Virology 468-470 (2014) 293–302

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Deletions in the fifth alpha helix of HIV-1 matrix block virus release

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ARTICLE INFO

Article history:
Received 27 May 2014
Returned to author for revisions
15 June 2014
Accepted 18 August 2014
Available online 15 September 2014

Keywords:
HIV-1 assembly
Matrix
Deletion mutagenesis
HIV-1 budding
NEDD4L

ABSTRACT

The matrix (MA) protein of HIV-1 is the N-terminal component of the Gag structural protein and is critical for the early and late stages of viral replication. MA contains five α-helices (α1–α5). Deletions in the N-terminus of α5 as small as three amino acids impaired virus release. Electron microscopy of one deletion mutant (MAΔ96-120) showed that its particles were tethered to the surface of cells by membranous stalks. Immunoblots indicated all mutants were processed completely, but mutants with large deletions had alternative processing intermediates. Consistent with the EM data, MAΔ96-120 retained membrane association and multimerization capability. Co-expression of this mutant inhibited wild type particle release. Alanine scanning mutation in this region did not affect virus release, although the progeny virions were poorly infectious. Combined, these data demonstrate that structural ablation of the α5 of MA inhibits virus release.

Introduction

Human immunodeficiency virus type 1 (HIV-1) is a member of the lentivirus subfamily of the *Retroviridae* family and the etiologic agent of acquired immune deficiency syndrome (AIDS). Similar to all retroviruses, HIV-1 has 3 major genes, *gag*, *pol* and *env*. The *gag* gene produces a polyprotein precursor $Pr55^{Gag}$ (Gag) that mediates the essential steps in virus assembly including plasma membrane binding, incorporation of the genomic RNA, multimerization and particle formation, and recruitment of the host endosomal sorting complex required for transport (ESCRT) pathway for release of the particles (Freed, 1998). During or shortly after budding, Gag is proteolytically cleaved by the viral protease into the four major structural proteins: matrix (MA), capsid, nucleocapsid, p6; and the two spacer proteins p1 and p2. This cleavage is required for efficient release of particles from cells (Kaplan et al., 1994). The MA protein is comprised of 132 amino acids and makes up the N-terminal subunit of the Gag polyprotein (Fiorentini et al., 2006). NMR and crystallography show that MA contains five alpha helices, the first four of which form an N-terminal globular head and the fifth (α^5), located at the C-terminus, projects away from the head beginning at amino acid 96 (Hill et al., 1996; Massiah et al., 1996).

MA plays important roles in both entry and egress stages of the viral lifecycle. During the early steps of replication, MA is associated with reverse transcription complexes (RTCs) and preintegration complexes (PICs) (Fassati and Goff, 2001; Gallay et al., 1995). Stemming from this observation, it was proposed that MA is involved in virus uncoating and PIC nuclear import (Haffar et al., 2000; Kaushik and Ratner, 2004). MA contains one nuclear export signal and two transferable nuclear localization signals, one in the N-terminus and one in the C-terminus of the protein (Bukrinsky et al., 1993; Dupont et al., 1999; Haffar et al., 2000). However, the role of MA during the afferent steps of virus replication remains controversial. Several studies refute the role of MA in the early steps of replication and show that mutation of the nuclear localization signals has no consequence on nuclear import of viral DNA (Fouchier et al., 1997; Freed et al., 1995; Freed and Martin, 1994; Hearps et al., 2008). Moreover, removal of the C-terminus of gp41 permits the replication of HIV with a large portion of MA deleted (Reil et al., 1998).

The production of progeny virions requires assembly of the viral proteins into immature particles at the plasma membrane and recruitment of the ESCRT (endosomal sorting complex required for transport) machinery for particle release (reviewed in Balasubramaniam and Freed (2011) and others). The MA domain targets the Gag and Gag-Pol polyproteins to the plasma membrane and mediates incorporation of the viral envelope protein (ENV). The first 6 amino acids are critical for proper PM targeting via an N-terminal myristic acid moiety that is added co-translationally to

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http://dx.doi.org/10.1016/j.virol.2014.08.017
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the N-terminal glycine residue following the removal of the leader methionine (Bryant and Ratner, 1990; Freed et al., 1994; Spearman et al., 1994). In addition of the N-terminus, amino acids 84–88 are essential for correct targeting of virus assembly to the plasma membrane (Freed et al., 1994). Numerous single amino acid substitutions in the N-terminal region of MA (amino acids 11–13, 16, 27–30, 34, 37, 63–65, and 77–80) as well as the beginning of α^5 (aa 98–100) abrogate ENV incorporation (Freed and Martin, 1995, 1996; Lee et al., 1997; Murakami and Freed, 2000; Ono et al., 1997; Yu et al., 1992).

MA α^5 includes amino acids 96–121. Previous mutagenesis studies of this region show effects on both early and late steps of virus replication. A K98E substitution caused an infectivity defect at an early post-entry step (Kiernan et al., 1998). Deletion of amino acids 98–100 abolished ENV incorporation (Dorfman et al., 1994). This phenotype was also observed with a single A99V substitution (Brandano and Stevenson, 2011). A related substitution (A99E) blocks virus production in CEM cells (Freed et al., 1994). Deletions of amino acids 105–114 or 116–123 exhibit no effect on late events and a mild impairment on infectivity attributed to decreased envelope incorporation (Bhatia et al., 2007). However, truncation of the intracellular tail of the viral transmembrane protein gp41 rescues the ENV incorporation defect (Bhatia et al., 2009).

In this study we investigated a panel of mutants with deletions within amino acids 96–120 of MA. Viruses containing deletions of amino acids 96–99 or 100–107 exhibited severely impaired virus release from cells compared to wild-type. Alanine scanning mutations in this area did not block release. Deletion of aa 96–120 did not affect Gag multimerization or membrane association, and the Gag proteins of all the deletion mutants showed complete proteolytic processing. Transmission electron microcopy (TEM) and confocal microscopy of the Δ96-120 mutant showed a phenotype and pattern of localization similar to a p6 late domain mutant. However, unlike the late domain mutant, over-expression of the NEDD4L ubiquitin ligase could not rescue its release. These results suggest that the deletions in this region create a structural defect that leads to a release deficiency distinct from late domain mutants.

