

Redundant late domain functions of tandem VP2 YPX₃L motifs in cellular egress of quasi-enveloped hepatitis A virus

Running title: VP2 YPX₃L motifs of HAV

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ABSTRACT

The quasi-envelopment of hepatitis A virus (HAV) capsids in exosome-like virions (eHAV) is an important but incompletely understood aspect of the hepatovirus life cycle. This process is driven by recruitment of newly assembled capsids to endosomal vesicles into which they bud to form multi-vesicular bodies with intraluminal vesicles that are later released at the plasma membrane as eHAV. The endosomal sorting complexes required for transport (ESCRT) are key to this process, as is the ESCRT-III associated protein, ALIX, which also contributes to membrane budding of conventional enveloped viruses. YPX_{1or3}L late domains in the structural proteins of these viruses mediate interactions with ALIX, and two such domains exist in the HAV VP2 capsid protein. Mutational studies of these domains are confounded by the fact that the Tyr residues (important for interactions of YPX_{1or3}L peptides with ALIX) are required for efficient capsid assembly. However, single Leu-to-Ala substitutions within either VP2 YPX₃L motif (L1-A and L2-A mutants) were well tolerated, albeit associated with significantly reduced eHAV release. In contrast, simultaneous substitutions in both motifs (L1,2-A) eliminated virus release, but did not inhibit assembly of infectious intracellular particles. Immunoprecipitation experiments suggested that the loss of eHAV release was associated with a loss of ALIX recruitment. Collectively, these data indicate that HAV YPX₃L motifs function as redundant late domains during quasi-envelopment and viral release. Since these motifs present little solvent accessible area in the crystal structure of the naked extracellular capsid, the capsid structure may be substantially different during quasi-envelopment.

IMPORTANCE

Nonlytic release of hepatitis A virus (HAV) as exosome-like quasi-enveloped virions is a unique but incompletely understood aspect of the hepatovirus life cycle. Several lines of evidence

indicate that the host protein ALIX is essential for this process. Tandem YPX₃L ‘late domains’ in the VP2 capsid protein could be sites of interaction with ALIX, but they are not accessible on the surface of an X-ray model of the extracellular capsid, raising doubts about this putative late domain function. Here we describe YPX₃L domain mutants that assemble capsids normally, but fail to bind ALIX and be secreted as quasi-enveloped eHAV. Our data support late domain function for the VP2 YPX₃L motifs, and raise questions about the structure of the HAV capsid prior to and following quasi-envelopment.

INTRODUCTION

Hepatitis A virus (HAV) is an unusual hepatotropic picornavirus. Classified within the genus *Hepatovirus*, it is an ancient human pathogen that remains a common cause of enterically-transmitted hepatitis globally. It infects susceptible individuals in a stealth-like manner, replicating efficiently in the liver and releasing newly replicated virus through the biliary track to the intestines from which it is shed in feces (1, 2). Virus-specific antibodies generally first appear after 3-4 weeks of clinically silent infection, and typically herald both the onset of acute liver injury and resolution of the infection (3). Sudden increases in serum alanine aminotransferase (ALT) activity are associated with necrosis and apoptosis of hepatocytes and intrahepatic portal inflammatory cell infiltrates comprised of lymphocytes, macrophages, and plasma cells (4, 5). Although the disease can be fulminant, leading to death in a small fraction of those infected, the acute inflammatory phase of the illness is typically short lived and self-limited. Fecal shedding and intrahepatic replication of the virus diminishes rapidly after the appearance of antibody, and ALT levels return to normal over several weeks. Persistent infections occur rarely, if ever, and neutralizing anti-HAV antibodies provide durable protection against second episodes of disease (5).

Despite the acute necroinflammatory changes within the liver that accompany HAV infection, wild-type virus is noncytopathic; the acute liver injury it causes results from innate and possibly also adaptive immune responses to the infection (6). Many mammalian cell lines are permissive for HAV replication (*e.g.*, Huh7, HepG2, MRC-5, BSC-1), but wild-type and low-passage isolates induce no cytopathology (7). Virus is released from such cells without lysis in small extracellular vesicles (EVs) that protect the HAV capsid from neutralizing antibodies (8).

These ‘quasi-enveloped’ virions (eHAV) are the only form of virus found in sera from infected humans. They have a specific infectivity similar to naked HAV virions, are ~50-110nm in diameter, and possess a buoyant density of ~1.100 g/cm³ in iodixanol (8). In many ways, these eHAV vesicles resemble ‘exosomes’, small EVs that function in intercellular communications (9). Recent quantitative proteomics studies have shown that HAV capsids are recruited for export in these vesicles via a highly specific sorting process, and that the eHAV membrane is associated with a variety of endolysosomal proteins typically found in exosomes (10). Also present in eHAV are multiple proteins associated with endosomal sorting complexes required for transport (ESCRT), including ALIX, CHMP1A and CHMP4B (10). In aggregate, these findings support an exosome-like mechanism of eHAV biogenesis, in which assembled intracellular capsids are recruited to the cytosolic surface of endosomes into which they bud in an ESCRT-mediated process to form multivesicular bodies (MVB) that ultimately fuse with the plasma membrane, releasing intraluminal eHAV to the extracellular environment (11).

