The unique role of domain 2A of the hepatitis A virus precursor polypeptide P1-2A in viral morphogenesis

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The initial step during assembly of the hepatitis A virus particle is driven by domain 2A of P1-2A, which is the precursor of the structural proteins. The proteolytic removal of 2A from particulate VP1-2A by an as yet unknown host enzyme presumably terminates viral morphogenesis. Using a genetic approach, we show that a basic amino acid residue at the C-terminus of VP1 is required for efficient particle assembly and that host proteases trypsin and cathepsin L remove 2A from hepatitis A virus particles in vitro. Analyses of insertion mutants in the C-terminus of 2A reveal that this part of 2A is important for liberation of P1-2A from the polypeptide. The data provide the first evidence that the VP1/2A junction is involved in both viral particle assembly and maturation and, therefore, seems to coordinate the first and last steps in viral morphogenesis. [BMB reports 2008; 41(9): 678-683]

INTRODUCTION

Picornaviruses are small RNA viruses with an icosahedral capsid composed of 12 pentamers that comprise five copies each of the structural proteins VP1, VP2, VP3, and VP4. All viral structural and functional proteins are derived from a large polypeptide by proteolytic cleavage. The top panel of Fig. 1 depicts the location of mature viral proteins within the polyprotein of the hepatitis A virus (HAV). HAV is unique among other family members with respect to its high physical stability and nonlytic replication mode. Furthermore, unlike most picornaviruses that employ at least two virus-encoded proteases, HAV uses only one proteinase (3Cpro) for the regulated liberation of mature viral proteins from the polyprotein. Initially, 3Cpro cleaves the 2A/2B junction in the polyprotein, thereby releasing the capsid protein precursor P1-2A. In a regulated processing cascade, intermediate and mature products are subsequently released by 3Cpro cleavage. While protein 2A of poliovirus (the prototypic picornavirus) is a proteinase, the respective domain in the HAV polyprotein represents the C-terminal extension of the structural protein VP1-2A that lacks any proteolytic activity. Domain 2A functions as the pri-
ary signal for viral particle assembly, being involved in P1-2A oligomerization to pentamers and subsequent particle maturation (1, 2). As part of VP1-2A, domain 2A is exposed to the surface of immature or empty HAV particles, while mature particles containing viral RNA are almost devoid of 2A. Empty and mature particles sediment with differing velocity of 70S and 160S, respectively. HAV 3C<sup>C</sup> does not liberate 2A from its precursor VP1-2A, suggesting that 2A is probably removed by an yet unknown host protease(s) during capsid maturation (3-5). The host protease factor Xa (FXa) is able to specifically remove domain 2A from the surface of empty HAV particles in vitro; it has been suggested that the VP1/2A cleavage site localizes to the Arg/Ile dipeptide at amino acid position 278/279 of VP1-2A (5; Fig. 1A). Additional experimental data showed that HAV assembly not only depends on the N-terminal part of domain 2A, but that also its C-terminus influences particle formation. In fact, deletions within the 2A C-terminal region alter the cleavability of the VP1/2A site, impairing proper assembly and capsid maturation (2). In spite of these unusual features, the existing evidence still does not provide clear indications for the various parts of domain 2A in HAV particle formation. In addition, understanding the structure and the molecular mechanisms of viral assembly provides valuable information pertinent for future anti-viral strategies (6, 7).

To better understand the role of host proteases in HAV morphogenesis, recombinant and native HAV particles were exposed in vitro to proteases that specifically cleave at the carboxy peptide bond of basic amino acid residues. We report that FXa, trypsin, and cathepsin L produce a polypeptide that comigrates with mature VP1, implying that these host proteases might be involved in HAV particle maturation. In addition, analysis of cleavage site mutants showed that substitution of the arginine residue at position 278 of VP1-2A does not influence primary cleavage at the 2A/2B site, but significantly affects subsequent P1-2A assembly and processing. Furthermore, we tested various insertion mutants to assess the role of the 2A C-terminal domain in particle assembly. Observation that insertions in the C-terminal part of 2A had no effect on cleavability at the 2A/2B and VP1/2A sites is evidence of the conformational flexibility of this part of 2A. Overall, the data indicate that the VP1/2A junction is involved in the primary and final steps of viral morphogenesis, while conformational alterations in the 2A C-terminus modulate polypeptide processing and subsequent HAV particle formation.

