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Homogenous Hepatitis A Virus Particles

PROTEOLYTIC RELEASE OF THE ASSEMBLY SIGNAL 2A FROM PROCAPSIDS BY FACTOR Xa*

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Among the *picornaviridae*, hepatitis A virus (HAV) is unique in that its assembly is driven by domain 2A of P1-2A, the precursor of the structural proteins (Probst, C., Jecht, M., and Gauss-Müller, V. (1999) *J. Biol. Chem.* 274, 4527–4531). Whereas infected individuals excrete in stool mature HAV capsids with VP1 as the major structural protein, its C-terminal extended form VP1-2A is the main component of immature procapsids produced in HAV-infected cells in culture. Obviously, a postassembly proteolytic step is required to remove the primary assembly signal 2A from VP1-2A of procapsids. Mutants of VP1-2A were expressed in COS7 cells to determine the cleavage site in VP1-2A and to test for the cleavage potential of viral and host proteinases (factor Xa and thrombin). Site-specific *in vitro* cleavage by factor Xa and thrombin occurred in procapsids that contained VP1-2A with engineered cognate cleavage sites for these proteinases. Interestingly, factor Xa but not thrombin liberated mature VP1 also from native procapsids in an assembly-dependent manner. The data show that domain 2A, which is required for pentamerization of its precursor polypeptides and thus for the primary step of HAV assembly, is removed from the surface of immature procapsid by a host proteinase. Moreover, our data open a novel avenue to produce homogenous HAV particles from recombinant intermediates by *in vitro* treatment with exogenously added proteases such as factor Xa or thrombin.

The mature capsid of picornaviruses has an icosahedral symmetry and contains 60 copies of the structural proteins VP1, VP2, VP3, and VP4 that enclose the viral RNA genome. All viral structural and functional proteins are derived from a large polyprotein by proteolytic cleavage. The P1 region of the polyprotein is the precursor of the structural proteins, and the regions P2 and P3 contain polypeptides involved in the replication of the viral genome. Similar to other RNA viruses, picornaviral gene expression is mainly governed by proteolytic processing of the polyprotein. Whereas most picornaviruses use at least two proteinases for primary and secondary cleavages, HAV[1] encodes only proteinase 3C, which is part of P3. Uniquely, HAV 3C catalyzes both the liberation of the precursor of the structural proteins P1-2A in a primary cleavage step as well as that of most mature viral proteins in secondary cleavages (1–5). Following proteolytic release of P1-2A from the polyprotein, viral particle formation is initiated by the oligomerization of this polypeptide to pentamers, which sediment at 14 S after 3C cleavage (6, 7). Subsequent assembly of 12 pentamers gives rise to procapsids, an icosahedral structure that sediments at 70 S. The formation of pentamers and procapsids is dependent on cleavage of P1-2A to produce the structural proteins VP0, VP3, and VP1-2A. For all picornaviruses, it is generally agreed that mature capsids sedimenting at 160 S are formed by the encapsidation of newly synthesized RNA and cleavage of VP0 to VP2 and VP4. Cleavage of HAV VP0 during particle maturation is greatly protracted when compared with other picornaviruses, and it possibly retards the spreading of this virus in cell culture (8, 9). Absolutely unique to HAV procapsids is the presence of VP1-2A (38 kDa), the C-terminal extended form of the structural protein VP1 that was demonstrated in viral particles produced in infected cells (6–9). In contrast, the virus fecally excreted by HAV-infected individuals contains VP1 (33 kDa) as the largest structural protein.

For HAV, polyprotein cleavage and the initial assembly steps have been assessed in detail by genetic studies using recombinant expression systems (1–5, 10–16). Clear evidence was provided that instead of P1, polypeptide P1-2A is the functional precursor of the HAV structural proteins, and only the viral proteinase 3C as part of the P3 domain is required for particle formation. It was shown that 2A as the C-terminal domain of P1-2A functions as the assembly signal for pentamer formation, whereas VP4 as its N-terminal domain is crucial for the assembly of 12 pentamers into the icosahedral capsid structure (1). During the assembly of the HAV precursor polypeptide and their subsequent proteolytic cleavage by the viral proteinase 3C, a conformational change occurs that is accompanied by the formation of the neutralizing epitope and that can be determined immunologically with a monoclonal antibody (1, 6). Whereas 2A is a crucial prerequisite for the first step of HAV particle assembly, its absence in fecally excreted particles suggests that its release may affect capsid maturation and/or export from the infected cells. In fact, nothing is known about the route by which HAV particles are released from cells during its non-lytic and persistent replication.

