Basic Residues in the Nucleocapsid Domain of Gag Are Required for Interaction of HIV-1 Gag with ABCE1 (HP68), a Cellular Protein Important for HIV-1 Capsid Assembly

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During human immunodeficiency virus, type 1 (HIV-1) assembly, Gag polypeptides multimerize into immature HIV-1 capsids. The cellular ATP-binding protein ABCE1 (also called HP68 or RNase L inhibitor) appears to be critical for proper assembly of the HIV-1 capsid. In primate cells, ABCE1 associates with Gag polypeptides present in immature capsid assembly intermediates. Here we demonstrate that the NC domain of Gag is critical for interaction with endogenous primate ABCE1, whereas other domains in Gag can be deleted without eliminating the association of Gag with ABCE1. NC contains two Cys-His boxes that form zinc finger motifs and are responsible for encapsidation of HIV-1 genomic RNA. In addition, NC contains basic residues known to play a critical role in non-specific RNA binding, Gag-Gag interactions, and particle formation. We demonstrate that basic residues in NC are needed for the Gag-ABCE1 interaction, whereas the cysteine and histidine residues in the zinc fingers are dispensable. Constructs that fail to interact with primate ABCE1 or interact poorly also fail to form capsids and are arrested at an early point in the immature capsid assembly pathway. Whereas others have shown that basic residues in NC bind non-specifically to RNA, which in turn scaffolds or nucleates assembly, our data demonstrate that the same basic residues in NC act either directly or indirectly to recruit a cellular protein that also promotes capsid formation. Thus, in cells, basic residues in NC appear to act by two mechanisms, recruiting both RNA and a cellular ATPase in order to facilitate efficient assembly of HIV-1 capsids.
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these basic residues could also act by other mechanisms to promote capsid assembly.

Here we demonstrate that the basic residues within NC are necessary to recruit endogenous ABCE1 into Gag-containing assembly intermediates. Furthermore, we find that the cysteine and histidine residues in NC are not required for the Gag-ABCE1 interaction and that large regions of the MA and CA domains of Gag are also dispensable. Velocity sedimentation analyses demonstrate that two NC mutants that fail to form fully assembled immature capsids (including a mutant that fails to bind ABCE1 and one that binds ABCE1 poorly) are arrested at early points in the assembly pathway in cells. Together, our findings reveal that the basic residues in NC that bind nonspecifically to RNA also are important for the interaction of Gag with the cellular protein ABCE1, which facilitates HIV-1 capsid formation. Thus, NC appears to act by two mechanisms to promote efficient HIV-1 capsid assembly in the complex environment of the cytoplasm.

EXPERIMENTAL PROCEDURES

Plasmids—For mammalian cell transfection plasmids, Gag mutations were engineered into the pSvGagRRE-R construct, which was obtained from David Rekosh (29) and encodes Gag and the Rev response element from the BH10 strain of HIV-1. Truncations were engineered by introduction of two stop codons after the amino acid in Gag indicated in the construct name, using site-directed mutagenesis (Stratagene). To make other constructs, a SacI site was engineered into the parental construct immediately downstream from the Gag coding region by site-directed mutagenesis. Gag mutations were engineered using standard PCR procedures and inserted into the SacI sites on either side of the Gag coding region in the modified pSvGagRRE-R plasmid. The KR10A construct was engineered in an analogous manner by fusing the NC-p6 domains from a template plasmid encoding the KR10A mutations (M1-2/BR (24)), obtained from Jeremy Luban, to the MA-CA domains in pSvGagRRE-R using standard PCR procedures.

Plasmids encoding WT Gag, Tr361, and Tr437 for in vitro transcription have been described previously (1–3). Plasmids encoding CH1A, CH2A, and CH1/2A for in vitro transcription were engineered using standard PCR procedures and inserted into the SacI sites on either side of the Gag coding region in the modified pSvGagRRE-R plasmid. The KR10A construct was engineered in an analogous manner by fusing the NC-p6 domains from a template plasmid encoding the KR10A mutations (M1-2/BR (24)), obtained from Jeremy Luban, to the MA-CA domains in pSvGagRRE-R using standard PCR procedures.