Results

Deletions within amino acids 96–107 of matrix block virus release

A series of HIV-1 NLX-derived (Brown et al., 1999) molecular clones were constructed with deletions in α^5 of MA (Fig. 1). The largest clone, MAΔ96-120, had all but a single amino acid of the C-terminal helix of MA removed. All subsequent deletions were smaller iterations of this clone for mapping studies (Fig. 1B). We also constructed a previously described PTAP to LIRL p6 mutant to use as a control in the release assays (Demirov et al., 2002b). Initially we investigated whether the deletions affected virus release. First we examined virus release from transiently transfected 293T cells by immunoblot. Supernatants and cell lysates were collected and virus particles concentrated by ultracentrifugation through a sucrose cushion. Samples were analyzed by immunoblot for p24 capsid and MA (Fig. 2A). There was substantially less p24 and MA detected in the supernatants of any mutant with either amino acids 96–99 or 100–107 deleted, which was comparable to the PTAP late domain mutant (lane 8). No release of MAΔ96-120 was observed when supernatants were directly centrifuged either (data not shown). To confirm these data, we also measured the levels of RT activity present in supernatants by exogenous RT assay (Fig. 2B). Consistent with the immunoblot data, only the Δ107-120 mutant exhibited efficient release of virus. The results were also the same when virus release was measured by p24 antigen ELISA (data not shown).

Deletions in proteins can cause structural problems. The proteolytic cleavage site for MA is at aa 132 and to explore the possibility that the deletions blocked Gag processing we examined the expression and processing of each mutant in cell lysates (Fig. 2C). Full length p55 Gag, p24, and MA were detected for all the mutants, although the level of MA detected varied among the clones and was consistently low for MAΔ96-99 and MAΔ100-107. This could suggest a loss of expression of these mutants; alternatively, the differences in expression levels might have been due to loss of antibody detection. The detection of both MA and p24 capsid protein demonstrated that complete protease processing occurred for each mutant. As expected, the Gag and MA proteins of the various deletion mutants migrated at different rates reflecting their different amino acid length. The only exception was the p55 Gag protein of MAΔ107-120 which migrated at a larger than expected size (lane 5). The expected proteolytic cleavage band p41 was readily detected for all the mutants except Δ96-99 and Δ100-107 (lanes 6, 7), which both showed lower overall expression in replicate experiments. Notably, in addition to the expected slightly smaller p41 intermediate band, a prominent alternative processing intermediate at 41 kDa was detected in the lysates of both MAΔ96-120 and MAΔ96-107 (indicated on blot with (*)). This data suggested that some alternative cleavage occurred with those mutants during processing. Similar to wild-type NLX, protease inhibitor treatment of MAΔ96-120 completely blocked all cleavage, indicating that any aberrant cleavage was not due to cellular proteases (Fig. 2D).

The virus release data indicated that all of the deletions in MA would likely block virus replication due to the release defect except for MAΔ107-120. Nevertheless we measured the infectivity of MAΔ107-120 to see if the deletion affected virus viability. Mutant and wild-type virus stocks were produced by transient transfection of 293T cells and normalized by RT activity. Infectivity was measured using TZM-bl indicator cells, which showed that MAΔ107-120 exhibited levels of infection comparable to NLX (Fig. 2E). This combined with the release data, indicates this region is dispensible for virus infection in vitro.

Microscopy analysis

To determine the nature of the MAΔ96-120 release defect, transmission electron microscopy (TEM) was performed on cells transiently transfected with pNLX or pMAΔ96-120. As reported in other studies, we observed electron dense sites of the initial formation of virus particles on the plasma membrane of cells and only a small number of mature virus particles that appeared to be released in NLX transfected cell grids (Fig. 3A; (Babe and Craik, 1994; Chrystie and Almeida, 1988; Lee and Linial, 1994; Pal et al., 1990)). In marked contrast, the MAΔ96-120 cells had clusters of virus particles accumulated at the plasma membrane of cells (Fig. 3B–F), suggesting that deletion of amino acids 96–120 caused a defect in virus budding and release. The clusters of particles numbered from a few to >50. The MAΔ96-120 particles appeared to undergo normal formation of electron dense budding sites at the surface of cells (Fig. 3C–E), and showed no gross change in morphology.

The notable phenotype observed with the MAΔ96-120 mutant was the presence of particles connected to cells by large budding stalks (Fig. 3D and E, black arrows). This suggested that the release defect was due to a failure of stalk closing and membrane scission. Stalks were also seen between particles and at sites of clustered virus particles. Occasionally particles with narrow stalks were also observed (Fig. 3F, asterisk), but with less frequency. Overall, the images suggested that the release defect of the MAΔ96-120

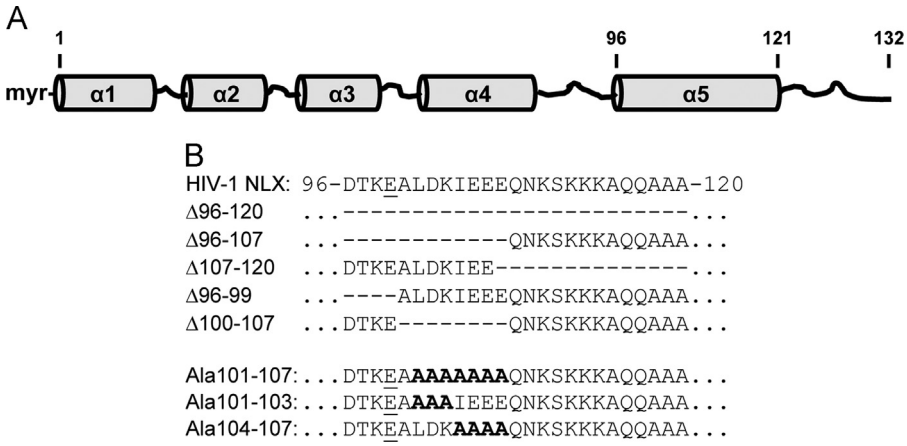

Fig. 1. MA deletion mutants used in this study. (A) Cartoon depiction of the α-helicies in MA. The position of the amino acids flanking α^5 is indicated. (B) Mutants used in this study. The sequence of amino acids 96–120 of the NLX molecular clone is shown. Names of mutants used in the study are indicated on the left and the amino acids deleted or mutated are shown on right. Deleted amino acids are denoted with a dash.

mutant was at a late budding step. To rule out that a protein factor, such as BST-2/tetherin, was responsible for tethering the particles to the cells, we performed subtilisin protease release experiments as described previously (Neil et al., 2006; Van Damme et al., 2008). In these experiments MAΔ96-120 transfected cells were treated briefly with subtilisin to release particles attached to cells via protein bridges. In repeated experiments we failed to detect any release of viral proteins by immunoblot (data not shown). This finding, combined with the fact that 293T cells do not express BST-2/tetherin (Sato et al., 2009; Van Damme et al., 2008) supports the supposition that the MAΔ96-120 particles are tethered via the membrane to cells.