Conflicting evidence has been put forward on the type of proteinase(s) that may catalyze the release of 2A from its precursors VP1-2A or P1-2A. From recombinant studies with mutated 3C and P1-2A substrates, it was concluded that HAV 3C does not liberate 2A (14, 15). In a similar experimental approach, yet with a high level expression system, we have

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[1] The abbreviations used are: HAV, hepatitis A virus; FXa, factor Xa; Th, thrombin; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; PBS-Tw, PBS containing Tween 20; ELISA, enzyme-linked immunosorbent assay.

shown that coexpression of P1-2A and VP1-2A with 3C-containing polypeptides yielded both VP1-2A and VP1 (1, 10). Furthermore, electrophoretic analyses of various truncated forms of recombinant VP1 suggested that the C-terminal amino acid of VP1 might be the glutamine residue at position 273 of VP1-2A (10, 16). This circumstantial evidence was questioned when C-terminal amino acid sequence analyses of VP1 from purified HAV particles showed heterogeneous termini with threonine, glutamine, and serine at positions 272–274, respectively (14). As these residues are not part of the generally accepted consensus sequence of HAV 3C substrates, the authors concluded that a host proteinase mediates the liberation of the VP1 C terminus.

To resolve these discrepancies and to directly test host proteinases for their ability to release 2A from HAV procapsids, particle formation and cleavage was studied for variants of HAV P1-2A carrying mutations at the proposed C terminus of VP1. In addition, P1-2A mutants carrying the consensus cleavage sequence of either factor Xa (FXa) or thrombin near the C terminus of VP1 were tested for their ability to assemble into subviral particles. These mutants were generated with the aim to assess the accessibility of the VP1-2A cleavage site and to enable proteolytic removal of 2A by direct and 3C-independent cleavage. Since FXa and thrombin are highly specific proteinases that are produced as inactive proenzymes in the liver, they were chosen as molecular tools for proteolytic cleavage. We show that FXa and thrombin cleaved particulate VP1-2A that carried cognate cleavage sites near the C terminus of VP1. Cleavage mediated by these host proteinases produced polypeptides that migrated with the apparent molecular mass expected. Surprisingly, FXa but not thrombin released 2A also from the surface of native procapsids in an assembly-dependent manner. Direct evidence for 2A-dependent pentamerization of VP1-2A is presented, confirming the notion that 2A is the first assembly signal of HAV particle formation that can be removed from the surface of the particle by FXa. The data suggest that host proteinase(s) such as FXa are involved in the conversion of viral procapsids to capsids.

EXPERIMENTAL PROCEDURES

cDNA Constructs—pET-HM-P1-2A(E-S), pET-HM-P1-2A(V-S), and pET-HM-P3 were prepared by inserting the appropriate HAV fragment derived from pEXT7-HM/HM-P1-2A(E-S), pEXT7-HM/HM-P1-2A(V-S), and pT7-HAV1, respectively (10), into pET11a (Novagen). By PCR with primers listed below, mutated fragments of 2.5 kb were created, restricted with *Nhe*I and BclI, and inserted into pET11a opened with *Nhe*I and *Bam*HI. To construct plasmids encoding the mutated sequences shown in Fig. 1, pET-HM-P1-2A(E-S) was used as template for site-directed mutagenesis with the following primers: for pET-HM-P1-2A(V-T), 5′-GCT ATG TTG TCC ACT GTT ACT ATG ATG AGT AGA ATT GC-3′ (sense V-T); for pET-HM-P1-(E-S)-(278), 5′-CC ATG ATG AGT AGA TAA GCT GCT GGA GAC TTG G-3′ (sense (278)); for pET-HM-P1-2A(E-S)-R278M, 5′-CC ATG ATG AGT ATG ATT GCT GCT GGA GAC TTG G-3′ (sense -R278M); for pET-HM-P1-2A(E/FXa/S), 5′-GCT ATG TTG TCC ACT GAG ATT GAA GGA AGA TCC ATG ATG AGT AGA ATT GCA GC-3′ (sense E/FXa/S); for pET-HM-P1-2A(FXa/S), 5′-CCA TTA AAT TCA AAT GCT ATG ATT GAA GGA AGA TCC ATG ATG AGT AGA ATT GCA GC-3′ (sense FXa/S); for pET-HM-P1-2A(E/Th/S), 5′-GCT ATG TTG TCC ACT GAG TTG GTT CCT AGA GGA TCC ATG ATG AGT AGA ATT GCA GC-3′ (sense E/Th/S); for pET-HM-P1-2A(Th/S), 5′-CCA TTA AAT TCA AAT GCT TTG GTT CCT AGA GGA TCC ATG ATG AGT AGA ATT GCA GC-3′ (sense Th/S). Antisense primers were complementary to the listed sense primers. Underlined nucleotides differ from the template sequence. Site-directed mutagenesis was carried out according to the instructions of the manufacturer (Stratagene) or by a modified protocol described elsewhere (17). The nucleotide sequences of the mutated regions were verified by DNA sequencing. The numbers of amino acid residues 273 and 274 refer to the position of Glu and Ser in VP1-2A and correspond to positions 764 and 765 in P1-2A (10, 11, 15). To construct infectious full-length genomes carrying the insertion mutations (E/Th/S and E/FXa/S), appropriate restriction fragments were inserted into the HAV full-length cDNA pT7-18f (13).

