were resolved by sucrose density centrifugation, and DRM and DSM fractions were analyzed for the presence of caveolin by Western blotting. The effect of β-methyl cyclodextrin on $p55^{gag}$ and envelope was tested on 293T cells cotransfected with pSVIIIenv and *env*$^-$ pNL4.3.

rafts (40). Nevertheless, the association of particular membrane proteins with DRMs has physiological relevance. Thus, putative lipid rafts have been implicated in the traffic and sorting of membrane proteins throughout the cell, including the endoplasmic reticulum, Golgi complex, cell membrane, and vesicles (18, 24). The role of lipid rafts in the trafficking of HIV-1 envelope to the sites of virus budding is unclear; however, recent studies have provided evidence that rafts support budding and assembly of many enveloped viruses, including HIV-1 (29, 38, 45). Previously, we showed that envelope incorporation into lipid rafts conferred optimal assembly onto virus particles and infectivity (4). In this study, we investigated whether the Gag precursor was required for envelope association with DRMs. Our results show that Gag is required for HIV-1 envelopes to associate with DRMs. When envelope was coexpressed with a mutated $p55^{gag}$ (L30E) that does not interact with envelope, then envelope association with DRMs was eliminated. Several studies have previously supported an interaction between Gag and envelope during assembly. Deletions and mutations in the matrix domain of *gag* abrogate envelope incorporation onto mature virions (12, 14–16). In addition, association of gp41 with $p55^{gag}$ prevents envelope from interacting with endocytic machinery (10). Wyma et al. (44) showed that in immature HIV-1 particles, the $p55^{gag}$ interaction with gp41 was shown to be persistent even in the presence of Triton X-100, suggesting that envelope-Gag interactions are stable in detergent. In addition, cores isolated from immature HIV-1 virions containing uncleaved p55 precursors partially retain gp41. Thus, Gag-envelope interactions are intricately involved in the recruitment of envelope proteins for assembly onto budding virions.

To further examine the role of the gp41 cytoplasmic domain in envelope association with lipid rafts, we investigated a patient-derived envelope (NA420 LN40) that was found to be defective for envelope incorporation onto virions. The NA420 LN40 envelope was derived by PCR from lymph node tissue of an AIDS patient (36). Sequence analyses revealed that LN40 envelope gp41 has several amino acid changes compared to

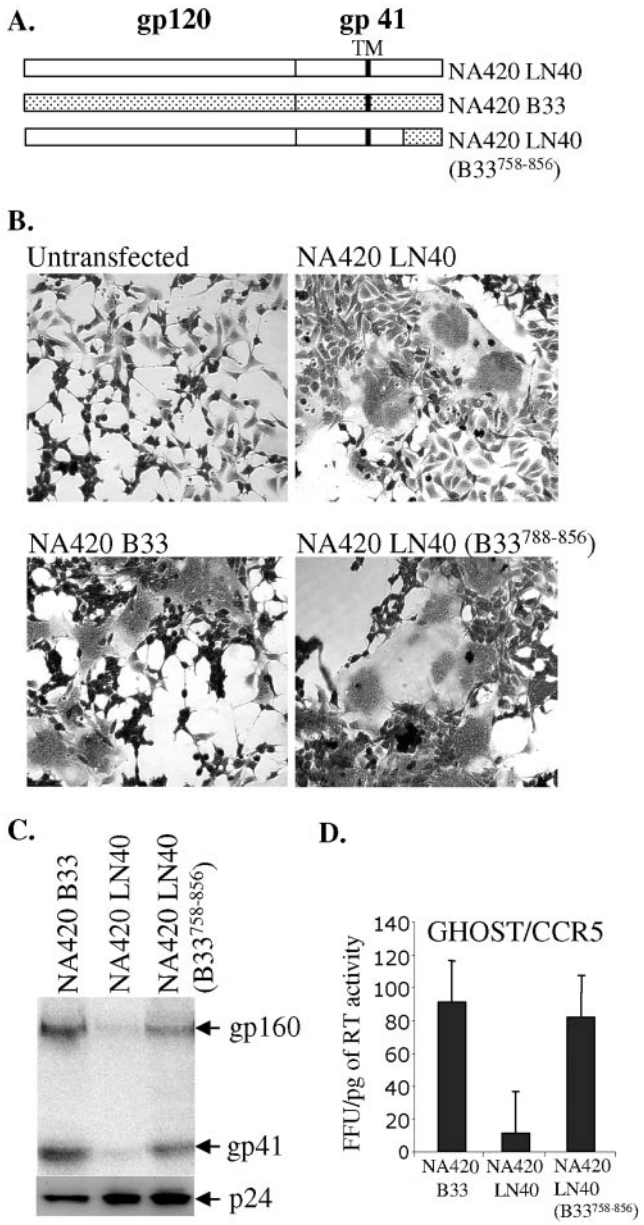


FIG. 6. Functionality, infectivity, and assembly of patient-derived NA420 envelopes. (A) Amino acids from position 758 to the C terminus (position 856) of NA420 B33 gp41 cytoplasmic domain were introduced into LN40 to produce the NA420 LN40 ($B33^{758-856}$) chimeric construct. (B) Functionality of LN40, B33, and NA420 LN40 ($B33^{758-856}$) was assessed by cell-cell fusion. (C) Envelope incorporation was measured by resolving virus pellets on SDS-polyacrylamide gels, followed by Western blotting. (D) Infectivity conferred by each of the patient-derived envelopes was measured in GHOST/CCR5 cells and plotted as infectivity (FFU) to particle (RT) ratios.