How HAV capsids are recruited to ESCRT is incompletely understood, but a tantalizing clue is the presence of tandem YPX₃L ‘late domain’ motifs, separated by 28 amino acid residues, in the VP2 capsid protein (8) (**Fig. 1A**). The structural proteins of conventional enveloped viruses typically contain ‘late domains’ (PPXY, P(S/T)AP, GPPX₃Y, or YPX_{1or3}L) that mediate interactions with ESCRT machinery (12, 13). The two YPX₃L motifs in VP2 are the only known late domain motifs in the 2227 amino acid (aa) sequence of the complete HAV polyprotein, and they are conserved in all 94 human HAV sequences in GenBank as well as novel hepatovirus species recently identified in bats and other small mammals (14). YPX₃L motifs mediate interactions with ALIX, which in turn binds CHMP4 (ESCRT-III complex) in a step that is critical for membrane scission (15). We have shown that both CHMP4 and ALIX are associated

with eHAV (10). Although previous studies of HAV mutants in which the Tyr residues in either YPX₃L motif were replaced with Ala suggest that anti-ALIX antibodies bind the capsid in a YPX₃L motif-dependent fashion (8, 10), the interpretation of these studies was confounded by disruption of capsid assembly by either Tyr-to-Ala substitution. A Tyr¹⁷⁷Ala mutant (Y2-A, **Fig. 1A**) produced minimal amounts of capsid reactive with the anti-capsid monoclonal antibody (mAb), K24F2, whereas a Tyr¹⁴⁴Ala mutant (Y1-A) produced none (8).

Further confusion about the role of these VP2 YPX₃L motifs in the HAV lifecycle stems from the crystallographic structure of the naked, nonenveloped extracellular HAV particle, solved shortly after the discovery of quasi-enveloped eHAV (16) (**Fig. 1B**). Both VP2 motifs are largely buried beneath the surface of the capsid in the structure, with little solvent accessible surface available for interaction with ALIX (**Fig. 1A,C**). This makes it difficult to understand how these putative late domains could function in ESCRT-mediated release of quasi-enveloped virus. However, the protein composition of the eHAV capsid differs from that of the naked capsid that has been studied crystallographically, as eHAV contains an unprocessed 345 aa-long VP1pX capsid protein rather than the 274 aa VP1 present in naked extracellular capsids (8). Whether this results in significant conformational differences between the capsids of quasi-enveloped and naked capsids, is unknown. There is no direct evidence for this, and the positioning of the YPX₃L motifs within the naked capsid calls into question the proposed role of these motifs in recruitment of ALIX.

With these issues in mind, we carried out additional mutational studies of the YPX₃L motifs of HAV using a newly developed, low cell culture-passage, noncytopathic infectious molecular HAV clone. We have assessed the impact of amino acid substitutions within these motifs that do not block capsid assembly on the nonlytic release of quasi-enveloped eHAV from cells and

ALIX interactions with the HAV capsid. These new studies provide additional support for a role of these conserved VP2 motifs in the biogenesis of quasi-enveloped virus, and suggest indirectly that there may be significant differences in the structures of quasi-enveloped and naked HAV capsids.

RESULTS

Tyrosine substitutions within the VP2 YPX₃L motifs disrupt capsid assembly. Previous reverse molecular genetics studies (8) demonstrated that Ala substitutions of the N-terminal Tyr residues of either of the two VP2 YPX₃L late domains motifs have no effect on replication of the HAV genome, but substantially reduce release of infectious virus from RNA-transfected cells. The interpretation of these experiments was complicated by limited evidence for capsid assembly, however, especially when the first late domain motif (VP2 residues 144-149) was mutated, as well as the use of a parental infectious molecular clone that is highly cell culture adapted and that replicates rapidly with a strong cytopathic effect (HM175/18f) (17, 18). To better assess the impact of such mutations on the release of virus without cell lysis over longer periods of time, we constructed a new infectious molecular clone using cDNA from a low-passage virus (HM175/p16) that replicates more efficiently than wild-type virus but without cytopathic effect (19). As we had found previously with mutants in the cytopathic 18f background (8), single Tyr-to-Ala substitutions in either putative late domain (**Fig. 1A**) eliminated the release of extracellular virus, which was first evident 3-4 days after electroporation of the parental p16 RNA containing wild-type motif sequences (**Fig. 2A**). Blind, cell-free passage of lysates prepared 7 days after transfection of the p16 mutants resulted in the emergence of robust virus release 8 to 9 days later (**Fig. 2A**). Sequencing of virus collected from supernatant fluids 14 days after blind passage revealed that the Y1-A mutant had reverted to the

wild-type sequence, restoring the first late domain motif. The VP2 Tyr¹⁷⁷Ala substitution persisted in the Y2-A mutant, but was now accompanied by a second substitution (His¹⁷¹Asn) in VP2. Viral RNA persisted in supernatant fluids of cells transfected with the double mutant, Y1,2-A, but only at levels 1000-fold less than the parental virus (**Fig. 2A**). We did not attempt to sequence this.