Expression and Immunological Analysis of Viral Proteins and Particles—3×10^5 COS7 cells grown overnight to ~70% confluency in wells of 35-mm diameter were transfected with a total of 1 μg of cDNA and 9 μl of LipofectAMINE™ according to the instructions of the manufacturer (Invitrogen). Three h after transfection, infection with the recombinant vaccinia virus vTF7-3 followed for 30 min at 37 °C (1, 18). After replacement of the medium by 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, incubation was continued for 18 h. Transfected cells were scraped off the plate in 250 μl of phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBS-Tw), resulting in the total cell extract. To separate soluble 70 S procapsids from insoluble overexpressed proteins, we centrifuged the crude cell extract for 5 min at 13,000 rpm. 10 μl of the crude and the soluble extracts were mixed with 3.3 μl of NuPAGE (Invitrogen) sample buffer and incubated at 70 °C for 10 min followed by ultrasonication. After separation on a 10% NuPAGE gel using MOPS-SDS running buffer, proteins were blotted onto nitrocellulose. The antisera used for the immunoblots were described elsewhere (anti-VP1, anti-VP0 (19), anti-2A (10), anti-3C (20)). Subviral particles were detected and quantified by a particle-specific ELISA using the neutralizing monoclonal antibody K2-4F2 as capture and detection antibody (1). Each transfection was independently analyzed three times. For the production of complete viral particles, Huh7 cells were infected with HAV strain 18f and incubated for 20 days. Due to its protracted replication, long incubation periods are required for sufficient antigen accumulation. The virus was harvested as described above for the transfected cells.

Proteinase Digest—Transfected cells of 6 wells were extracted with 250 μl of PBS-Tw. The crude cell extract was clarified by centrifugation, and 30 μl of the soluble extract were digested with either 1 unit of factor Xa (Novagen) or ½₅ unit of thrombin (Novagen) for 16 h at 20 °C. 10 μl of the digests were separated by SDS-PAGE and analyzed by immunoblot.

Characterization of Subviral Particles—For rate zonal centrifugation, 1×10^7 COS7 cells grown on 175 cm^2 were transfected with a total of 17.5 μg of cDNA and 157 μl of LipofectAMINE™ (Invitrogen) in 17.5 ml of OptiMEM. Infection with vTF7-3 was performed as described above. Cells were lysed by passing the extracts in 700 μl of PBS-Tw through a 26-gauge needle. 500 μl of the soluble extract were incubated with the proteinase or its buffer. Ultracentrifugation was performed in a SW41 rotor (Beckmann Instruments) at 35,000 rpm, 4 °C for 3 h using a linear 5–30% (w/w) sucrose gradient in 100 mM NaCl, 10 mM Tris-HCl, pH 7.3. The gradient was fractionated from the bottom of the tube, and the sucrose concentration of each fraction was measured with a refractometer. 50 μl of each fraction (1 ml) were diluted 1:1 with PBS-Tw and analyzed by ELISA. For immunoblot analysis of the proteins of the particles, 500 μl of each fraction were concentrated to 30 μl with Microcon YM-30 (g_{max} 14,000) according to the instructions of the manufacturer (Millipore) and adjusted to a final volume of 30 μl in 1× NuPAGE sample buffer. Samples were incubated for 10 min at 70 °C and separated on a 10% NuPAGE gel using MOPS-SDS running buffer. The subsequent immune reaction with anti-VP0, anti-VP1, and anti-2A was carried out as described above.

RESULTS

3C Cleavage of Assembled and Non-assembled HAV P1-2A—Due to the slow and asynchronous replication in cell culture, details of HAV protein expression and particle formation cannot be assessed in infected cells, yet both can be efficiently analyzed in a mammalian recombinant system using vaccinia virus vTF7-3. As described previously, virions, empty viral procapsids, and the viral proteinase 3C were found in the soluble extract of infected or transfected cells, and other functional proteins and uncleaved intermediate processing products were mostly insoluble (1, 10, 21, 22). Here, we made use of these earlier observations on the solubility of correctly assembled viral proteins. By comparing the protein pattern in the soluble and insoluble cell fractions, assembled particles were distinguished from uncleaved and presumably incorrectly folded precursor polypeptides and their processing products.