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Quantitative RT-PCR—RNA was purified from cell lysates by adding 20 μl of lysis to 200 μl of RNaseqeous lysis buffer (Ambion). After mixing, 20 μl of a control lystate (murine EL4 cells; see below) was spiked into the reaction to serve as a control for RNA purification and reverse transcription efficiency. RNA was then isolated per the manufacturer’s protocol. Eluates were then treated with rNase 1 (Ambion) and subsequently subjected to reverse transcription using random DNA primers and Superscript II reverse transcriptase (+RT; Invitrogen). Quantitative PCR was performed using iQ SYBR green supermix (Bio-Rad). Serial dilutions of a corresponding DNA template were run in parallel. A standard curve of Cₚ versus log[DNA] was calculated, in which the [DNA] of the highest dilution was arbitrarily set at 1, and sample values were extrapolated from the standard curves. Reactions minus RT (−RT) were processed in parallel, and −RT values were subtracted from the +RT values. Values are reported as 0 if the −RT value was greater than the +RT value. Sequences for the primers are 5′-gactagt- tagacgccctctat-3′ (forward) and 5′-cacaacgtctgcctcctrccctc-3′ (reverse) for HIV-1 Gag, 5′-cagctggcccccagc-3′ (forward) and 5′-ggaagttgtaagagta-3′ (reverse) for human actin, and 5′-cagctgctctcctct-3′ (forward) and 5′-ggaagttgctccag-3′ (reverse) for murine actin. For EL4 cell lysate, EL4 cells were lysed in lysis buffer (RNaseqeous; Ambion) at a concentration of 5 × 10⁴ cells/μl.

Cell-free Assembly Reactions—In vitro transcription and cell-free translation using wheat germ extract and Tran32S-label (ICN Biochemicals) were performed as described previously (1, 2). Cell-free reactions were programmed using a mixture of 40% FLAG-ABCE1 transcript and 60% WT or mutant Gag transcripts, as described previously (1). Cell-free translations were diluted 300-fold in Nonidet P-40 buffer and subjected to immunoprecipitation with FLAG antibody coupled to beads (Sigma) or mouse IgG (Sigma) with protein G beads (Pierce), as described previously (1). Immunoprecipitations were analyzed by SDS-PAGE and autoradiography in parallel with aliquots of total cell-free reaction representing 5% of input.

GST-ABCE1 Pull-down Assays—Competent BL21 Escherichia coli (Novagen) were transformed with GST-ABCE1. E. coli were grown to
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FIGURE 1. The Gag-ABCE1 interaction is progressively reduced upon truncation of Gag in NC. A, diagram showing NC residues in BH10 Gag, with arrows indicating the last amino acid present in each truncation construct. Lines demarcate where the NC domain meets the spacer regions, SP1 and SP2. Basic residues in NC are shown in gray.

B, Gag truncation constructs are diagrammed on the right with major domains labeled above and Cys-His boxes (CH1 and CH2) shown in gray. Lysates of COS-1 cells transfected with the indicated constructs were subjected to immunoprecipitation with α-ABCE1 (immune, I) or nonimmune control antibody (N) and followed by immunoblotting with an antibody to Gag. Equivalent aliquots of total input (T) are shown to indicate migration and level of expression. Immunoprecipitations were performed under either native conditions (Native) or after denaturation (Denat). For each construct, all lanes shown were taken from a single exposure. Experiments in each panel were repeated three times, and representative data are shown.