To more fully assess the impact of the original mutations on the capacity of the mutated genomes to express HAV proteins and direct the intracellular assembly of capsids, we used laser-scanning confocal fluorescence microscopy to image cells 48 hrs following transfection of viral RNA. For these short-term imaging experiments, we used mutants derived from the HM175/18f clone, as it provides substantially higher levels of viral protein expression than the HM175/p16 clone 48-72 hrs after electroporation of the RNA. Importantly, the amino acid sequences of the capsid proteins (including the pX domain of VP1) are identical in these clones, with the exception of a VP1 Ser²⁷¹Pro substitution in 18f, 4 residues upstream of the VP1/pX cleavage site. We stained cells either with polyclonal, postconvalescent human antibodies (JC serum) or a capsid-specific mAb (K34C8) that has been shown previously to recognize a conformational epitope present on 70S capsids, but not 14S pentamer subunits (20). Under these conditions, the polyclonal JC antibody recognized only structural proteins, as it did not label cells containing a replicating subgenomic RNA replicon (18f-FLuc) that does not encode capsid proteins but expresses abundant double-stranded RNA (dsRNA) identified with a dsRNA-specific mAb, J2 (21) (**Fig. 3**). Labeling with the polyclonal human antibodies revealed readily detectable antigen in cells transfected with each of the Try-to-Ala mutants, confirming capsid protein expression, whereas K34C8 stained only cells transfected with the parental virus containing wild-type (wt) late domain motif sequences (**Fig. 2B**).

Collectively, these data confirm that Tyr-to-Ala substitutions in either late domain motif strongly impair capsid assembly. However, the ability of the Y2-A/H¹⁷¹N second-site revertant to replicate and efficiently egress from cells in the absence of cytopathic effect (**Fig. 2A**) indicates that the second late domain motif is not essential for eHAV release. This suggests that these late domains could function redundantly, if in fact they do function to recruit ALIX during quasi-envelope of capsids.

Because the late domain Tyr residues are buried in the structure of the naked virus particle (**Fig. 1A**) (16), we considered that it might be important for assembly or stability of the capsid to substitute them with residues that have side chains of approximately equal size and hydrophobicity. We thus created additional sets of mutants in which Tyr¹⁴⁴ was substituted with Glu (Y1-E mutant), or Tyr¹⁷⁷ was substituted with Trp (Y2-W), or both (Y1,2-E/W) (**Fig. 1A**). Each of the mutants created in the 18f background expressed capsid proteins that were readily detected 48 hrs after transfection by staining with the polyclonal JC antibody, but none expressed capsid antigen detectable with K34C8 (**Fig. 4A**). Moreover, in the p16 background, the release of extracellular virus was reduced more than 99% by either the single or double mutation, as determined by RT-qPCR (**Fig. 4B**). Intracellular viral RNA abundance was similarly reduced with each of the mutants 9 days after transfection (data not shown). Thus, these substitutions of the N-terminal Tyr residues also appear to prevent efficient capsid assembly.

Capsid assembly is not impaired by Leu-to-Ala substitutions in the late domain motifs.

As an alternative to amino acid substitutions involving the N-terminal Tyr residues in the YPX₃L motifs, we constructed mutants with single Leu-to-Ala motifs in the C-terminal Leu residues of the motifs (L1-A and L2-A, **Fig. 1A**) and as well as a double mutant with substitutions in both (L1,2-A) (**Fig. 1A**). Each of these mutants replicated well as RNA in the 18f background,

generating strong staining for dsRNA with the J2 antibody 48 hrs after transfection, as well as strong fluorescence with both JC and K43C8 labeling, the latter of which is indicative of capsid assembly (20) (**Fig. 5A**). Both the proportion of transfected cells staining positively with K34C8 (**Fig. 5B**), as well as the corrected total K34C8 fluorescence intensity per cell (CTFC) (**Fig. 5C**), were similar for cells transfected with viral RNA containing wt late domain motifs (18f), as well as each of the three mutants. We confirmed that the L1,2-A double mutant is capable of assembly and production of intracellular infectious virus by lysing cells 48 hrs after electroporation of the RNA, and demonstrating the presence of replicating viral RNA in naïve cells inoculated subsequently with the lysate (**Figs. 5D,E**). Lysates from cells electroporated with wt or L1,2-A RNAs generated similar numbers of infected cells upon passage, suggesting that the double mutant is fully capable of capsid assembly.

Leu to Ala substitutions in both late domain motifs ablate viral egress. Single Leu-to-Ala substitutions in either the first or second late domain motif resulted in significant reductions in the nonlytic release of p16 virus from RNA-transfected cells (**Fig. 6A**). Eight to 9 days post-transfection, the amount of L1-A mutant released into supernatant culture fluids was 24-26% of the parental virus, and the L2-A mutant 55-64%, based on RT-qPCR quantitation of HAV RNA (L1-A versus p16 $p=0.0009$, and L2-A versus p16 $p=0.0089$, by two-way ANOVA with Sidak's correction for multiple comparisons, from 5-9 days post-transfection). Interestingly, however there was no detectable release of virus from cells transfected with the L1,2-A double mutant (**Fig. 6A**), despite readily detectable K34C8 capsid antigen and recovery of virus from lysates of cells prepared 48 hrs after transfection (**Fig. 5**). The abundance of L1,2-A RNA in supernatant fluids at 8-9 days was 3% that of the parental p16 virus ($p=0.0004$), which was indistinguishable

from the amount of viral RNA present in supernatant fluids of cells transfected with a replication-incompetent 3D^{pol} mutant (**Fig. 6A**).