Conflicting evidence has been presented as to what proteinase is responsible for cleavage within the precursor polypeptide VP1-2A that is found in HAV procapsids (10, 14–16, 20). To distinguish between VP1/2A cleavage catalyzed by a putative

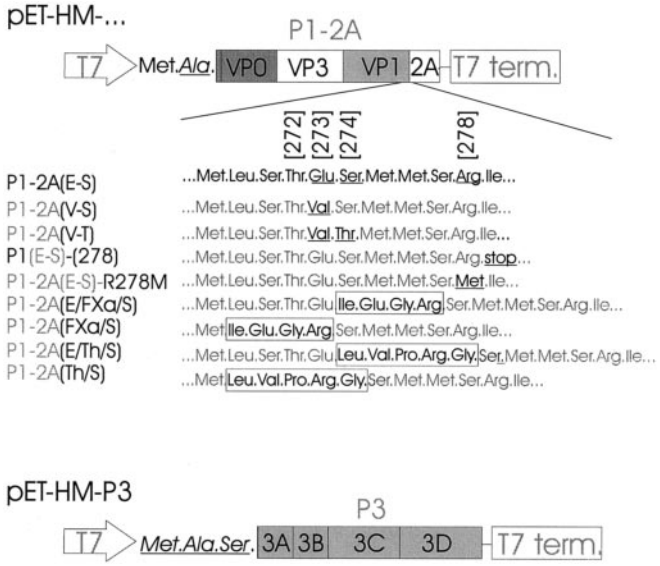

FIG. 1. **Schematic representation of wild type and mutated HAV cDNAs and their amino acid sequences.** The T7 promoter precedes the HAV coding sequence, and the T7 terminator is located downstream. Termination of translation is ensured by a stop codon. At the proposed site for HAV 3C cleavage in VP1-2A (10), the ES sequences were replaced by the dipeptide sequences VS and VT in mutants P1-2A(V-S) and P1-2A(V-T), respectively. The VS sequence is found in the attenuated HAV strain adapted to cell culture, whereas ES is found in the wild type strain isolated from stool (see Ref. 10). In P1-2A(E-S)-(278), the amino acid sequence is terminated at position 278 of VP1-2A; in P1-2A(E-S)-R278M, the arginine residue at position 278 is replaced by a methionine. The consensus sequences of FXa (IEGR) or thrombin (LVPRG) were inserted at the proposed VP1/2A cleavage site, either replacing the sequences upstream of the cleavage site or in addition to the HAV sequences. The P1-2A constructs with the various VP1/2A cleavage sites were prepared by site-directed mutagenesis. pET-HM-P3 was coexpressed to provide the viral proteinase 3C. *Boxed residues* mark the insertions or replacements, and *underlined residues* vary among the upper five constructs.

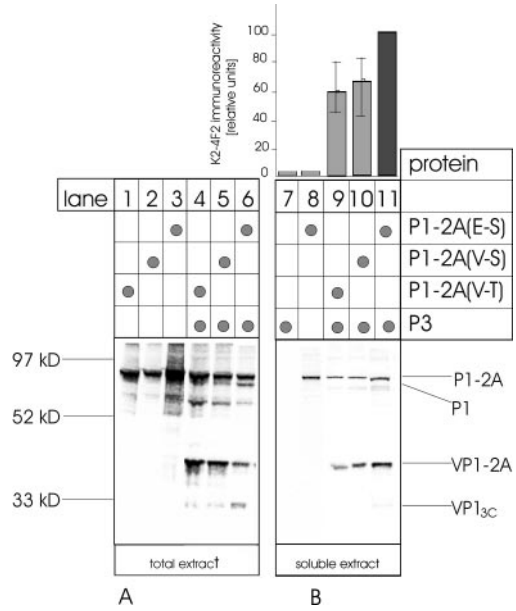

FIG. 2. **Protein pattern and HAV antigen produced after expression of constructs with various amino acid sequences at VP1-2A position 273/274.** As indicated schematically *above* the *lanes*, P1-2A proteins were either expressed alone (*lanes 1–3* and *8*) or expressed in the presence of HAV P3 encoding the viral proteinase 3C^{pro} (*lanes 4–11*). In *lanes 1–6* of *panel A*, total extracts are shown, whereas the soluble extracts containing subviral particles are depicted in *lanes 7–11* of *panel B*. Proteins were identified by immunoblot with anti-VP1, as indicated on the *right*. Molecular mass standards are shown on the *left*. In the *upper panel* of *lanes 7–11*, viral particle formation as determined by ELISA with the neutralizing monoclonal antibody K2–4F2 is shown. For the ELISA, each lysate was analyzed three times. The amount of antigenicity detected for the wild type structural proteins (*lane 11*) was always higher than for the mutant proteins, possible due to a change in the presentation of the antigenic epitope. As negative control for the ELISA, the extract of the precursor protein P1-2A expressed alone was used (*lane 8*). In *lane 7*, the extract of P3-expressing cells demonstrates the specificity of the immunoblot.