C, the reduction in ABCE1 interaction relative to wild-type Gag upon progressive truncation in NC was quantitated from three repeats of this experiment (statistical significance: *, p < 0.05; **, p < 0.01). Indicated below the bar graph for each construct are the number of lysines and arginines in NC (#KR) and the presence or absence of the first and second Cys-His boxes (CH1/CH2).
FIGURE 2. Altering Lys and Arg residues but not Cys and His residues in NC eliminates the Gag-ABCE1 interaction. Lysates from COS-1 cells expressing the indicated mutants were subjected to immunoprecipitation (IP) under native conditions with α-ABCE1 (immune, I) or nonimmune control antibody (N) and followed by immunoblotting with an antibody to Gag. Constructs examined include mutants containing Cys-His box deletions (A) and amino acid substitutions (B). Equivalent aliquots of total input (T) are shown to indicate migration and level of expression. All lanes were taken from a single exposure. Diagrams in A show amino acids in NC for each construct, with major domains labeled above, lysines and arginines in gray, cysteines and histidines outlined, and substituted amino acids indicated with black dots. Experiments in each panel were repeated three times, and representative data are shown. C, the reduction in ABCE1 interaction relative to wild-type Gag for each construct was quantitated from three repeats of each experiment (statistical significance; *, p < 0.05; **, p < 0.01). Indicated below the bar graph for each construct are the number of lysines and arginines in NC (#KR) and presence or absence of first and second Cys-His boxes (CH1/CH2).
Gag is important for the Gag-ABCE1 interaction in mammalian cells (1). Further, our deletion of both Cys-His boxes (Gag^CH1/2) associated with ABCE1, Tr405, contained one Cys-His box and seven basic residues. The CH2 construct did not allow us to precisely define the contribution of the Cys-His boxes. Quantitation revealed that these differences in ABCE1 interaction were significant (Fig. 1C). When lysates were denatured to disrupt protein-protein interactions, ABCE1 immunoprecipitated WT Gag or any of the Gag truncation mutants with previous findings (1, 4). Together, these data demonstrate that loss of NC residues correlates with a reduction in association of Gag with ABCE1.

Analysis of features in these truncated constructs revealed that the two constructs that interacted with ABCE1 as well as wild-type (Tr427 and Tr437) contained both Cys-His boxes and 14 or 15 basic residues. The four constructs that interacted to an intermediate extent (Tr405, Tr409, Tr410, and Tr412) contained one Cys-His box and 7–11 basic residues. Finally, the two constructs that failed to interact with ABCE1 (Tr361 and Tr388) contained no Cys-His boxes and four or fewer basic residues. The shortest truncation mutant from our series that associated with ABCE1, Tr405, contained one Cys-His box and seven basic residues in NC (see Fig. 1, A and C [bottom]).

Substitution of Lysines and Arginines but Not Cysteines and Histidines in NC Eliminates ABCE1 Association—Since both the zinc fingers and the basic residues in NC were altered upon truncation of NC, the mutations described above failed to distinguish whether only one of these features was critical for the Gag-ABCE1 interaction. Therefore, we engineered additional mutations to determine whether the Cys-His boxes or the dispersed basic residues in NC govern association with ABCE1. First, we assessed the effect of deleting the Cys-His boxes. Gag mutants containing deletions of either the first Cys-His box or the second Cys-His box (Gag^ΔCH1 versus Gag^ΔCH2) associated with ABCE1, as indicated by immunoprecipitation of COS-1 lysates with α-ABCE1 (Fig. 2A). Deletion of both Cys-His boxes (Gag^ΔCH1/2) reduced the Gag-ABCE1 interaction to very low but detectable levels (Fig. 2A).

Each Cys-His box in NC contains not only the cysteine and histidine residues that are critical for zinc finger formation but also a few basic residues. Thus, our deletion of both Cys-His boxes (Gag^ΔCH1/2) resulted in the loss of five of the 15 lysines and arginines present in NC (see diagrams in Fig. 2A). Consequently, results obtained using the Gag^ΔCH1/2 construct did not allow us to precisely define the contrib-
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We engineered point mutants that would allow us to completely dissociate the contribution of cysteines and histidines from that of lysines and arginines in NC. In constructs CH1A and CH2A, all of the cysteines and histidines in either the first or second Cys-His box were replaced with alanines, whereas CH1/2A contained the same substitutions in both Cys-His boxes. In all of these constructs, the lysines and arginines present in NC were unaltered. As shown in Fig. 2B, when expressed in COS-1 cells, all three constructs were co-immunoprecipitated by α-ABCE1, indicating that the Gag-ABCE1 interaction was maintained even upon substitution of all the cysteines and histidines in NC. Co-immunoprecipitation of CH1A and CH2A by α-ABCE1 was similar to wild-type Gag. In contrast, substitution of all the cysteines and histidines in NC (CH1/2A) resulted in reduced association with endogenous ABCE1, but the association was consistently detectable (Fig. 2C).