Both L1-A and L2-A virus populations present in the supernatant fluids of RNA-transfected cells were predominantly quasi-enveloped, with peak densities in isopycnic iodixanol gradients of $\sim 1.09 \text{ g/cm}^3$ (**Fig. 6B**). Thus, the defect in the release of these mutant viruses from cells was quantitative, not qualitative. The intracellular abundance of the Leu-to-Ala mutant RNAs was also reduced, compared to the parental p16 RNA-transfected cells, 9 days following transfection (**Fig. 6C**), consistent with reductions in the spread of virus within the cultures. The Leu residues that were altered in these mutants (Leu¹⁴⁹ and Leu¹⁸²) are relatively closely positioned within the structure of the naked capsid, but have no direct interactions with each other (16) (**Fig. 6D**). These results are thus consistent with the mutations having independent, albeit redundant effects on viral egress, with Leu-to-Ala substitutions in both late domains for nonlytic egress of the virus to be disrupted. Consistent with this, an additional mutant containing a Leu¹⁴⁹Ala substitution in the first motif and Tyr¹⁷⁷Ala substitution in the second motif, together with the stabilizing second-site His¹⁷¹Asn substitution that rescued replication and extracellular release of the Y2-A mutant (**Fig. 2A**), failed to generate extracellular virus when transfected into cells (data not shown).

To better understand the handicap in release of the L1,2-A double mutant, we characterized the distribution of viral RNA in isopycnic iodixanol gradients loaded with lysates harvested 72 after transfection of the RNA (18f virus background). RNA present in lysates of cells transfected with the parental 18f RNA or the 18f-FLuc subgenomic replicon banded at a predominant density of $\sim 1.25 \text{ g/cm}^3$, with a somewhat lighter “shoulder” at $\sim 1.19 \text{ g/cm}^3$ (**Fig. 7A**, top and bottom, respectively). Similar RNA species were present in lysates from cells transfected with

the L1,2-A mutant, but also present was a unique minor species banding at 1.093 g/cm³ (**Fig. 7A**, bottom) which is similar to the density of extracellular quasi-enveloped virus (8). Similar light RNA peaks (at slightly higher densities) were evident in gradients loaded with lysates of cells supporting replication of the L1-A and L2-A mutants (**Fig. 7B**, middle and bottom respectively). The magnitude of this peak was greater with the L1-A than the L2-A mutant, which correlates with the greater handicap in release of L1-A versus L2-A virus (**Fig. 6A,B**). The viral RNA present in these peaks is likely associated with membranes, and may represent a step in the egress pathway at which release is retarded (L1-A and L2-A mutants) or completely arrested (L1,2-A) by mutations in the late domains. Capsid proteins were not present in sufficient abundance in fractions to ascertain their positions in these gradients by immunoblotting or ELISA.

Leu-to-Ala mutations in the late domain YPX₃L motifs ablate capsid interactions with ALIX. YPX₁₋₃L late domain motifs in the structural proteins of enveloped viruses engage ALIX, an ESCRT-III associated protein, during budding of virus from cellular membranes. Our previous work shows that antibodies to ALIX can precipitate both intracellular and extracellular HAV capsids, and that a VP2 Tyr¹⁷⁷-Ala mutation (Y2-A, **Fig. 1A**) disrupts this association (8, 10). However, this amino acid substitution also severely impacts capsid assembly (8) (**Fig. 2**), as described above, clouding the interpretation of immunoprecipitation experiments. To assess whether Leu-to-Ala mutations in the putative late domains also interfere with ALIX binding to the HAV capsid, we used RT-PCR to quantify virus precipitated by anti-ALIX from cell lysates prepared 48 hrs after transfection of cells with parental (18f), L1-A (the single mutant with the greater defect in viral release), and double mutant, L1,2-A, viral RNAs. Antibody to ALIX robustly precipitated virus (>10-fold that precipitated by control IgG) from lysates of cells

transfected with the wt and L1-A mutant RNAs (**Fig. 8**). However, anti-ALIX failed to precipitate more virus than control IgG from the lysates of cells transfected with the L1,2-A double mutant (**Fig. 8**). These results are consistent a loss of capsid interactions with ALIX in the double mutant.

DISCUSSION

The quasi-envelopment and extracellular release of nascent HAV capsids in exosome-like virions represents a key aspect of the hepatovirus life cycle (8, 11). It allows for nonlytic egress of the virus, and thus noncytopathic replication with stealthy spread of virus within the liver as well as extended shedding of infectious virus in feces prior to the onset of liver injury. The ESCRT III-associated protein, ALIX (apoptosis-linked gene 2 (ALG2)-interacting protein X, otherwise known as PDCD6IP, programmed cell death 6 interacting protein) plays a central role in this process. RNAi-mediated depletion of ALIX results in a loss of extracellular release of the virus, but has no impact on replication of the viral RNA within infected cells (8). Furthermore, immunoprecipitation and quantitative proteomics experiments have demonstrated that ALIX is associated with encapsidated viral RNA in lysates of infected cells (8, 10). ALIX is also present within quasi-enveloped eHAV virions released from infected cell cultures or circulating in the blood of infected humans (8, 10). YPX_{1or3}L late domains represent important ALIX interaction motifs in the structural proteins of many conventional enveloped viruses (12, 13). The presence of two such YPX₃L motifs, separated by only 28 aa in the VP2 capsid protein is consistent with the role played by ALIX in quasi-envelopment of the HAV capsid. These motifs are highly conserved among disparate hepatoviruses infecting a wide variety of small mammalian species, although Pro-to-Lys or -Arg substitutions are present in the second (downstream) motif in some bat viruses (22). As discussed above, however, the limited accessible surface area presented by

these YPX₃L residues within the crystallographic model of the naked extracellular capsid structure appears incompatible with a direct ALIX interaction (**Fig. 1A**) (16).