RESULTS AND DISCUSSION

VP1-2A cleavage by host proteinases

FXa is able to remove domain 2A from particulate VP1-2A in an assembly-dependent manner (5). The product of FXa cleavage at the Arg/Ile dipeptide site (residues 278/279 of VP1-2A; see Fig. 1) is indistinguishable from VP1 of mature HAV particles, implying that host protease-mediated removal of domain 2A is associated with viral maturation. To test whether an intracellular protease might be involved in HAV particle maturation, cathepsin L was compared with trypsin and FXa for its ability to liberate 2A from particulate VP1-2A. Cathepsin L was chosen as it cleaves at the carboxy peptide bond of basic amino acid residues, similar to FXa. Extracts of HAV-infected cells and extracts of trans-infected cells were treated with FXa, trypsin, and cathepsin L (Fig. 1C). Empty recombinant particles contained exclusively VP1-2A (Fig. 1C, lane 1), whereas RNA-containing virions produced in infected cells were comprised mostly of VP1 with small amounts of VP1-2A (lane 7). After incubation with FXa and cathepsin L in vitro, VP1-2A of both empty recombinant (lanes 2 and 3, respectively) and infectious particles (lanes 6 and 8, respectively) was converted to mature VP1. Trypsin treatment of empty (not shown) and full particles also produced conversion of VP1-2A to VP1 (lane 7). HAV 3C<sup>C</sup> was not involved in 2A removal from particulate VP1-2A (lane 5), consistent with previous observations (3-5). Under the chosen proteolytic conditions, none of the other structural proteins (VP0/VP2 and VP3) were degraded and the particles’ integrity or their antigenicity were not affected when treated by rate-zonal centrifugation and enzyme-linked immunosorbent assay (ELISA) (data not shown). Combined with earlier results, these data clearly demonstrate that the VP1/2A cleavage site is accessible to various host proteases independent of the presence of viral RNA in the particle. Moreover, the specific 2A removal catalysed by cathepsin L indicates that the lysosomal or similar proteases might be involved in the final step of HAV morphogenesis.

In HAV-infected cells, mature particles contained mostly VP1 and only low levels of VP1-2A (Fig. 1C, lane 7), indicating that the unknown host protease is active on immature particles during viral replication in vivo. Surprisingly, conversion of VP1-2A to VP1 did not occur in vivo on the empty recombinant particles (Fig. 1C, lane 1) that were produced by transient expression in the same cells in the absence of viral genome replication. As determined by the 7E7 ELISA and by immunoblot, these empty particles were indistinguishable from immature particles that represented morphogenetic intermediates in HAV-infected cells. As the recombinant empty particles were resistant to cleavage in vivo by the host proteases, yet could be cleaved in vitro, it must be assumed that they differ from native immature procapsids in their intracellular location and/or surface conformation. Further structural studies are required to distinguish between these possibilities.

Our finding that the lysosomal protease cathepsin L was able to specifically cleave VP1-2A in vitro was striking and might shed new light on the intracellular site of HAV particle maturation and/or export. Cathepsins are active at acid pH, a condition on which HAV is stable (8). During the course of HAV particle assembly and RNA packaging, immature procapsids might be targeted to early lysosomes where they encounter cellular proteases including cathepsin L that remove 2A and thereby fully mature the particles. The lysosome or related autophagosome might also be involved in viral cell egress. For

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poliovirus, it was recently hypothesized that autophagosome-like membranes might provide a mechanism for the non-lytic release of cytoplasmic viruses (9). In vivo experiments will be required to verify the proposed role of the cathepsins or other acid proteinases and the general role of the lysosome in HAV maturation and release from infected cells.