Having established that substitution of cysteine and histidine residues critical for zinc finger formation did not eliminate the Gag-ABCE1 interaction, we next addressed the contribution of basic charge substitutions. Previously, Cimarelli et al. (24) found that mutation of the 10 arginine and lysine residues located in and between the Cys-His boxes resulted in loss of viral replication, virion production, and Gag-Gag interactions. When we substituted the same 10 lysines and arginines in NC with alanines (KR10A), complete elimination of the Gag-ABCE1 interaction was observed, despite expression levels similar to wild-type
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Gag (Fig. 2B, T lanes). Even very long exposures of immunoblots failed to detect co-immunoprecipitation of KR10A by α-ABCE1. These results were confirmed by quantitation (Fig. 2C). Together, these data indicate that the association of HIV-1 Gag with ABCE1 is dependent on basic residues in NC and does not require intact zinc fingers in NC.

To evaluate the number of basic charge substitutions that are required to abolish the interaction of Gag with ABCE1, we engineered Gag constructs containing substitutions of fewer than 10 basic residues. Because initial experiments revealed that alanine substitution of three or four basic residues in the region between the two Cys-His boxes only had small effects on the ABCE1 interaction (data not shown), we engineered a more extreme charge disruption by substituting 3 or 4 lysines and arginines in this linker region of NC with glutamic acids (KR3E and KR4E, respectively). Upon expression of these constructs in COS-1 cells, association with endogenous ABCE1 was observed by co-immunoprecipitation, albeit at reduced levels (Fig. 3A). These data suggest that between 5 and 10 basic residues in NC need to be mutated (leaving 5–10 basic residues intact) to completely eliminate association of full-length Gag with endogenous ABCE1. The results are similar to findings obtained using truncation mutants in Fig. 1, in which constructs encoding seven or more basic residues were found to associate with ABCE1. Since the exact number of residues required for loss of the Gag-ABCE1 interaction is likely to vary with the exact position of the mutated residues within NC, the choice of substituting amino acid, the length of the Gag construct used, and the exact coding sequence of the Gag strain, we did not attempt finer resolution mapping of basic residues.

Deletion of Large Regions of MA and CA Does Not Eliminate the Gag-ABCE1 Interaction—Others have reported that deletions of large regions of MA and CA do not have significant effects on infectivity, as long as the myristoylation signal and associated charged residues in MA are left intact (35, 36). Consistent with this, we found that deletion of a large region of CA (residues 143–276 in Gag; ΔCA) or deletion of most of MA and CA (residues 12–282 in Gag; ΔMACA) reduced but did not eliminate the ability of Gag to associate with ABCE1 (Fig. 3B). In addition, deletion of the major homology region (residues 285–304 in Gag; ΔMHR) within CA had little effect on the Gag-ABCE1 interaction (Fig. 3B). Together, these findings indicate that most of MA and CA are not essential for the Gag-ABCE1 interaction. However, the reduction in association observed with the large deletions raises the possibility that these domains may contain residues that modulate the Gag-ABCE1 interaction.

Confirmation of the Role of Basic Charge in NC for Gag-ABCE1 Interaction Using Other Approaches—The interaction of ABCE1 with Gag was initially identified by biochemical dissection of a cell-free system that supports de novo assembly of capsids closely resembling immature HIV-1 capsids produced in cells (1, 2, 4, 37). Immunodepletion-reconstitution experiments in this system were also used to demonstrate the critical role of ABCE1 in post-translational events in HIV-1 capsid formation (4). To validate findings obtained using α-ABCE1 in COS-1 cells, we examined the co-immunoprecipitation of HIV-1 Gag mutants in this cell-free HIV-1 capsid assembly system. FLAG-tagged human ABCE1 was co-translated either with HIV-1 wild-type Gag or with selected Gag mutants in parallel reactions and then subjected to immunoprecipitation under native conditions with antibody to FLAG (α-FLAG). Consistent with our previous observations, α-FLAG co-immunoprecipitated wild-type Gag and Tr437 but not Tr361 (Fig. 4A). In addition, α-FLAG co-immunoprecipitated CH1A, CH2A, and CH1/2A to varying extents (Fig. 4A) but did not immunoprecipitate the KR10A mutant (Fig. 4B). Thus, these NC mutations have comparable effects on the Gag-ABCE1 interaction when assessed in different cellular contexts (primate cells versus a cell-free system programmed with wheat germ extract) or using different antibodies (α-ABCE1 to detect endogenous ABCE1 versus α-FLAG to detect epitope-tagged ABCE1).