To determine the overall localization pattern of the MAΔ96-120 mutant, we inserted the deletion into the previously described Gag-EGFP construct (Hermida-Matsumoto and Resh, 2000) and compared its localization to a wild-type as well as a PTAP mutant Gag-EGFP by confocal microscopy of transfected HeLa cells. Two distinct patterns of Gag-EGFP localization were observed in cells transfected with wild-type Gag-EGFP, diffuse staining or cells full of distinct puncta (Fig. 4A and B). Quantification of the localization patterns indicated that Gag-EGFP was diffuse in the majority of cells (Fig. 4G). In contrast, the Δ96-120 mutant showed primarily punctate localization (Figs 4C and D), very similar to that observed with the PTAP mutant (Fig. 4E and F). This conclusion was supported when the localization patterns were counted (Fig. 4G) – both mutants exhibited a substantial increase in the proportion of cells with punctate staining. These results, combined with the TEM and biochemical data, indicate that the deletions in MA result in a localization phenotype similar to the late domain mutant.

MAΔ96-120 Gag associates with membranes

The inability of the deletion mutants to release from cells could also result from a loss of other critical functions of MA during virus assembly or release. MA is required for targeting of Gag to sites of assembly at the plasma membrane. To test if the deletions in MA affected this function, we performed membrane floatation assays. The lysates of cells expressing MAΔ96-120 or wild-type NLX were separated into membrane and soluble fractions using step gradients. Gradient fractions were collected by positive displacement (fraction 1 = top) and viral proteins detected by SDS-PAGE and western blot (Fig. 5). Gag protein was detected in fractions 2 and 3 from gradients of NLX transfected cells, indicative of protein associated with membranes, and in fractions 8 and 9, indicative of soluble proteins (Fig. 5, bottom panel). The pattern of viral protein distribution was similar in cells transfected with the MAΔ96-120 mutant (Fig. 5, top panel), indicating the MAΔ96-120 Gag protein associated with membranes. These data were consistent with the TEM results.

Co-expression of MAΔ96-120 inhibits wild-type virus release

Another critical step during virus assembly is the multimerization of Gag monomers to form immature virus particles. To test whether MAΔ96-120 Gag was capable of multimerizing, we took advantage of its size difference with wild-type MA and performed co-transfection assays to see if MAΔ96-120 could be incorporated with wild-type Gag and released. To do this, equal amounts of pNLX and pMAΔ96-120 were transiently transfected into 293T cells and protein expression and virus release detected by western blot (Fig. 6A). Both NLX and MAΔ96-120 proteins were detected in the lysates and supernatants of the co-transfected cells indicating that MAΔ96-120 multimerized with wild-type Gag were released. However, as shown in Fig. 6A, the amount of p24 detected in the supernatants of co-transfected samples was substantially decreased compared to the NLX sample despite approximately equal amounts of p24 in cell lysates. From this data, it appeared that expression of MAΔ96-120 inhibited the release of NLX.

To further test the supposition that MAΔ96-120 was inhibiting wild-type release, we performed a similar experiment in which a constant amount of NLX was co-transfected with increasing amounts of MAΔ96-120 (Fig. 6B). Consistent with the previous experiment, the amount of capsid and MA released in supernatants decreased as the amount of MAΔ96-120 increased (top panels). Examination of the cell lysates showed a slight decrease in the amount of p55 Gag protein and a slight increase in the amount of p24 CA. From these experiments we concluded that expression of MAΔ96-120 inhibits wild-type NLX release in a *trans*-dominant manner.

The budding defect of MAΔ96-120 is distinct from late domain mutants

HIV-1 budding is facilitated by the ESCRT pathway, including ESCRT-I, –III, Vps4, and ALIX. Recruitment of the ESCRT pathway is mediated by a late domain present in the p6 region of Gag. This domain recruits the ESCRT-I component TSG101 to sites of virus assembly. Mutations in the late domain of HIV Gag impair membrane scission and show a phenotype similar to that observed in our EM studies (Carlson et al., 2008; Demirov et al., 2002a;