Several studies have examined the structural basis of interactions between ALIX and the Gag proteins of retroviruses (23-25). ALIX is a large 89 kDa multi-domain protein that contributes to ESCRT functions in cytokinesis, endocytosis, exosome biogenesis, and budding of enveloped viruses (26, 27). Gag proteins interact through their YPX_{1or3}L motifs with a central V-shaped domain in ALIX (the “V” domain) that is formed by two multi-helix bundles and flanked on its two sides by an N-terminal Bro1-like domain that binds ESCRT-III and a C-terminal Pro-rich protein interaction domain (23). Crystal structures of the V domain complexed with Gag peptides show binding is stabilized by hydrophobic interactions involving a deep groove in the larger of the two helical bundles (24). The late domain Tyr residue is particularly important in this interaction, as its side chain extends deep into this hydrophobic pocket where it forms a hydrogen bond with a conserved Asp residue in ALIX. Additional hydrophobic interactions between the Gag late domains and ALIX involve conserved Leu residues immediately preceding the YPX_{1or3}L motif (hence the retroviral late domain motif is LYPX_{1or3}L) (24). Interestingly, the first VP2 late domain motif in VP2 is preceded in almost all hepatoviruses by Val¹⁴³ (Ile or Met in a few non-primate virus species) (**Fig. 1A**), which could contribute to similar stabilizing hydrophobic interactions with ALIX.

Mutational studies show that the late domain Tyr-Pro residues are critical for efficient HIV-1 release as well as the interaction of the HIV p6Gag YPX_{1or3}L motif with ALIX (25). Similar studies aimed at determining whether this is also true for HAV have been confounded by a lack of capsid assembly when either late domain Tyr residue is substituted with an alternative amino acid (28) (**Fig. 2B**). Both of these Tyr residues are structurally important for capsid stability, but

this is particularly true for Tyr¹⁴⁴ as it stabilizes the protein core and a short stretch of 3₁₀ helix in an adjacent loop (16). It is not surprising that the Tyr¹⁴⁴Ala substitution inhibits capsid assembly, as Ala cannot fulfil the same role. The importance of Tyr¹⁴⁴ to capsid structure is consistent with the reversion to wild-type sequence we observed on passage of the Y1-A mutant (**Fig. 2A**).

Tyr¹⁷⁷ in the second late domain stabilizes the conformation of the VP2 GH loop in the capsid structure by making bridging interactions across the loop (16). His¹⁷¹ is at the far end of the loop, and its substitution with Asn in the second-site revertant we observed upon passage of the Y2-A mutant (**Fig. 2A**) would seem likely to increase the number of loop-stabilizing interactions at this position, achieving a similar effect in the absence of Tyr¹⁷⁷. Importantly, the viability of the L2-A/H¹⁷¹N revertant and its efficient release from infected cells demonstrates that both late domain Tyr residues are not required for quasi-envelopment of HAV, and that the VP2 late domains thus act redundantly. Similar redundancy has been noted among the late domains of enveloped viruses (13, 29).

The C-terminal Leu residues of the retroviral Gag YPX_{1or3}L late domain motifs are also important for the interaction with ALIX, as they rest in hydrophobic contact with Phe and Ile residues in a shallow groove in the ALIX V domain (24). Ala substitution of the Leu⁴⁴ residue in the HIV p6 Gag domain substantially impairs binding to ALIX (30). Consistent with this, single Ala substitutions at either Leu¹⁴⁹ (L1-A mutant) or Leu¹⁸² (L2-A) resulted in modest reductions in the extracellular release of eHAV, while dual substitutions at both Leu residues (L1,2-A) resulted in a complete loss of extracellular virus release (**Fig. 6A,B**). Importantly, these substitutions, either singly or together, had no apparent impact on the expression of K34C8-specific fluorescence or production of infectious intracellular virus in RNA-transfected cells (**Fig. 5**). These mutations thus do not inhibit capsid assembly, but rather the quasi-envelopment

and extracellular release of capsids. Gradient analyses demonstrated the presence of a novel RNA-containing particle banding at a density similar to extracellular eHAV in lysates of cells transfected with the dual motif L1,2-A mutant RNA (**Fig. 7A**), but such cells did not contain encapsidated RNA precipitable with anti-ALIX antibody (**Fig. 8**). Collectively, these data provide strong support for the VP2 YPX₃L motifs functioning as late domains in the recruitment of ALIX to the HAV capsid and the subsequent budding of quasi-enveloped virus into MVBs. As suggested by the Y2-A/H₁₇₇N revertant (**Fig. 2A**), these domains are functionally redundant, with loss of either one resulting in only minor deficits in virus release (**Figs. 2A** and **6A**).