host or the viral proteinase, a genetic strategy was chosen, and several mutants of the proposed site (Glu-273–Ser-274 in VP1-2A) were analyzed (10). P1-2A(V-S) carries a valine residue at the amino acid position 273 of VP1, whereas in P1-2A(V-T), the dipeptide sequence V-T replaces E-S at the proposed cleavage site (Fig. 1). The three P1-2A variants were transiently expressed in the presence and absence of P3 that provided the active proteinase domain 3C. As reported earlier (1), coexpression of P1-2A with P3 or 3C is a prerequisite for particle formation as shown by rate zonal centrifugation (see below). In addition, assembly of 70 S procapsids and mature virions is accompanied by the formation of the HAV neutralization epitope that is recognized by the monoclonal antibody K2-4F2. We used an ELISA with this antibody to assay for procapsids and their assembly precursors (Fig. 2*B*, *columns* above the *lanes*). When either P1-2A or P3 were expressed alone, no ELISA signal was detectable, proving the specificity of the assay (Fig. 2*B*, *lanes 7* and *8*). HAV particle formation was detected in the soluble fraction of cells coexpressing P3 with all P1-2A variants (Fig. 2*B*, *columns* above *lanes 9–11*), with P1-2A(E-S) yielding somewhat higher levels of antigenicity than the other precursor polypeptides.

To assess proteolytic processing in detail, the P1-2A and P3 processing products were visualized by immunoblots. Expression and processing of P3 proteins was efficient (not shown), confirming our results obtained before (13, 21). Both the soluble fractions and the total cell extracts were analyzed to distinguish assembled proteins from unassembled and precursor polypeptides. In the immunoblot of the total extracts of all variants coexpressed with P3, the uncleaved precursors P1-2A and VP1-2A were the predominant polypeptides besides products of various molecular mass (Fig. 2*A*, *lanes 4–6*). Both P1 and VP1$_{3C}$ were also detected as products of all mutants tested, with VP1$_{3C}$ being more efficiently liberated from P1-2A(E-S) (*lane 6*) than from the other variants (*lanes 4* and *5*). This finding confirms our earlier observation that 3C is able to liberate VP1 from P1-2A (10). As VP1$_{3C}$ found in the total extract was liberated from all variants of the precursor polypeptide, 3C processing of VP1-2A does not appear to be very sensitive to mutations at the cleavage site proposed by us earlier (10). This suggests that either 3C does not cleave at the proposed site or that 3C cleavage within VP1-2A is less restricted in its substrate specificity. 3C cleavage at other sites in P1-2A (VP0/VP3 and VP3/VP1) was unaffected by these mutations as equal amounts of mature VP0 were liberated (not shown; see Fig. 1 for the order of viral proteins in the precursor P1-2A). When the P1-2A variants were expressed in the absence of P3 (Fig. 2*A*, *lanes 1–3*), neither P1 nor other specific cleavage products were found in the total cell extracts, confirming our earlier observation that the viral proteinase 3C and/or its precursors are necessary to produce both VP1-2A and VP1$_{3C}$ (1). No proteins were detected by anti-VP1 in P3-expressing cells, proving the specificity of the antiserum (*lane 7*). To distinguish VP1 produced by 3C from that produced by exogenously added host proteinases (see below), this protein is now called VP1$_{3C}$. From these data, it is also clear that intracellular host proteinases do not directly cleave recombinant P1-2A.

To test whether VP1$_{3C}$ produced under these expression conditions was part of the HAV procapsids, the soluble frac-

tions of the extracts were analyzed (Fig. 2*B*, *lanes 9–11*). Only tiny amounts of P1 and $VP1_{3C}$ were detectable among the soluble products of P1-2A(E-S)+P3-expressing cells (*lane 11*). In contrast to the respective total extracts, no VP1 was found among the products of P1-2A(V-T) and P1-2A(V-S) (*lanes 9* and *10*). The presence of $VP1_{3C}$ in the total, but not in the soluble extract, suggests that $VP1_{3C}$ is derived from unassembled precursor proteins and does not present a component of assembled procapsids (see Fig. 5) (15). It is conceivable that the lack of $VP1_{3C}$ in assembled particles is due to inaccessibility of the potential cleavage site for 3C after assembly.