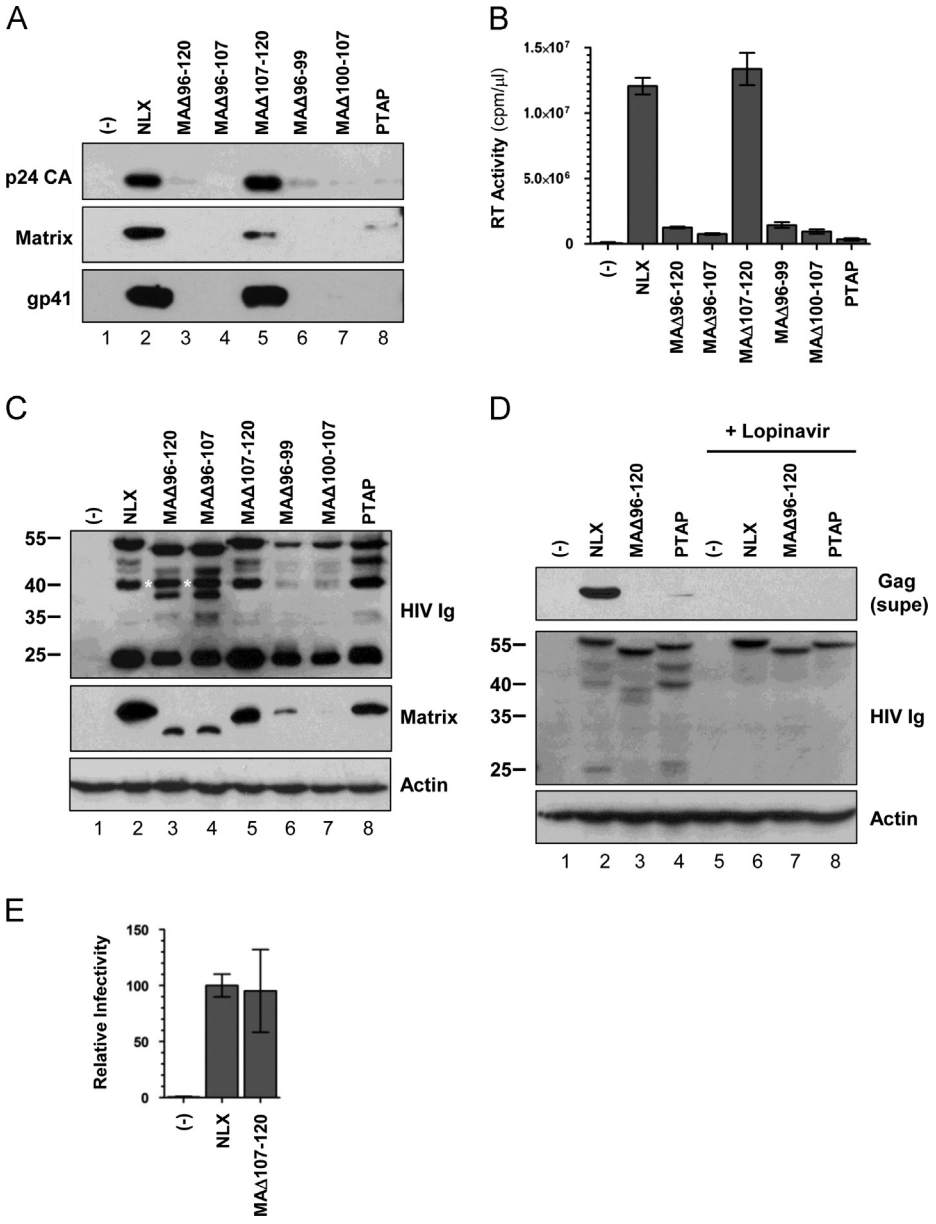

Fig. 2. Expression and release of MA deletion mutants. (A) Virus release. Supernatants were collected from 293T cells transiently transfected with the HIV-1 clones indicated at the top and directly resolved by SDS-PAGE. The indicated viral proteins detected by western blot. All blots are representative of at least three independent experiments. (B) Reverse transcriptase activity released from cells. Supernatants were assayed by in vitro [^{32}P]–TTP incorporation assay. Error bars show standard error of triplicate experiments. (C) Immunoblot of cell lysates. Gag products were detected with HIV Ig (top panel). Aberrant processing intermediates are indicated with a (*). (D) Effects of Lopinavir treatment. Supernatants were collected from 293T cells transiently transfected with the HIV-1 clones and directly resolved by SDS-PAGE and immunoblotted to detect release (top panel). Lower panels show immunoblots of cell lysates. (E) Infectivity of MAΔ107-120. Virus stocks were normalized by RT activity and infectivity measured using TZM-bl indicator cells. The results were normalized relative to wild-type NLX. Data represents the mean of three independent experiments and the error bars denote the combined SEM of all experiments.

Garrus et al., 2001; Göttlinger et al., 1991; Morita et al., 2011). Therefore, we next investigated if MAΔ96-120 was able to recruit ESCRT pathway. First we tested whether the Gag protein of MAΔ96-120 interacted with endogenous TSG101 by co-immunoprecipitation assay using the Gag-EGFP constructs. Cells were transfected with wild-type or MAΔ96-120 Gag-GFP, lysed, and immunoprecipitated with HIV immunoglobulin. As shown in Fig. 7A, TSG101 co-immunoprecipitated with both wild-type and MAΔ96-120 Gag-EGFP, indicating that the mutant was capable of interacting with TSG101.

Previous studies have also shown that over-expression of ALIX or the HECT domain E3 ubiquitin ligase NEDD4L can rescue budding of p6 late domain mutants, (Chung et al., 2008; Fisher et al., 2007; Usami et al., 2007, 2008). The mechanism is not completely understood although recent data shows that Gag ubiquitination is required for virus release (Sette et al., 2013). To further test if the MAΔ96-120 defect was related to the defect seen with p6 late domain mutants, we assessed whether the over-expression of NEDD4L could rescue its release. The molecular clones (NLX, MAΔ96-120, or NLX-PTAP) were co-transfected with a NEDD4L expression vector and virus release monitored by western blot analysis of supernatants as in previous experiments (Fig. 7B). Despite the fact that NEDD4L over-expression was able to facilitate release of the PTAP mutant (top panel, compare lanes 8–4), the release of MAΔ96-120 was still blocked (lane 7). These results suggested that the defect caused by the deletion in MA was distinct from that observed with p6 late domain mutants.