We also examined the interaction of WT and mutant Gag with a recombinant GST-ABCE1 fusion protein (encoding human ABCE1) produced in E. coli using a pull-down assay. Lysates of COS-1 cells expressing either wild-type or mutant Gag constructs were incubated with GST-ABCE1 purified from E. coli and bound to glutathione beads.

FIGURE 4. Other approaches validate the finding that basic charge in NC is critical for the Gag-ABCE1 interaction. A and B, the indicated Gag constructs and FLAG-tagged human ABCE1 (if ABCE1) were co-translated in a cell-free translation and assembly system containing [35S]methionine. Reactions were subjected to immunoprecipitation with α-FLAG (immune, I) or nonimmune control antibody (N). Migrations of WT Gag and Gag mutants are indicated to the left and the right. Equivalent aliquots of total input (T) are shown to indicate migration and level of expression. Lower bands represent radiolabeled polypeptides that terminated early or initiated late. Asterisks indicate the predicted migration of each Gag construct in the immune lane. All lanes were taken from a single exposure. C, recombinant GST-ABCE1 fusion protein from E. coli was bound to glutathione-agarose columns. Lysates of COS-1 cells expressing WT Gag, KR10A, CH2A, or CH1/2A were applied to GST-ABCE1 columns in parallel. Columns were washed, and three elutions (E1, E2, and E3) were performed using buffer containing glutathione. Equivalent amounts of the last wash (W), E1, E2, and E3 were analyzed for the presence of GST-ABCE1 bound to WT or mutant Gag from the cell lysate by immunoblotting (WB) with α-ABCE1 (top panels) and antibody to Gag (bottom panels). The first lane of each panel (T) shows an aliquot of total input cell lysate to indicate migration and level of expression. Note that the cell lysate does not contain GST-ABCE1, which is derived only from the column. Experiments in each panel were repeated three times, and representative data are shown.
GST-ABCE1 and proteins bound to GST-ABCE1 were eluted from the beads using glutathione, and the presence of Gag and ABCE1 in eluates was assessed by immunoblotting. Following incubation of lysates containing WT Gag, final washes contained no Gag or ABCE1, whereas glutathione eluted both GST-ABCE1 and Gag, indicating that WT Gag was bound to GST-ABCE1 (Fig. 4C). In contrast, after incubation with lysates expressing the negative control Tr361 (Gag truncated proximal to NC; see Fig. 1A), recombinant GST-ABCE1 was detected in eluates, but Tr361 was not (data not shown). KR10A expressed in COS-1 cell lysates also failed to bind to GST-ABCE1, whereas CH1A, CH2A, and CH1/2A from cell lysates bound to intermediate levels (Fig. 4C). Thus, comparable results were obtained when constructs that separately abolish zinc finger motifs versus basic residues in NC were examined by three different approaches, including co-immunoprecipitation of endogenous ABCE1 in COS-1 cells, co-immunoprecipitation of epitope-tagged human ABCE1 expressed in a cell-free assembly system and pull-down from COS-1 cells with recombinant GST-ABCE1. Therefore, we conclude that the basic residues in the NC domain of Gag are critical for association of Gag with ABCE1.

The Interaction between Gag and ABCE1 Is Resistant to RNase A Treatment—Many studies have shown that the basic residues in the NC domain of Gag are responsible for binding to nonspecific RNA (reviewed in Ref. 23). Association of Gag with other proteins, such as Apobec3G, via an RNA bridge has been demonstrated (e.g. see Refs. 38 and 39). Because ABCE1 may have RNA binding elements (6, 15), it is possible that the charged residues in NC allow Gag to associate with ABCE1 by an RNA bridge.