P1-2A Cleavage by Proteinases Factor Xa (FXa) and Thrombin—To test the accessibility of the VP1-2A cleavage site to 3C or to exogenous host proteinases, further P1-2A mutants were expressed in the presence of P3. To this end, P1-2A variants were constructed that contained cleavage consensus sequences for proteinase FXa or thrombin. The consensus sequences were introduced near the site that had been proposed as C terminus of mature VP1 (14). In P1-2A(FXa/S), the VP1 C-terminal amino acid residues LSTE were substituted by the FXa consensus sequence IEGR; in P1-2A(Th/S), they were replaced by the thrombin consensus sequence LVPRG (Fig. 1). In polyproteins P1-2A (E/FXa/S) and P1-2A(E/Th/S), the appropriate 4 or 5 amino acid residues, respectively, were inserted between Glu-273 and Ser-274 of VP1-2A. The insertion and substitution mutants of P1-2A were expressed in the absence or presence of P3, and the soluble cell extracts were analyzed by ELISA and immunoblot. As determined by the ELISA, HAV antigenicity was found in all soluble extracts being somewhat lower in those of cells coexpressing the mutated forms of P1-2A with P3 as compared with native P1-2A(E-S) (not shown). It is conceivable that formation of the neutralizing epitope on the surface of the procapsid is modulated by mutations near the proposed C terminus of VP1.

Mutations (E/FXa/S) and (E/Th/S) were inserted into the background of an infectious HAV cDNA to test for their effect on viral infectivity. Transcripts of both cDNAs were fully infectious, and the insertions were found to be stable for at least three viral passages (data not shown). This finding suggests that small insertions at the C terminus of VP1 not only did not interfere with viral assembly but also did not abrogate other steps in the viral life cycle, such as viral entry and egress.

Soluble processing products that were generated by coexpression of the mutated precursor polypeptides with P3 were identified by immunoblot (Fig. 3). 3C cleavage at the VP3/VP1 site was unaffected in all mutants as the anti-VP1 blot revealed almost equal amounts of the structural protein VP1-2A (Fig. 3*A*, *lanes 1–5*). As equal amounts of VP0 were detected (not shown), the cleavability at the VP0/VP3 site apparently was also unaffected by the mutations. Due to the additional amino acid residues, VP1-2A of the insertion mutants P1-2A(E/FXa/S) (*lane 3*) and P1-2A(E/Th/S) (*lane 4*) migrated more slowly than VP1-2A, containing amino acid substitutions (P1-2A(FXa/S) and P1-2A(Th/S) (in *lanes 2* and *5*, respectively). Taken together, the data clearly show that the mutations neither affected proteolytic cleavage of the precursor proteins nor had a detrimental effect on the structure and function of the assembly signal as the mutant precursor polypeptides assembled into procapsids (see sucrose gradient in Fig. 5).

To test whether the inserted cleavage sites in assembled procapsids were accessible to FXa or thrombin, the extracts shown in Fig. 3*A* were treated with the proteinases *in vitro* and analyzed by immunoblot with anti-2A, anti-VP0 (not shown), and anti-VP1 (Fig. 3*B*). In all instances, except P1-2A(Th/S), the mutated VP1-2A polypeptides contained in the HAV procapsids were cleaved by the respective proteinases yielding

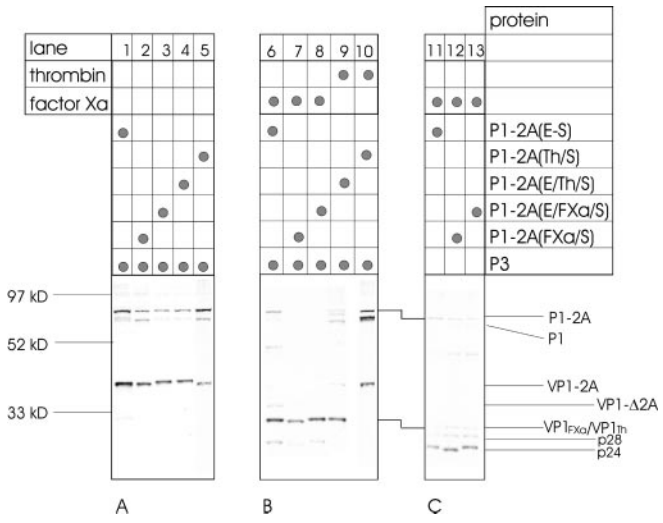

FIG. 3. **Proteolytic processing by factor Xa and thrombin of P1-2A variants containing surrogate cleavage sites.** P1-2A cDNA constructs indicated *above* the *lanes* were expressed either in the presence (*A* and *B*, *lanes 1–10*) or in the absence of the viral proteinase encoded by P3 (*C*, *lanes 11–13*). Viral proteins in the soluble extracts were identified by immunoblot with anti-VP1. After coexpression of the various P1-2A constructs with P3, the extracts were digested with FXa and thrombin (*B*, *lanes 6–10*). As control, extracts containing P1-2A expressed alone and digested with FXa are also shown (*lanes 11–13*). Proteolytic processing products are marked on the *right side*, and molecular mass standards are shown on the *left*.