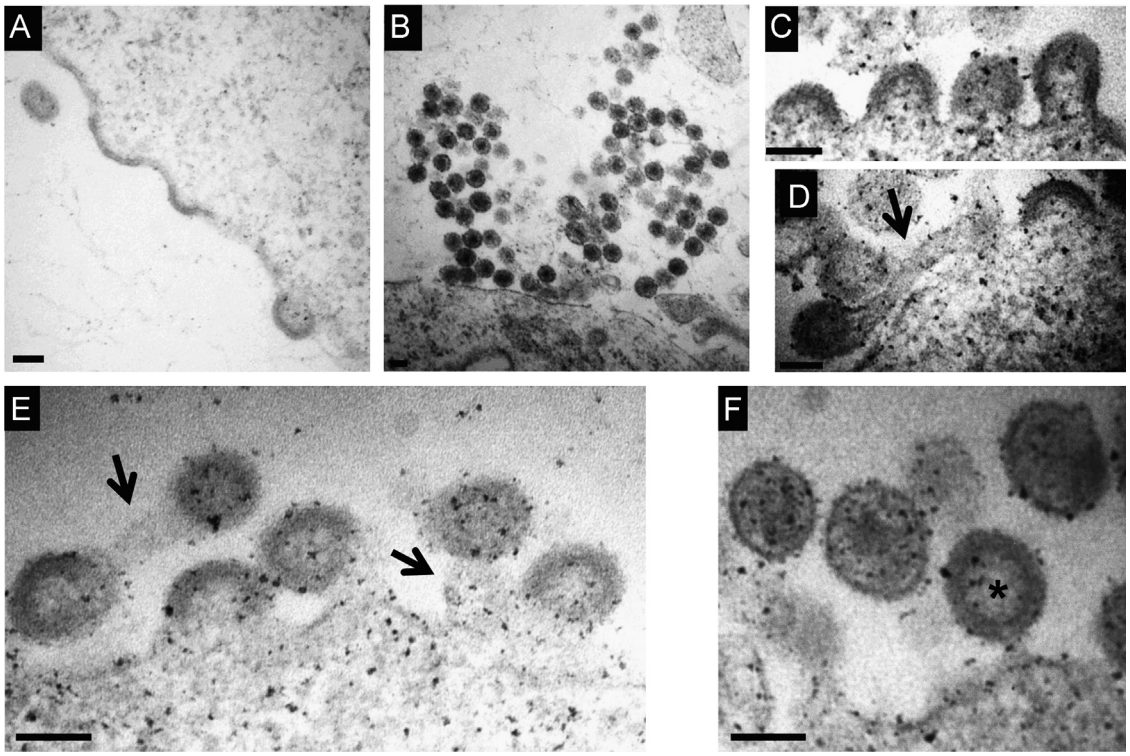

Fig. 3. Transmission electron microscopic images showing viral particles in the surface regions of cells expressing wild-type NLX (A) and MAΔ96-120 (B-F). Arrows indicate visible membrane stalks. Scale bars: 0.1 μm.

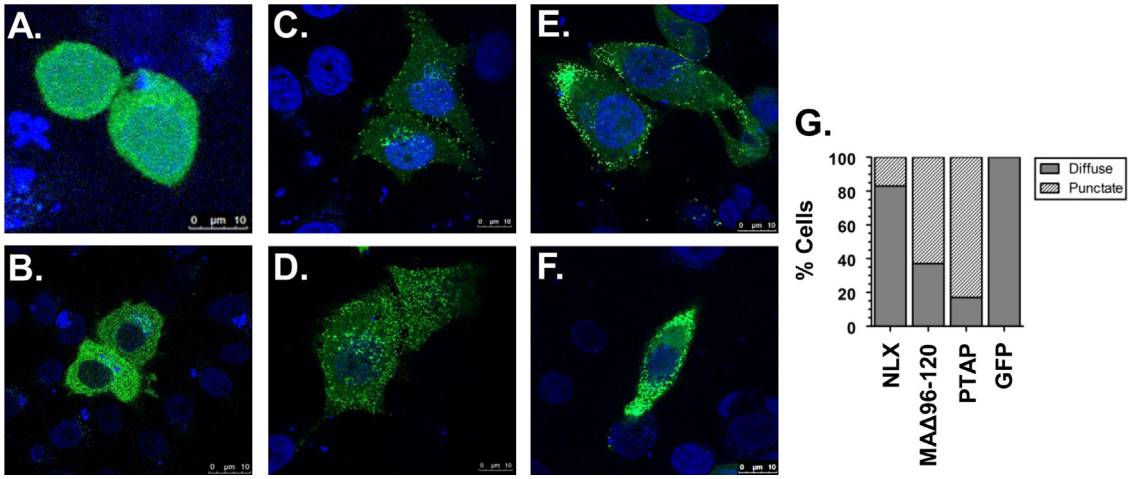

Fig. 4. Localization of Gag-GFP constructs. (A) Confocal microscopy of HeLa cells transiently transfected with wild-type (a, b), MAΔ96-120 (c, d), or PTAP mutant Gag-GFP (e, f) constructs. (G) Graphical representation of observed localizations of proteins.

Amino acid mutagenesis does not block release

Finally, to test the supposition that structural ablation was causing the release defect we constructed three alanine scanning mutants within amino acids 100–107 (Fig. 1B). Notably, immunoblot of cell lysates and superantants showed that all three mutants were readily expressed and released from cells (Fig. 8A and B). These data support the theory that deletions in α^5 of MA cause a structural ablation and result in an inhibition of virus release. The viruses were then tested for infectivity on TZM-bl cells (Fig. 8C). All three mutants showed a loss of infectivity, indicating that this region of MA is important for the afferent steps of virus replication.

Discussion

The HIV-1 MA protein plays important roles in both early and late stages of the viral lifecycle (Fiorentini et al., 2006; Freed, 1998; Ghanam et al., 2012). The experiments described herein show that deletions in the N-terminal portion of the 5th alpha helix of the MA protein impair virus release. Mutants lacking either amino acids 96–99 or 100–107 showed a reduction in virus production compared to wild-type NLX. Moreover, co-expression of MAΔ96-120 blocked wild-type virus release in a *trans*-dominant manner. Notably, despite a 13 amino acid closer to the MA–CA cleavage site (amino acids 107–120) did not appreciably affect virus egress. TEM of MAΔ96-120 showed fully formed particles tethered to cells by

membranous stalks. Consistent with this, protease treatment of cells did not permit release of particles. Moreover, the majority of these studies were performed with 293T cells which do not express the restriction factor BST-2/tetherin (Sato et al., 2009; Van Damme et al., 2008). In contrast to the deletion mutants, site-directed mutagenesis of this region did not affect release. Combined these data indicate that this region of MA contains structural elements critical for virus release. To our knowledge, this is the first studies to describe a release defect phenotype associated with the MA protein.

Efficient virus production requires multiple steps, including targeting and multimerization of Gag/Gag-Pol polyproteins and recruitment of the ESCRT pathway. The floatation experiments and western blot analysis of transfected cell lysates demonstrated that MAΔ96-120 is targeted to membranes and proteolytically processed. The defect was evident in the TEM analysis. The MAΔ96-120 viruses formed particles that failed to abscise from the plasma membrane. This resulted in clusters of viruses present at the cell surface. The presence of incompletely closed virus particles (Carlson et al., 2008) and membrane stalks suggests that the MAΔ96-120 tethering results from a failure of membrane abscission. The final step of virus budding requires recruitment of the ESCRT-III components including CHMP2A, CHMP4B, and Vps4 (Garrus et al., 2001; Morita et al., 2011), and the ubiquitination of Gag (Sette et al., 2013). Recruitment of ESCRT is facilitated by the interaction of the p6 region of Gag with the ESCRT-I component TSG101. MAΔ96-120 Gag retained interaction with TSG101 suggesting that ESCRT recruitment is not the cause of the budding defect. Furthermore, over-expression of NEDD4L, which can compensate late domain mutants, failed to promote release of MAΔ96-120. Combined these data suggest that the deletions in MA result in a structural ablation of Gag that impairs budding via a novel mechanism.