Cleavage site mutants

The present and previous immunological data and biochemical evidence imply that the C-terminus of VP1 and the N-terminus of domain 2A contain oligomerization and procapsid maturation signals (2, 5). In particular, the arginine residue at position 278 of VP1-2A seems to be involved in both functions, as substitution of arginine 278 by methionine interferes with oligomerization of P1-2A and maturation of procapsids, probably by hindering the correct structure of the assembly and maturation signals. To further characterize these overlapping functions, arginine 278 was substituted by either lysine or glutamic acid. To determine the effect of these mutations on HAV particle formation, parental and mutant cDNAs were expressed with the help of vaccinia virus. Extracts of trans-infected cells were tested using ELISA for particle formation (Fig. 2, lanes 1-5) and by immunoblotting for viral structural proteins. The parental (WT) cDNA and all cleavage site mutants produced P1-2A (Fig. 2, lanes 2-9) indicating that the 2A/2B primary cleavage was unaffected by the amino acid substitutions. Using anti-VP1, substantial amounts of VP1-2A were detectable in WT and R278K mutant extracts, but less in mutants R278M and R278E (Fig. 2, lanes 2-5). In the immunoblot with anti-2A, P1-2A and VP1-2A were detectable in all extracts (Fig. 2, lanes 6-9). While WT and R278K extracts were positive in the ELISA (Fig. 2, lanes 2 and 3), extracts of mutants R278M and R278E were negative (Fig. 2, lanes 4 and 5), implying that P1-2A with these mutations are unable to assemble and are inefficiently processed by 3Cpro. In fact, VP0 was only detected in the WT extract (Fig. 2, lane 11), but was virtually undetectable in the extracts of cells expressing the mutants (Fig. 2, lanes 12-14). As VP1-2A but not VP0 was liberated from P1-2A of the mutants, it can be concluded that the VP3/VP1 cleavage sites (for their location in the viral polyprotein, see Fig. 1) are distinctly affected by the mutations and are thereby partially inaccessible to 3Cpro, an observation similarly made for poliovirus (10). Lack of assembly of mutants R278M and R278E suggests that a basic residue at position 278 has a “sine qua non” assembly signal function of this domain.

In addition, the cDNAs were transfected into HuH-T7 cells that constitutively express T7 RNA polymerase with the aim to rescue infectious virus. No infectious virus could be rescued from any mutant (data not shown). This was expected for mutants R278M and R278E, which cannot initiate particle formation by the assembly of P1-2A. The inability of mutant R278K to replicate and complete the infectious cycle, in spite of its capacity to form particles, might imply that replication necessitates efficient particle formation and/or efficient maturation cleavage by a host protease. Probst et al. (1) and Rachow et al. (5) suggested that the assembly signal for pentamerization maps to the junction domain spanning the VP1 C-terminus and the 2A N-terminus, and thereby overlaps with the cleavage site targeted by the host protease. As the parental and mutant R278K only differ in the molecular mass of the basic residue at position 278, it can be concluded that the assembly signal function is highly restricted in its conformation. Due to low amounts of assembled particles of R278K, their susceptibility to host proteases was not tested.

Expression of 2A insertion mutants: cleavage of P1-2A and particle formation

Genetic studies suggested that the C-terminal part of 2A is dispensable for viral replication (2, 11). In line with these findings, we have presented evidence that a short foreign sequence (seven amino acids) of HIV gp41 (2F5) can be inserted into domain 2A (12). No apparent interference with virus capsid assembly, infectivity, or immunogenicity has been observed (13). Similar results were reported for papaya ringspot virus (14). Extending our earlier work, we analyzed the influence of longer inserts on polyprotein processing and the physical stability of recombinant HAV particles, by replacing the amino acid residues 307 to 339 of VP1-2A with 27 residues representing antigenic epitopes of HIV or HCV, or with 18 residues corresponding to N-terminal sequences of the HAV polymerase gene (Fig. 1B). To assess particle formation of the mutants, they were expressed with the help of vaccinia virus vTF7-3 and compared with the parental (WT) genome.
insertion of the extended gp41 epitope significantly effected In contrast to pHAV-F78, pHAV-gp41 and pT7-18f-2Ains, by the 2Ains, HCV, or HIV insertion.