$VP1_{FXa}$ and $VP1_{Th}$ (*lanes 7–9*). VP1-2A of mutant P1-2A(Th/S) was not cleavable by thrombin, indicating that the engineered cleavage site was not accessible to this proteinase (*lane 10*). Due to their insertions, VP1 derived from P1-2A(E/FXa/S) and P1-2A(E/Th/S) migrated more slowly than VP1 of P1-2A(FXa/S) (*lanes 7–9*). The extracts treated either with FXa or thrombin contained an amount of antigen similar to that present before treatment (not shown), implying that the removal of 2A from the mutated procapsids neither affected the neutralization epitope nor the structure of the particle.

To further assess the substrate specificity of the exogenous proteinases, the extract of cells expressing native P1-2A(E-S)+P3 was also digested with FXa and thrombin. Surprisingly, FXa liberated $VP1_{FXa}$ (Fig. 3*B*, *lane 6*), whereas thrombin was unable to cleave (not shown). This polypeptide, as well as VP1 liberated by FXa from P1-2A(E/FXa/S) (Fig. 3*B*, *lane 8*) that contains an insertion of 4 amino acid residues, comigrated with VP1 of mature HAV particles (Fig. 4, *lane 10*). Since FXa cleaves at the C terminus of basic amino acid residues, this suggests that the Arg-Ile sequence at position 278/279 of VP1-2A(E-S) might be the FXa cleavage site for the release of 2A (Fig. 1 and below). For particles carrying the engineered sites, the comigration data suggest that the host proteinases cleaved at the inserted sites rather than at the native site (Arg-Ile) located 5 residues downstream. If FXa cleavage in VP1-2A(FXa/S) occurred at the native site (Arg-Ile at position 278/279), its product, which comprised the same number of amino acid residues, would comigrate with the cleavage product derived from P1-2A(E-S). This is not the case (*lane 7*), but rather the VP1 product of P1-2A(E/FXa/S) (*lane 8*) that comprised 4 additional amino acid residues comigrated with wild type VP1 (*lane 6*). We therefore assume that in the recombinant particles the engineered, but not the native site, is cleaved by FXa.

To test for the conformation specificity of FXa cleavage, the FXa cleavage pattern was assessed for non-assembled proteins produced in the absence of P3. Soluble extracts containing uncleaved P1-2A were exposed to FXa. As shown in Fig. 3*C*, very little $VP1_{FXa}$ was generated under these conditions, and

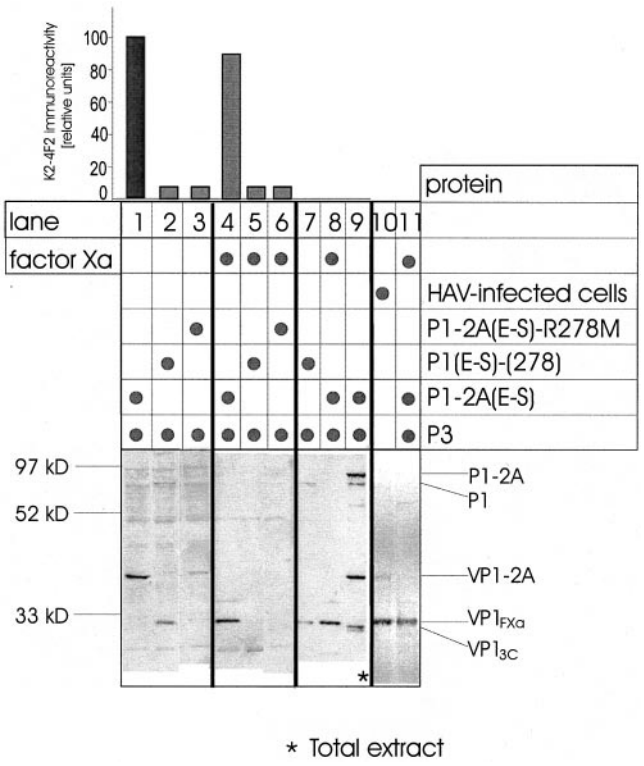

FIG. 4. **Assessment of the FXa cleavage site in VP1-2A.** As indicated *above* the *lanes*, cDNAs (P1-2A(E-S), P1-2A(E-S)-(278), P1-2A(E-S)-R278M, and P3) were coexpressed, and the soluble extracts were incubated with FXa as indicated. Proteins were identified by immunoblot with anti-VP1. Polypeptides of the total cell extract (*lane 9*) and the soluble extract of HAV-infected cells (*lane 10*) are also shown. *Above lanes 1–6*, viral particle formation as determined by ELISA with the neutralizing monoclonal antibody K2–4F2 is depicted.

anti-VP1-reactive polypeptides (p28 and p24) of 28 and 24 kDa, respectively, were liberated from uncleaved P1-2A precursors (*lanes 11–13*). Since p24 was also found among the FXa cleavage products of P1-2A(E-S), P1-2A(FXa/S), and P1-2A(E/FXa/S) coexpressed with P3 (Fig. 3*B*, *lanes 6–8*), we conclude that FXa is able to cleave within assembled and unassembled VP1, yet with different specificities. Taken together, the data clearly show that some amino acid residues can be inserted between VP1 and 2A without affecting protein processing and assembly. Furthermore, the inserted cleavage sites were accessible to exogenously applied proteases FXa or thrombin. FXa was also able to cleave VP1-2A in assembled wild type procapsids, indicating that the native cleavage site is exposed to the surface of the procapsids.