Although the TEM studies showed clusters of virus-like particles on the surface of cells, the results thus far cannot rule out the possibility that the deletion of residues 96–120 creates a structural constraint that mistargets Gag or blocks an essential modification of Gag necessary for processing and/or release. The misfolding of proteins can lead to the targeting of proteins to the proteosome or other intracellular structures such as aggresomes or inclusion bodies. However, healthy mitochondria were seen in MAΔ96-120 transfected cells, we did not observe any intracellular structures containing virus like particles, nor did we observe any structures fused to lysosomes, which would be indicative of autophagsome formation (data not shown). Finally, p24 CA and p17 MA were observed by immunoblot in both cell-free virus and lysates of MAΔ96-120 transfected cells. Combined, these results argue against misfolding or blockage of processing as the mechanism of release inhibition.

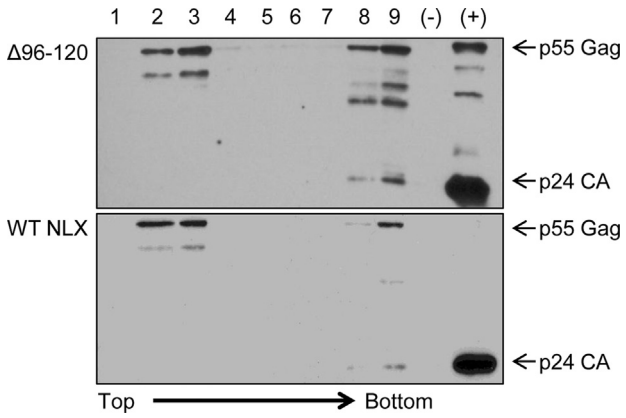

Fig. 5. Membrane floatation assay showing MAΔ96-120 is associated with membranes. 293T cells were transfected with either MAΔ96-120 (top panel) or wild-type NLX (bottom panel) for 24 h. Cells were lysed by hypotonic swelling/dounce homogenization and ultracentrifuged on a sucrose step gradient. Fractions were collected from the top and analyzed by SDS-PAGE and western blot with anti-Gag antiserum. Data is representative of two independent experiments.

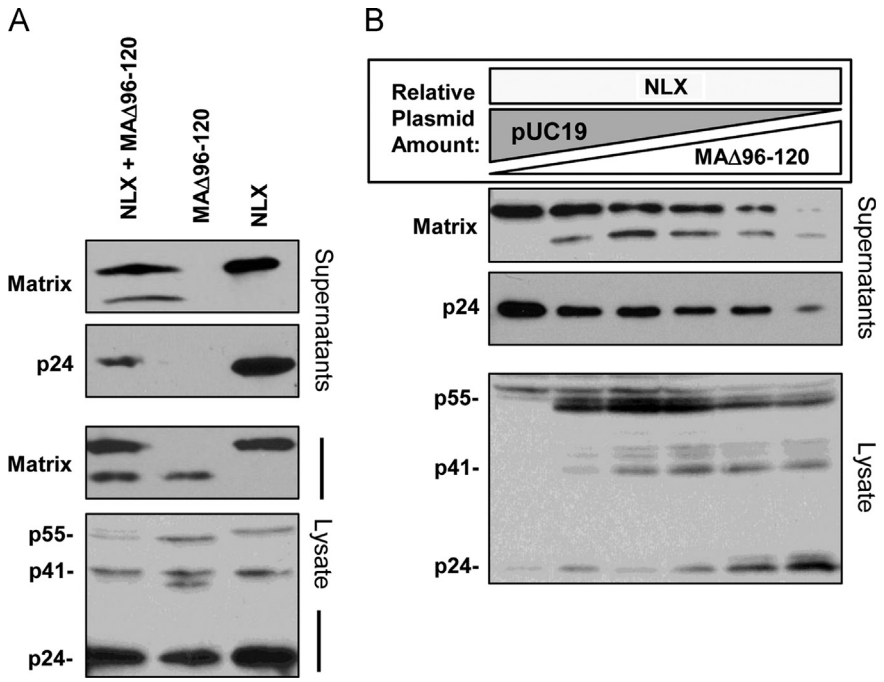

Fig. 6. Co-expression of MAΔ96-120 and NLX. (A) MAΔ96-120 is capable of multimerization with wild-type NLX. 293T cells were transfected with both NLX and MAΔ96-120, or either clone alone as indicated at the top. (B) MAΔ96-120 trans-dominantly inhibits wild-type virus release. Cells were transfected transiently with a constant amount of NLX plasmid, increasing amounts of MAΔ96-120, and pUC19 to normalize the overall amount of plasmid DNA. Supernatant and lysate samples were isolated and the indicated viral proteins detected as described in Fig. 2 legend. Panels are a representative of three independent experiments.