To address this, we examined the effect of RNase A on the Gag-ABCE1 interaction, using the Gag-Apobec3G interaction as a control for RNase sensitivity. Cellular lysates expressing the nearly complete HIV genome (HIV-1ΔEnv) and Apobec3G were treated in parallel with different concentrations of RNase A and then subjected to immunoprep-
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To further confirm that RNase A was effective in these experiments, we subjected an aliquot of selected lysates used for immunoprecipitation in Fig. 5A to reverse transcription followed by quantitative PCR. HIV-1-specific RNAs and actin mRNA were virtually eliminated upon treatment with even 1 μg/ml RNase A (Fig. 5B), indicating that RNase A was indeed active. Murine actin, from untreated murine cell lysate that was mixed in after RNase treatment to serve as a positive control for RNA extraction and RT efficiency (see “Experimental Procedures”), remained relatively constant between all samples (Fig. 5B). Thus, the Gag-ABCE1 interaction is maintained even when lysates are treated with concentrations of RNase in excess of that required to release cellular and HIV-1 RNA to undetectable levels.

Gag Mutants That Fail to Release Completed Capsids Also Fail to Progress through the Capsid Assembly Pathway—Studies by Cimarelli et al. (24) showed that Gag mutants encoding the KR10A substitutions do not support Gag-Gag interactions. Furthermore, they demonstrated that cells expressing these mutants in the context of the complete HIV genome do not release significant numbers of virions. The few virions released have abnormal cores by electron microscopy (24). To determine whether mutants that exhibited reduced ABCE1 binding were able to release immature capsids, we examined the media from cells expressing wild-type and mutant constructs. Immunoblotting of lysates from transfected COS-1 cells revealed that the NC mutants expressed to similar levels (Fig. 6A). Media from these cells were treated with detergent to remove the envelopes of released viral particles and then subjected to velocity sedimentation as previously described (1, 2, 4). As expected, wild-type immature capsids were detected in the fractions corresponding to ∼750 S by immunoblotting with antibody to Gag (Fig. 6B). Since the HIV-1 protease was not expressed, only immature capsids were released. Cells expressing the negative control construct Tr361 did not release immature capsids. Cells expressing the KR10A construct also failed to release immature capsids into the medium as judged by velocity sedimentation, although KR10A contains an intact p6 domain required for budding. Both CH1A and CH2A released ∼750 S capsids into the medium. In contrast, CH1/2A did not release immature capsids into the medium (Fig. 6B).

Immature capsid release by CH1A and CH2A is consistent with the findings of others showing that mutations in only one Cys-His box have minimal effects on particle release (41). In addition, capsid release by these constructs fits with our observation that CH1A and CH2A interact well with ABCE1 (Fig. 2, B and C). Conversely, the absence of capsid release by CH1/2A and KR10A, which interacted with ABCE1 poorly or not at all (Fig. 2, B and C), suggests defects in intracellular events during immature capsid formation. Previously, we have demonstrated that wild-type Gag and assembly-competent Gag mutants progress through the entire assembly pathway, progressively forming the ∼10, ∼80, ∼150, and ∼500 S intracellular assembly intermediates before forming completed ∼750 S capsids (2). In contrast, assembly-defective Gag mutants are arrested at different points along the immature capsid assembly pathway with accumulation of assembly intermediates that precede the point of blockade (1–3, 30). To determine why KR10A and CH1/2A failed to produce virions, we examined intracellular capsid assembly intermediates formed by KR10A and CH1/2. Lysates of COS-1 cells expressing WT and mutant Gag constructs were analyzed using velocity sedimentation gradients in which early assembly intermediates (∼10 and ∼80 S) migrate at the top of the gradient, and late (∼500 and ∼750 S) assembly intermediates migrate at the bottom. Analysis of the assembly-competent WT Gag and CH2A constructs revealed the presence of both early and late assembly intermediates at steady state (Fig. 7A, left panels). Similar results were obtained for CH1A (data not shown). In contrast, the assembly-defective KR10A and CH1/2A constructs formed only early assembly intermediates and accumulated
these in large amounts (Fig. 7A, left panels). These data indicate that both KR10A and CH1/2A fail to produce completed capsids because they are arrested at early points in the immature capsid assembly pathway.