dicates the presumed P1-2AB. #Particle formation as indicated be-

probed with: anti-VP1 (lanes 1-6), anti-gp41 (lanes 7-10), or anti-F78 fractionated by 12% SDS-PAGE, transferred onto nitrocellulose, and pHAV-gp41, pHAV-gp41ext, or the empty vector (mock) and in-

protein carrying various insertions in the C-terminus of 2A. Huh-T7 
mature cleavage products VP0 and VP3. Collectively, these re-

ti-VP3, anti-VP0, and anti-2C (data not shown). These results 
were specifically detected (Fig. 3, lane 12). For all extracts, im-
munoblot analyses were also carried out with anti-2A, an-
ti-VP3, anti-VP0, and anti-2C (data not shown). These results 
confirmed the identity of parental and mutated P1-2A and the 
lymunoreactivity, respectively, and imply that proteolytic proc-

F78 or pHAV-gp41 contain both HAV and F78 or gp41 im-

assembly using the particle-specific ELISA with monoclonal an-
tibody 7E7 (1, 13). Particle formation was clearly demonstrable for pT7-18f, pT7-18f-2Ains, pHAV-gp41 and pHAV-F78 (Fig. 3, lanes 1-6). In addition, presentation of the foreign epit-

ope on the chimeric particles was analyzed with a chimeric 
ELISA detailed in the supplementary data. The results obtained 
with monoclonal antibodies 7E7 and 2F5 for pHAV-gp41, and 
with 7E7 and R209 for pHAV-F78, respectively, strongly sug-

gest that the formation of chimeric viral particles in cells trans-

fected with these constructs (data not shown). Extracts of cells 
expressing pHAV-gp41ext were negative in both the 7E7 and 
2F5 ELISA, indicating that this insertion abrogated particle 
assembly using the particle-specific ELISA with monoclonal an-
tibody 7E7 (1, 13). Particle formation was clearly demonstrable for pT7-18f, pT7-18f-2Ains, pHAV-gp41 and pHAV-F78 (Fig. 3, lanes 1-6). In addition, presentation of the foreign epit-

ponent derived from pHAV- 
gp41ext. Whereas P1-2A of gp41ext was present in similar amounts as the respective parental polypeptide (compare lanes 
and 4 of Fig. 3), the amount of VP1-2A was significantly lower. Furthermore, a prominent polypeptide larger than 
P1-2A (marked §) was detected (Fig. 3, lanes 3 and 4). Based on 
its molecular mass and the lack of reactivity with anti-2C (data not shown), this polypeptide might represent P1-2AB. This finding implies that the gp41ext insertion impedes cleav-

age at the 2A/2B site, possibly by severely altering protein folding.

All extracts from trans-infected cells were tested for particle 
assembly using the particle-specific ELISA with monoclonal antibody 7E7 (1, 13). Particle formation was clearly demonstrable for pT7-18f, pT7-18f-2Ains, pHAV-gp41 and pHAV-F78 (Fig. 3, lanes 1-6). In addition, presentation of the foreign epit-

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gest that the formation of chimeric viral particles in cells trans-

fected with these constructs (data not shown). Extracts of cells 
expressing pHAV-gp41ext were negative in both the 7E7 and 
2F5 ELISA, indicating that this insertion abrogated particle 
formation. To verify the formation of chimeric particles, rate-

centrifugation on a sucrose gradient was performed with extracts of Huh-7 cells trans-infected with pHAV-gp41, 
pHAV-F78 and pT7-18f. In all cases, the major antigenic pro-

ducts detected by the 7E7 ELISA sedimented with approximately 
705 and, therefore, corresponded to empty viral particles (data 
not shown).