How can the lack of accessible surface area presented by the late domains in the crystal structure of the naked extracellular capsid (**Fig. 1A**) be reconciled with the notion that they interact with ALIX? The YPX_{1or3}L Tyr residues in Gag proteins are critical for interactions with the V domain of ALIX (24), but in HAV they play equally important roles in stabilizing the capsid structure, as discussed above, and are not available to be bound into the hydrophobic pocket of the V domain (16). Several possibilities could account for this apparent paradox. One is that the VP2 interaction with ALIX could occur prior to assembly of the capsid. This seems unlikely, however, as it fails to explain how all 4 capsid proteins would be selectively sorted for export if fully processed, and would suggest that polyprotein processing and capsid assembly occurs within vesicles destined for release. A second possibility is that the assembled intracellular capsid might “breathe” in a fashion that renders one of more of the 60 copies of each late domain transiently accessible on the surface of the capsid. However, this seems equally unlikely given the exceptionally high stability of the HAV capsid to heat and low pH (16, 31).

Yet a third scenario is that the structure of the intracellular capsids that are sorted for export might differ from that of the naked, extracellular capsid that has been studied

crystallographically. Rather than containing the 30.2 kDa VP1 protein present in the naked capsid, quasi-enveloped eHAV virions contain unprocessed 38.5 kDa VP1pX (8), first described by Anderson and Ross in more slowly sedimenting intracellular particles they named “pre-provirions” (32). The 8 kDa pX domain (sometimes referred to as the hepatovirus ‘2A’ protein) plays an essential role in pentamer assembly (33, 34), but much of it is surprisingly dispensable for replication (33, 35). It is rapidly lost from eHAV virions following exposure to detergents, although the identity of the cellular protease responsible for cleaving it from VP1 remains uncertain. Whether the structure of the naked capsid differs from that of the quasi-enveloped capsid, with the tandem VP2 YPX₃L motifs accessible on the surface for interactions with ALIX, and whether the capsid undergoes a final major conformational rearrangement following loss of pX are important questions to be addressed.

MATERIALS AND METHODS

Cells. Huh-7.5 human hepatoma cells were maintained in Dulbecco’s modified Eagle medium supplemented (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin at 37°C in a 5% CO₂ atmosphere.

Generation and transfection of HAV VP2 mutants. Mutations altering the putative VP2 late domains (Fig. 1A) were made in two closely related infectious molecular HAV clones: pHM175/18f.2 (high cell culture passage, rapid replication and cytopathic, GenBank KP879216.1) and pHM175/p16.2 (low cell culture passage, noncytopathic, GenBank KP879217.1). The Quick Change II Site-directed Mutagenesis Kit (Agilent) was used to generate both single and double mutants shown in Fig. 1B. Newly generated infectious clones were sequenced to confirm the mutations. For RNA transcription, plasmids were linearized at the *Mlu*I site at the 3’ end of the HAV sequence. RNA transcription was carried out using T7 RiboMAX™

Express Large-Scale RNA Production System (Promega) according to the manufacturer's protocol. A total of 5 μ g of synthetic RNA was electroporated into 2.5×10^6 Huh-7.5 cells using 4-mm cuvettes at 250V, 950 μ F, 50 Ω in a Gene Pulser Xcell Total electroporation system (Bio-Rad). Following electroporation, cells were maintained in DMEM supplemented with 3% FBS.

Viral RNA harvest and quantification. Infected cell supernatant fluids were cleared of dead cells and debris by centrifugation at 10,000 \times g for 5 min, followed by RNA extraction as previously described (36). For quantitation of intracellular virus, total RNA was extracted using the RNeasy Kit (Qiagen). Viral quantification was carried out using a two-step RT-qPCR as previously published (36). To confirm the production of intracellular infectious virus in cells transfected with L1,2-A RNA (see Results), approximately 1×10^6 electroporated cells were seeded into a 35 mm culture dish. Forty-eight hrs later, cells were subjected to freeze-thaw lysis in 200 μ L of 1X PBS. Naïve Huh-7.5 cells were inoculated with 50 μ L of lysate, and fixed and stained with anti-HAV antibody for fluorescence microscopy (see below) 48 hrs later.

Immunofluorescence microscopy. 8-well chamber slides containing Huh-7.5 cells were fixed with 4% paraformaldehyde followed by blocking with 10% goat serum. The cells were then incubated with primary antibodies at the appropriate dilution in Saponin solution (1 mg/mL in water, Sigma) for 2 hrs at room temperature. Antibodies used for immunofluorescence were: anti-HAV capsid K24F2 (1:300) and K34C8 (1:600) and hepatitis A convalescent human plasma antibody "JC" (1:1000). Cells were washed and incubated with secondary antibodies: Alexa Fluor® 488-goat anti-mouse, Alexa Fluor® 594-goat anti-mouse and Alexa Fluor® 488-goat anti-human for 1 hr at room temperature, followed by additional washing and sealing under coverslips using VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories). Images were collected using an Olympus FV10000 laser-scanning confocal microscope equipped

with a super-corrected 60×/1.4 NA oil-immersion objective (PLAPON60XOSC) and a dichroic mirror DM405/488/543/635. The pinhole was maintained at 1 Airy unit and laser power and image acquisition parameters were maintained at constant levels within independent experiments. The excitation/emission wavelengths were 405 nm/425-520 nm for DAPI, 488 nm/500-520 nm for Alexa Fluor 488, and 543 nm/555-647 nm for Alexa Fluor 594. Mean corrected total cell fluorescence (CTCF) values were obtained using ImageJ software and calculated as equal to Integrated Intensity – (Cell Area × Mean Background Fluorescence). Co-localization indexes were obtained with the Just Another Colocalisation Plugin (JACoP) module for ImageJ (37). Images were processed for presentation using Photoshop CS4.