Since KR10A fails to interact with ABCE1, whereas CH1/2A interacts with ABCE1 poorly, it is likely that they exhibit different defects during early events in immature capsid formation. We have previously shown that the ~10 S complex does not contain ABCE1, whereas the ~80 S complex represents the first assembly intermediate in which ABCE1 binds to Gag (1, 4). Moreover, the ~80 S assembly intermediate is a critical one, since depletion of ATP causes arrest of Gag in the assembly pathway, with accumulation of the ~80 and ~150 S assembly intermediates (2). Thus, formation of and/or exit from the ~80/150 S assembly intermediates is likely to be rate-limiting and critical for assembly.

To distinguish between early assembly intermediates, cell lysates were analyzed by velocity sedimentation using a gradient that completely separates the first two assembly intermediates (~10 S from ~80 S). WT Gag and CH2A, both of which are assembly-competent, contained clear peaks corresponding to both the ~10 and ~80 S assembly intermediates at steady state (Fig. 7B). In contrast, the KR10A mutant formed only the ~10 S complex. The failure of KR10A to form the ~80 S assembly intermediate (Fig. 7B) is consistent with its inability to interact with ABCE1 (Fig. 2, B and C). CH1/2A formed a small but reproducible peak in the ~80 S region of the gradient (Fig. 7B), superimposed on a trail of the ~10 S assembly intermediate. The significantly reduced binding of CH1/2A to ABCE1 (Fig. 2, B and C) with abnormal or inadequate formation of the ~80 S assembly intermediate (in which Gag associates with ABCE1) could explain the failure of CH1/2A to progress beyond the ~80 S stage of the assembly pathway. Thus, these data suggest that although the cysteines and histidines in the zinc fingers are not required for minimal binding of ABCE1, the presence of at least one intact Cys-His box in NC may be important for reaching threshold levels of ABCE1 binding and consequently for proper ABCE1 function in the intracellular capsid assembly pathway.

FIGURE 7. Velocity sedimentation analysis reveals that KR10A and CH1/2 are arrested in the form of early assembly intermediates. A, COS-1 cell lysates of wild type and the indicated mutant Gag constructs were analyzed by velocity sedimentation on gradients that separate early from late assembly intermediates. Fractions were immunoblotted, and the amount of Gag in each lane was quantitated and graphed as percentage of total Gag. Positions of early (~10 to ~80 S) and late (~500 to ~750 S) assembly intermediates are shown. B, the same lysates were analyzed as in A but using a different velocity sedimentation gradient that separates the ~10 S assembly intermediate from the ~80 S assembly intermediate, as indicated. Positions of early ~10 and ~80 S assembly intermediates are shown. Experiment was repeated twice, and representative data are shown.
DISCUSSION

Studies presented here demonstrate that basic residues in the NC domain of Gag are critical for the association of the cellular ATP-binding protein ABCE1 (previously known as HP68 or RLI) with HIV-1 Gag. Substitution of 10 of 15 lysines and arginines in NC completely abolished the interaction of Gag with endogenous ABCE1 present in COS-1 cells. In contrast, substitution of all of the cysteines and histidines in NC did not eliminate the Gag-ABCE1 interaction, indicating that the Gag-ABCE1 interaction can occur in the absence of intact zinc fingers. Large regions of MA and CA are also not essential for the Gag-ABCE1 association per se. Taken together with our previous finding that the p6 domain of Gag is dispensable for this interaction (1, 4), these data indicate that basic residues in NC are important determinants of the Gag-ABCE1 association in primate cells. More detailed mapping revealed that Gag constructs containing a minimum of 6–10 lysines and arginines in NC were able to interact with endogenous ABCE1 in primate cells, albeit at reduced levels.

Whereas basic charge in NC appears to be critical for the Gag-ABCE1 interaction, one caveat to note is that other motifs and regions of Gag could modulate this interaction and could also be needed for ABCE1 to function properly during assembly. Notably, the data presented here do not address whether the N terminus of MA plays a role in Gag-ABCE1 binding. Furthermore, the reduction in the Gag-ABCE1 interaction seen with deletions in MA and CA raise the possibility that residues in these regions influence recruitment and function of ABCE1 during capsid formation. Similarly, substitution of as few as four basic residues with glutamic acids (KR4E) and substitution of all the cysteines and histidines (CH1/2A) resulted in significant reduction in ABCE1 binding. Substitution of 10 of 15 lysines and arginines in NC completely abolishes the interaction of Gag with endogenous ABCE1 present in COS-1 cells, albeit at reduced levels.