B33, a functional envelope encoded by sequences amplified from brain tissue of the same patient. The amino acid substitutions included (i) C764F that eliminates the single (palmitoylation targeted) cysteine residue from the gp41 cytoplasmic region of this envelope and (ii) RR and HS substitutions at positions 787 and 788, respectively. These latter substitutions are located in the LLP-2 domain of gp41, a region previously

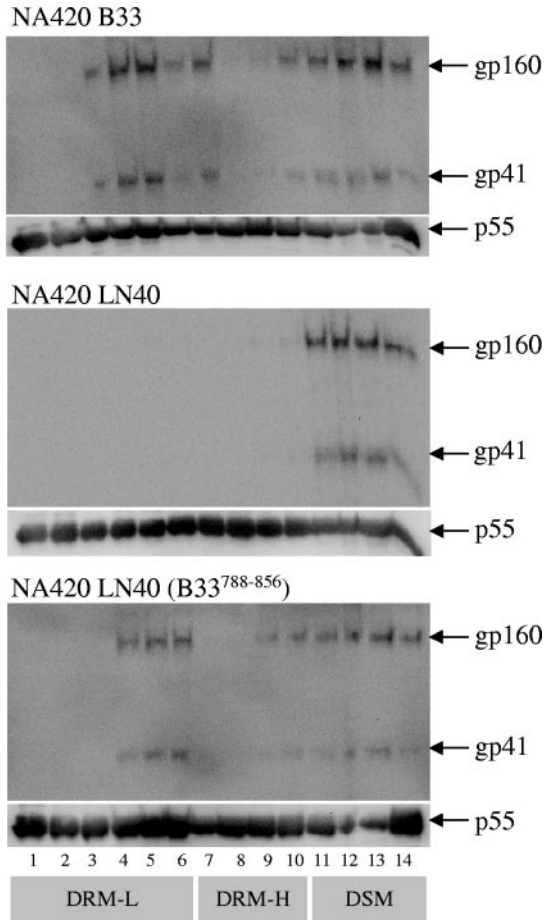


FIG. 7. Lipid raft association of patient-derived envelopes. 293T cells expressing NA420 LN40, NA420 B33, and chimeric NA420 LN40 ($B33^{758-856}$) envelopes were lysed with cold Triton X-100, and the postnuclear supernatants were subjected to sucrose density gradient centrifugation. DRM and DSM fractions were immunoprecipitated with serum from a HIV patient as described in Materials and Methods and resolved by SDS-PAGE. Envelope and Gag proteins were detected by Western blotting using Chessie 8 and 183-H12-5C MAbs, respectively. Note that the substitution of the $B33^{758-856}$ sequence in the LN40 gp41 cytoplasmic domain fully rescued envelope recruitment to DRMs.

implicated in envelope-Gag interactions and assembly onto virus particles (12, 21). LN40 envelope failed to associate with DRMs, even in the presence of Gag, thus firmly implicating the importance of this envelope region for assembly.

The envelope must traffic from the site of synthesis in the endoplasmic reticulum to sites of virus budding. However, the roles of envelope-Gag interactions and lipid raft association in this process are unknown. Neither is it known where Gag and envelope interact, whether at the sites of virus budding or during envelope traffic. Nor it is known if the envelope-Gag interaction is required for envelope trafficking before association with lipid rafts. The site of envelope palmitoylation is also unclear, and it will be interesting to test whether envelope excluded from rafts is palmitoylated. We cannot rule out whether H787/S788 or other LN40 mutations might interfere with envelope trafficking to a site in the cell where it meets Gag. Interestingly, substitution of F764C in the gp41 cytoplasmic domain of LN40 envelope did not rescue the envelope incorporation and infectivity nor facilitate envelope association with rafts in the presence of H787 and S788 (not shown). These observations are consistent with our previous conclusions that gp41 cysteines are neither sufficient nor entirely essential for envelope association with DRMs and assembly onto virions.

Our observations show that HIV-1 Gag is required for envelope glycoproteins to associate with DRMs. In contrast, the reciprocal arrangement has been reported for other viruses. Thus, for respiratory syncytial virus, the association of the matrix protein with DRMs is dependent on the expression of envelope glycoproteins (19). Similarly, influenza virus matrix protein associated with DRMs only if hemagglutinin and neuraminidase glycoproteins were coexpressed (1).

In conclusion, we have demonstrated that the $p55^{gag}$ protein is required to recruit envelope into DRMs. Identification of critical motifs regulating envelope trafficking and assembly onto virions will help the design of strategies to prevent release of infectious virions.

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