Isopycnic gradient centrifugation of virus. Isopycnic gradient fractionation of virus released into cell culture supernatant fluids was carried out as previously described (8). For gradient analysis of intracellular virus, infected cells were subjected to freeze-thaw lysis in 1X PBS. The lysate was cleared by centrifugation for 5 min and loaded onto an 8–40% iodixanol (Opti-Prep) step gradient identical to that described previously (8).

Co-immunoprecipitation. Cell extracts were prepared for immunoprecipitation (IP) using RadioImmunoPrecipitation Assay lysis buffer (RIPA, Thermo). A total of 30 µL extract was used for each IP assay, adjusting the volume where necessary with 1X Phosphate-buffered saline (PBS) supplemented with 1X Protease inhibitor cocktail (Sigma). IP was carried out using anti-ALIX (1:50, sc-53540, Santa Cruz Biotechnologies) or control immunoglobulin, incubating the immunoglobulin with extracts overnight at 4°C in constant rotation. For antibody elution, 25 µL of Pierce™ Protein A/G Magnetic Beads (Thermo) was added to extracts and incubated overnight at 4°C in constant rotation. Antibody-virus-bead complexes were collected with a magnet and washed three times with Washing buffer (Tris-buffered saline containing 0.05%

Tween-20). Antibody-virus complexes were then eluted from the magnetic beads by adding 100 μ L Low-pH Elution buffer (0.1M glycine, pH 2.0) for 10 min, followed by neutralization with 15 μ L of 1M Tris, pH 8. RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen) and quantified using two-step RT-qPCR .

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LEGENDS TO FIGURES

Figure 1. HAV VP2 YPX₃L late domain motifs. **(A)** Schematic representation of late domain motifs in VP2. The organization of the HAV genome is shown at the top with the polyprotein coding region displayed as an extended box. Below is shown a segment of the VP2 protein with the two conserved YPX₃L motifs in red font and with the accessible surface area (ASA) of each plotted above along with the ratio to calculated GXG value (surface area of the residue relative to that in a peptide in which it is flanked on each side by Gly) based on the X-ray model of the naked HAV capsid (16). At the bottom are shown the YPX₃L mutants constructed for this study in the background of either HM175/p16 or HM175/18f virus with amino acid substitutions highlighted in red font. **(B)** Protomer subunit of the HAV capsid showing the adjacent, anti-parallel orientation of the two late domain motifs (VP2 residues 144-149 and 177-182, highlighted in magenta) within the VP2 (green) β -barrel (16). VP1 is shown in blue, and VP3 in red. **(C)** Section through the HAV capsid wall, showing the buried position of residues within the late domain motifs (magenta).

Figure 2. Tyr-to-Ala mutations in the HAV VP2 YPX₃L motifs ablate capsid assembly. **(A)** Extracellular virus release following electroporation of Huh-7.5 cells with wt (HM175/p16) or related Y1-A, Y2-A and Y1,2-A mutants. Media was replaced daily, and virus quantified by RT-qPCR. Cell-free virus was passaged from lysates harvested on day 7 post-electroporation, with supernatant fluid virus titers followed for an additional 14 days. Sequencing of viruses at day 14 post-passage revealed reversion of the Y1-A mutation to wt (Tyr¹⁴⁴) and the presence of a second-site substitution in Y2-A (VP2 H¹⁷¹N). **(B)** Laser-scanning confocal fluorescence microscopy of Huh-7.5 cells electroporated with the indicated wt (HM175/18f) or mutant RNA, and fixed and stained 48 hrs later with either JC

polyclonal human (top row) or K34C8 murine monoclonal anti-capsid (bottom row) antibodies. The absence of K34C8 fluorescence despite abundant JC fluorescence in cells transfected with the mutant RNAs is consistent with the absence of capsid assembly. Nuclear counterstaining was with DAPI (blue). ‘ $\Delta 3D^{pol}$ ’ is a genome-length HAV RNA in which GAA has replaced the GDD motif in the $3D^{pol}$ RNA-dependent RNA polymerase. Scale bars = 10 μ m.

Figure 3. JC polyclonal human convalescent antibody labels only HAV structural proteins in viral RNA-transfected cells examined by confocal fluorescence microscopy. **(A)** Schematic representation of the subgenomic 18f-FLuc RNA derived from the HM175/18f virus used in this study, in which the firefly luciferase has replaced most of the VP2-VP1 coding sequence. Gly-Ala-Ala has replaced the $3D^{pol}$ Gly-Asp-Asp sequence in the replication-incompetent 18f-FLuc/ $\Delta 3D^{pol}$ mutant. **(B)** Cells were either mock electroporated, or electroporated with wt (HM175/18f) virus RNA, the replicon 18f-FLuc, or 18f-FLuc $\Delta 3D^{pol}$, then fixed and stained 72 hrs later with either J2, a murine monoclonal antibody that binds dsRNA in a sequence-independent fashion (21), or the JC polyclonal antibody. The absence of JC fluorescence in cells transfected with 18f-FLuc RNA, despite abundant J2 fluorescence indicative of RNA replication, indicates that JC fails to detect nonstructural proteins of the virus under the conditions used. Scale bars = 10 μ m.