Precise $C^{\text{exo}}$ cleavage within P1-2A requires 2A-mediated P1-2A pentamerization, whereas unassembled P1-2A or P1 do not serve as specific 3C substrates (1). During pentamerization, a conformational change seems to occur that is a prerequisite for specific P1-2A cleavage into VP1-2A, VP3, and VP0. Based on 
these preconditions and the partial processing defect at the 
2A/2B site exerted by the gp41ext insertion, the data imply that 
insertions in the 2A C-terminus are tolerated without disruption of the functions of 2A in assembly and maturation, as long as the inserts do not interfere with the overall and functional folding of the polyprotein, and the ability of 2A to mediate P1-2A pentamerization and subsequent P1-2A cleavage.

In conclusion, the data presented here extend our understanding of the unique properties and functions of domain 2A as part of the HAV precursor polypeptide P1-2A. The previously identified assembly function that maps to the VP1/2A junction seems to be highly restricted in its structure and to require an arginine residue at position 278 of VP1-2A. Besides the extracellular proteinases FXa and trypsin, cathepsin L was identified as an enzyme that can convert the major structural protein to its mature form, supporting the hypothesis that the lysosome is involved in HAV maturation and/or egress. In con-

trast to the conformational rigidity of the VP1/2A junction, the

![Fig. 3. P1-2A processing and particle assembly of the HAV polyprotein carrying various insertions in the C-terminus of 2A. Huh-T7 cells were transfected with pT7-18f, pT7-18f-2Ains, pHAV-F78, pHAV-gp41, pHAV-gp41ext, or the empty vector (mock) and in-

fected with vTF7-3. Proteins obtained 48 h post trans-infection were fractionated by 12% SDS-PAGE, transferred onto nitrocellulose, and probed with: anti-VP1 (lanes 1-6), anti-gp41 (lanes 7-10), or anti-F78 (lanes 11 and 12). Viral proteins are marked on the right. § indicates the presumed P1-2AB. #Particle formation as indicated be-

low lanes 1-6 was determined with the 7E7 ELISA.](http://bmbreports.org)
C-terminus of 2A seems to be flexible and to abide insertions of foreign epitopes, unless they do not interfere with cleavage of the 2A/2B site. Overall, our findings shed further light on features of HAV that are unique among the picornaviruses and account for the evolutionary distance between HAV and other picornaviruses.

**MATERIALS AND METHODS**

cDNA constructs
Details of the construction of mutants (Figs. 1A and B) are given in the supplementary data. The mutagenized cDNAs were sequenced by the dideoxy-termination method to verify their identity and the correctness of the reading frame.

cDNA expression and generation of recombinant virus particles
Wild type and mutant cDNAs were transiently expressed in Huh7-T7 cells with the help of the vaccinia virus T7F-7 that provided T7 RNA polymerase (15). Recombinant viral proteins and particles obtained 48 h after trans-infection were analysed as described in the supplementary data and previously (1, 12). For the generation of infectious virus, pT7-18f and mutant cDNAs were transfected into Huh7-T7 cells. Transfected cells were incubated for 20 days and analyzed as previously described (1, 12).

Proteolytic cleavage in vitro
Cells trans-infected with pT7-18f and T7F-7, or infected with HAV strain 18f were extracted with 250 μl PBS-Tween. The crude cell extracts were clarified by centrifugation for 5 min at 13,000 rpm to obtain soluble fractions that contained either empty or mature viral particles. Thirty microliters of the extracts were digested with either HAV 3Cpro (70 ng/ml), FXa (30 U/ml), or trypsin (10 ng/ml) in neutral buffer, or cathepsin L (10 nM) at pH 5 for 16 h at 20°C. Ten microliters of the digests were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblot.

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