Whereas basic charge in NC appears to be critical for the Gag-ABCE1 interaction, one caveat to note is that other motifs and regions of Gag could modulate this interaction and could also be needed for ABCE1 to function properly during assembly. Notably, the data presented here do not address whether the N terminus of MA plays a role in Gag-ABCE1 binding. Furthermore, the reduction in the Gag-ABCE1 interaction seen with deletions in MA and CA raise the possibility that residues in these regions influence recruitment and function of ABCE1 during capsid formation. Similarly, substitution of as few as four basic residues with glutamic acids (KR4E) and substitution of all the cysteines and histidines (CH1/2A) resulted in significant reduction in ABCE1 binding. In the case of CH1/2A, assembly is arrested at the ~80 S assembly intermediate, raising the possibility that reduced ABCE1 binding prevents ABCE1 from functioning properly during capsid assembly. Further investigation of this possibility will require a better understanding of how ABCE1 acts to promote capsid formation.

NC from HIV-1 and other retroviruses is known to interact nonspecifically with RNA as well as in a highly specific manner with genomic RNA (reviewed in Ref. 23). The nonspecific RNA interaction is important for promoting Gag multimerization and capsid formation and appears to be governed by basic charge in NC (24, 25, 42–45). In contrast, packaging of the genome of HIV-1 and many other retroviruses requires intact zinc fingers within NC (46–51). These and other findings support a widely accepted model in which association of basic residues with RNA promotes multimerization of the ~5000 Gag polypeptides that are needed to form a single immature capsid, whereas the Cys-His boxes govern encapsidation of genomic RNA (24, 26, 27, 52, 53). However, the finding that RNA plays a critical role in assembly does not exclude the possibility that other factors may also play an important role in promoting proper assembly. Recruitment of proteins that promote capsid assembly could be important in cells, where the concentration of Gag is typically quite low, barriers to efficient assembly are likely to exist, and efficiency of virion production is critical for propagation.

Although it remains to be determined whether primate ABCE1 has RNA-binding properties, our data suggest that the Gag-ABCE1 interaction is not dependent on an RNA bridge. We have previously found that the Gag-ABCE1 interaction is relatively resistant to 1 μg/ml RNase A (4). Here we demonstrate that the interaction is resistant at to RNAse A at 1000 μg/ml, which greatly exceeds the concentration of RNase A required to fully degrade cellular and HIV-1 RNA and disrupt another known RNase-sensitive interaction in the same extract. One explanation for these findings is that the Gag NC domain may be associated with ABCE1 largely through protein-protein interactions. An alternate possibility is that RNA binding by NC may promote Gag-Gag interactions that in turn alter the conformation of Gag, thereby exposing a binding site for ABCE1 elsewhere in Gag. In this model, after ABCE1 is bound to Gag, RNA is no longer required to maintain the altered conformation that exposes the ABCE1 binding site. Further investigation will be required to distinguish between these models in which the basic charge in NC acts either directly or indirectly to promote the Gag-ABCE1 interaction. Note that other primate lentiviral Gag proteins interact with ABCE1 during assembly (1). Studies suggest that HIV-2 and SIV Gag proteins also utilize basic residues to recruit endogenous ABCE1 in primate cells, although the exact residues involved have not been mapped (1, 30).

In summary, our data suggest the following model. Whereas a wide variety of unrelated viruses bind nonspecifically to RNA to promote capsid formation, HIV-1 and other primate lentiviruses appear to have evolved a mechanism in which the same RNA-binding residues also act directly or indirectly to recruit ABCE1, a cellular protein that further facilitates the capsid assembly process. By promoting efficient capsid formation in cells, ABCE1 may act as a molecular chaperone in concert with RNA to ensure Gag multimerization under circumstances where assembly is not favored. Since ABCE1 is involved in promoting ribosome assembly (5–9), it would be present in an ideal location to associate with and act on newly synthesized Gag polypeptides.

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