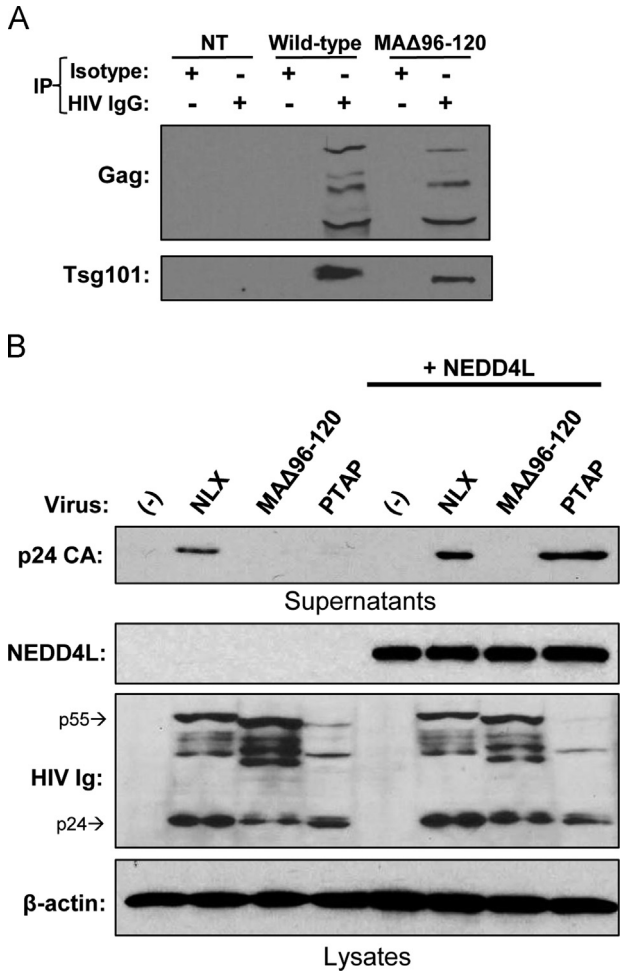

Fig. 7. The MAΔ96-120 phenotype is distinct from late domain mutants. (A) Co-immunoprecipiation assay showing MAΔ96-120 Gag interacts with TSG101. Cells transfected with wild-type or MAΔ96-120 Gag-GFP were lysed, immunoprecipitated with HIV IgG, and immmunoblotted for TSG101 and Gag as indicated. (B) NEDD4L over-expression does not rescue MAΔ96-120 release. The indicated molecular clones were co-transfected with a NEDD4L expression construct. Supernatants and lysates were tested for the indicated proteins as described in Fig. 2. β-actin was detected as a control. Data is representative of two independent experiments.

Finally, we identified that the amino acid sequence in this region was critical for the early steps of virus replication. The Los Alamos National Laboratory sequence database indicates that with the exception of E^{107}, amino acids 100–107 (–ALDKIEEE-) are highly conserved across all virus clades. Two previous studies investigated deletions in the 5th α-helix of MA. Yu et al. (1992) found that deletion of amino acids 105–115 moderately impaired virus replication, whereas deletion of 116–128 was more deleterious. Both mutants exhibited reductions in viral DNA synthesis. However, Bhatia et al. (2007) saw only a small (3- to 4-fold) reduction in infectivity upon deletions of amino acids 105–114 or 116–123. They found that the drop in infectivity correlated with a reduction in envelope incorporation and reduced membrane fusion (Bhatia et al., 2007). The loss of virus release precluded testing of the infectivity of the deletion mutants, however the alanine scanning mutagenesis showed that amino acids 100–107 are critical for the early steps of virus replication. Combined with all the previous work, our data confirms that C-terminal α-helix of MA plays a critical role during the afferent steps of virus replication. Identification of the function(s) of MA during virus infection could lead to the development of novel inhibitors.

Materials and methods

Cell lines

293T, HeLa, and TZM-bl cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Clone III (Hyclone, Logan, UT USA), and 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown at 37 °C with a 5% CO_2 atmosphere.

DNA constructs

Deletions in MA and p6 as well as the alanine substitutions were made in the pNLX clone (Brown et al., 1999) of HIV-1 or pGag-EGFP (Hermida-Matsumoto and Resh, 2000) using standard cloning techniques. Briefly, the deletions and substitutions in the MA region were constructed by PCR overlap mutagenesis spanning the unique *Bss*HII and *Spe*I restriction sites within *gag*, whereas the PTAP to LIRL mutation in p6 was made using the unique *Spe*I and *Sbf*I restriction sites. For the Gag-EGFPΔ96-120 and Gag-EGFP-PTAP clones the restriction sites utilized were *Bam*HI and *Kpn*I. A list of primers can be provided upon request. The deletion fragments were gel purified, digested, and ligated into pNLX cut with the same enzymes. The complete region inserted into each clone was verified by DNA sequencing at the Molecular Biology Core at Creighton University Medical Center. The NEDD4L expression construct was obtained from ATCC (Manassas, VA USA).

Virus production and purification

Virus stocks were produced by transient transfection of 293T cells with molecular clones using PEI and purified as previously described (Schweitzer et al., 2012). Briefly, supernatants were collected at 24 h intervals for 3 days, pooled, clarified through a 0.45 μm pore size filter, aliquoted, and stored at −80 °C. Virus release was monitored and virus stocks normalized by reverse transcriptase (RT) assay as previously described (Belshan et al., 2009). For western blot experiments, supernatants and cells lysates were collected at 24 h post-transfection. Supernatants were clarified by centrifugation and virus particles concentrated by ultracentrifugation through a 20% (w/v in PBS) sucrose cushion. Lopinavir (1 μM) was added two hours prior to transfection in indicated experiments. Cells were washed with PBS and lysed with M-PER lysis buffer as directed by the manufacturer (Pierce Biotechnology, Rockford, IL). For the NEDD4L rescue experiments, 5 μg of NEDD4L expression plasmid was co-transfected with 10 μg of molecular clone.

Immunoblots

Cell or viral lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane with a semi-dry blotter. Membranes were incubated for 1 h with the following primary antibodies: Anti-Gag 1/2000, anti-Matrix 1/2500 (both kind gifts of Lee Ratner), HIV immunoglobulin 1/20,000 (NIH AIDS Research & Reference Program) or anti-gp41 Chessie-8 1/1000 ((Abacioglu et al., 1994); NIH AIDS Research & Reference Program). Blots were incubated for 45 min with secondary antibodies conjugated with species specific anti-IgG antibodies conjugated to horseradish peroxidase. Proteins were detected using SuperSignal West Pico chemiluminesence substrate (Pierce Biotechnology), exposed and visualized with film. Images were acquired by flatbed scanning, cropped, and, if necessary, adjusted for brightness/contrast.