Figure 4. Late domain mutants with Y1-E, Y2-W, and Y1,2-E/W substitutions are unable to efficiently assemble capsids and thus heavily handicapped in replication. **(A)** Confocal fluorescence microscopic images of cells mock electroporated, or electroporated 48 hrs previously with wt (HM175/18f) or related mutant RNAs as shown. Absence of K34C8 fluorescence despite readily detectable expression of structural proteins labelled with JC

polyclonal antibody suggests a failure of capsid assembly. Scale bars = 10 μ m. **(B)**

Extracellular HAV RNA in supernatant of cells 9 days after electroporation with wt (HM175/p16) or related Y1-E, Y2-W, or Y1,2-E/W RNAs. Cells transfected with each of the mutants released less than 1% of the amount of virus released by cells transfected with the wt control.

Figure 5. Capsid assembly is not impaired by carboxy-terminal Leu to Ala substitutions within the VP2 YPX₃L motifs of HAV. **(A)** Confocal immunofluorescence microscopy of Huh-7.5 cells mock electroporated or electroporated wt (HM175p16) or related L1-A, L2-A, or L1,2-A double mutant RNAs. Cells were fixed 48 hrs post-electroporation and stained with J2 (monoclonal anti-dsRNA), JC (polyclonal human anti-HAV), or K34C8 (monoclonal HAV anti-capsid) antibodies. Strong K34C8 fluorescence in cells transfected with each of the mutants is indicative of efficient capsid assembly (20). Nuclear counterstaining was with DAPI (blue). Scale bars = 10 μ m. **(B)** Percentage of cells stained with the K34C8 anti-capsid monoclonal antibody following transfection with each of the mutants. Data shown are mean \pm s.d. from 4 independent experiments. **(C)** Intensity of K34C8 fluorescence (CFTC, corrected total fluorescence intensity per cell) in cells transfected with wt or the indicated mutant viral RNA. All comparisons between cells transfected with mutant versus wt control RNAs were nonsignificant statistically ($p > 0.29$ by one-way ANOVA). **(D)** Assay for infectious virus produced in cells transfected with wt (18f) or L1,2-A RNA. Results shown represent percent cells staining positively with JC antibody 48 hrs post RNA electroporation, or 48 hrs after inoculation with lysates of electroporated cells. Data are from 7-10 low power microscopy fields of cells in each condition, and are representative of two independent experiments. **(E)** Confocal microscopic images of Huh-7.5 cells inoculated with cell-free

lysates prepared from cells 48 hrs after electroporation with wt or L1,2-A RNA, or no RNA (mock). Cells were stained with polyclonal JC antibody to HAV 48 hrs after inoculation.

Figure 6. Viral egress is reduced by Ala substitutions at the carboxy-terminal Leu of the VP2 YPX₃L late domain motifs. **(A)** Extracellular virus quantified by RT-qPCR following electroporation of wildtype (HM175/p16) or the related L1-A, L2-A, L1,2-A, or replication incompetent $\Delta 3D^{pol}$ mutants. See legend to Fig. 2A for details. **(B)** Viral RNA present in fractions of an isopycnic iodixanol gradient showing the buoyant density of virus present in supernatant fluids of cultures 9 days following electroporation of the single L1-A or L2-A motif mutants. Virus present in the peak fractions (fraction 12, 1.086 g/cm³) was sequenced and the presence of each mutation confirmed. **(C)** Intracellular viral RNA abundance 9 days post-transfection of the indicated wt or mutant virus. Abundance was normalized to that present in cells transfected with the replication-incompetent $\Delta 3D^{pol}$ RNA. **(D)** Orientation of the side chains of Leu¹⁴⁹ (L149) and Leu¹⁸² (L182) (magenta) within the HAV capsid, showing lack of a direct interaction between these residues.

Figure 7. Isopycnic iodixanol gradient profiles showing density distributions of intracellular wt and mutant viral RNAs. **(A)** Gradients loaded with lysates from cells electroporated 72 hrs previously with (top) wt (HM175/18f) or (bottom) the related L1,2-A mutant. Also shown in the bottom panel is the density distribution of subgenomic replicon 18f-FLuc RNA. A unique peak of viral RNA is present in the L1,2-A gradient (1.093 g/cm³). **(B)** Gradients loaded with lysates from cells electroporated 9 days previously with (top) wt (HM175/p16) or the related (middle) L1-A and (bottom) L2-A mutants. Unique, lesser density RNA peaks are present in gradients containing lysates of cells transfected with either mutant.

Figure 8. Immunoprecipitation of encapsidated viral RNA present in lysates of cells prepared 48 hr following electroporation with wt (HM175/18f), L1-A, or L1,2-A mutant HAV RNAs. Input HAV RNA present in each lysate is shown on the left, quantified by RT-qPCR, followed (left to right) by the percent of each RNA precipitated by anti-ALIX antibody and nonspecific immunoglobulin (IgG). Data shown represent mean values from 3 independent experiments.