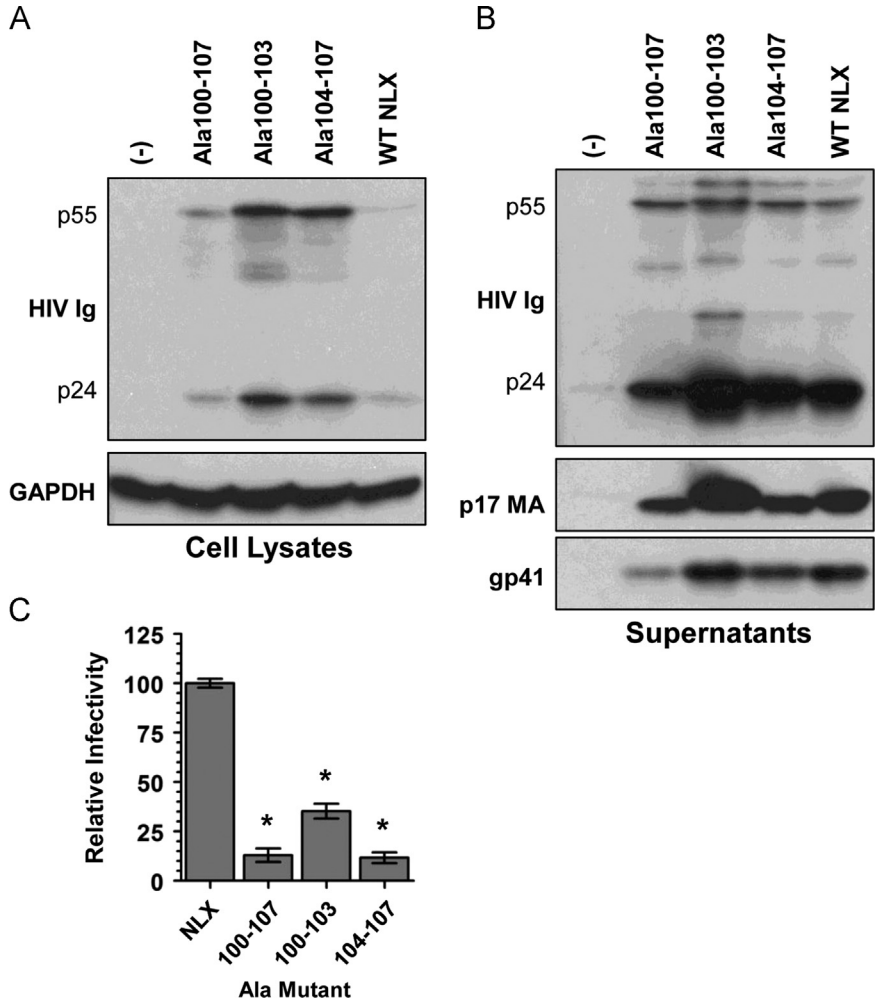

Fig. 8. Alanine scanning mutagenesis of MA aa 100-107. Virus expression (A) and release (B) from 293T cells. Proteins were resolved by SDS-PAGE and the indicated viral proteins detected by western blot. (C) Virus infection of Ala mutants measured on TZM-bl indicator cells. Data is a combination of two independent experiments with infections performed in triplicate. Error bars denote standard error of the mean; $^{*}p < 0.001$ by unpaired two-tail *T*-test.

Membrane association assay

293T cells were transfected as described above with either pNLX or pMAΔ96-120 and at 24 h post-transfection the cells were collected and pelleted by centrifugation. The pellet was resuspended in a complete protease inhibitor cocktail (Calbiochem, La Jolla, CA, USA) and dounce homogenized. The lysates were treated with 5 M NaCl and TNE (25 mM Tris–HCl (ph 7.5), 150 mM NaCl, 4 mM EDTA) +0.5%Tx-100, equilibrated to 73% sucrose (w/v), loaded onto a sucrose step gradient (85.5%, 65%, 10% w/v) between 85.5% and 65% layers, and ultracentrifuged for 16 h at 167,242*g*. Fractions were collected from the tube by positive displacement using the BioComp Gradient Master system (Fredericton, New Brunswick, Canada), mixed 1:1 with 2 × sample buffer, separated by SDS-PAGE and analyzed by western blot as described above.

Microscopy

For the TEM experiments HeLa cells were transfected with either pNLX or pMAΔ96-120 plasmid using GeneJet transfection reagent by following the manufacturer's protocol (SignaGen Laboratories, Rockville, MD, USA). At 24 h post transfection the cells were washed once with PBS, fixed in 2.5% glutaraldehyde/2.5% paraformaldehyde, and incubated for 1 h at room temperature then overnight at 4 °C. The cells were scraped from the plates, embedded and prepared for imaging as previously described (Rodriguez-Rocha et al., 2013; Wen et al., 2010). For the Gag-GFP imaging HeLa cells were seeded on glass coverslips at 50% confluency and transfected the next day with Gag-EGFP, Gag-EGFP-MAΔ96-120, or Gag-EGFP-PTAP as described above. At 18 h post-transfection the cells were washed with PBS, fixed with 3.7% formaldehyde, and mounted onto slides using Prolong Gold antifade reagent with DAPI to stain nuclei (Life Technologies, Grand Island, NY). Confocal microscopy was performed in the Creighton University Microscopy core facility using a Zeiss LSM 510 META NLO confocal scanning system. Images were cropped and processed for brightness/contrast using Adobe Photoshop (Adobe Systems Inc., San Jose, CA USA).

Infectivity assays

TZM-bl cells were seeded at 2×10^5 cells per well in 24-well plates. Triplicate wells were inoculated with equivalent amounts of virus and incubated for 48 h. Mock infections were performed in parallel as negative controls. Cells were lysed with M-PER lysis buffer and luminescence measured with a luminometer using One-Glo substrate as directed by the manufacturer (Promega, Madison, WI). The results were normalized to the average of the WT infected samples. Statistical analyses were performed using Graphpad Prism Software.

Co-immunoprecipitation

1×10^7 293 T cells were transfected in 10 cm dishes as described above with either Gag-EGFP or Gag-EGFP-MAΔ96-120. At 24 h post transfection the cells were lysed with IP lysis buffer (Tris buffered saline pH 7.8, 1% TritonX-100, 0.5% NP-40, 1 × protease inhibitor cocktail). Lysates were pre-cleared with protein G Sepharose beads (Life Technologies) and rotated overnight at 4 °C with HIV immunoglobulin. The following day protein G Sepharose beads were added and the samples rotated an additional 3 h. Beads were washed thrice with IP lysis buffer for 15 min. at 4 °C, pelleted and the captured proteins solubilized by boiling in 1 × sample buffer. Proteins were separated by SDS-PAGE and analyzed by western blot as described above.

Acknowledgments

The authors wish to thank Lee Ratner for the anti-MA and anti-Gag polyclonal antisera and the Creighton University Integrated Biomedical Imaging Core Facility for help with the confocal microscopy. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV immunoglobulin; HIV-1 gp41 hybridoma (Chessie 8) from George K. Lewis; and pGag-EGFP from Marilyn D. Resh and George Pavlakis. This work was funded by the United States National Institutes of Health, awards GM103509 (the Nebraska Center for Virology) and AI080348 to MB.

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