Assessment of the Potential FXa Cleavage Site in VP1-2A—Our observation that VP1 was produced by FXa treatment of particulate VP1-2A(E-S) (Fig. 3) was striking and further extended by comparing the FXa cleavage products derived from various mutated polypeptides. To test the Arg-Ile sequence at position 278/279 in VP1-2A as a target site of FXa cleavage, arginine 278 was mutated to methionine. As a control and comigration standard, P1(E-S)-(278) that terminates with the arginine residues at position 278 (Fig. 1) was coexpressed with P3. The capacity of P1-2A-R278M and P1(E-S)-(278) to assemble into procapsids after coexpression with P3 was first assessed by ELISA (Fig. 4, *columns* above the *lanes*). Due to the absence of 2A in P1(E-S)-(278), no particle formation was observed (Fig. 4, *lane 2*), again confirming our earlier observations that 2A as the C-terminal domain of P1-2A is required for assembly (1). Surprisingly, no particles were found in extracts of P1-2A-R278M+P3-expressing cells (*column* above *lane 3*), suggesting that the R278M mutation interfered with the correct structure of the assembly signal.

The 3C cleavage products of P1-2A(E-S), P1-2A-R278M, and P1(E-S)-(278) were analyzed in soluble extracts by immunoblot (Fig. 4, *lanes 1–3*). As no procapsids assembled from either the 2A-truncated precursor P1(E-S)-(278) or from P1-2A-R278M, only small amounts of soluble proteins were detected that were not part of assembled particles. Either VP1 or VP1-2A was found in the extracts of P1(E-S)-(278)+P3 (*lane 2*)- or P1-2A-R278M+P3-expressing cells (*lane 3*), respectively. The presence of these polypeptides (albeit in small amounts) indicates that both the R278M mutation and the deletion of 2A were not detrimental to proteolytic processing by 3C at the VP0/VP3 and the VP3/VP1 sites, but to particle assembly. To test whether the conformational changes accompanying particle formation might affect FXa cleavability, the soluble extracts were digested with FXa. Whereas VP1-2A(E-S) as a component of 70 S procapsids was specifically cleaved into $VP1_{FXa}$ (*lane 4*), VP1(E-S)-(278) (*lane 5*) and VP1-2A-R278M (*lane 6*) that are not particulate were degraded by FXa such that either no (*lane 6*) or small amounts (*lane 5*) of an immunoreactive polypeptide of 24 kDa were produced, respectively. The data indicate that only particulate VP1-2A(E-S), but not VP1-2A-R278M, adopted a conformation that allowed specific FXa cleavage. These findings also indicate that assessment of the conformation-dependent FXa cleavage site in wild type HAV is hampered by the fact that the expected cleavage site seems to lie in or near the domain of 2A that mediates assembly.

To distinguish between VP1 produced by 3C and that produced by FXa, the electrophoretic mobility of VP1 produced either in the absence or presence of FXa was compared. In addition, the protein pattern found in the complete and soluble extract of P1-2A(E-S)+P3-expressing cells was analyzed (Fig. 4, *lanes 8* and *9*). The soluble extracts of HAV-infected cells (*lane 10*) and cells expressing P1(E-S)-(278)+P3 (*lane 7*) were taken as comigration standards. $VP1_{FXa}$ liberated by FXa (*lane 8*) comigrated with VP1(E-S)-(278) (*lane 7*) and mature VP1 of HAV particles from infected cells (*lane 10*). Interestingly, $VP1_{3C}$ liberated by viral proteinase 3C and detectable in the total cell extract migrated slightly faster (*lane 9*). The data suggest that FXa cleaves within VP1-2A(E-S) at a position C-terminal to the proposed 3C cleavage site, most likely at site 278/279. Combined with data depicted in Fig. 3, these results show that the specificity of FXa-mediated VP1/2A cleavage depends on the conformation of the substrate, and they suggest that the Arg-Ile sequence represents a dominant and conformation-dependent FXa cleavage site. Since P1-2A-R278M+P3-expressing cells did not form particles, we conclude that residue 278 is a critical determinant for folding and assembly.

Characterization of Recombinant HAV Procapsids—To directly show that particles were formed after expression of wild type and engineered precursors in the recombinant system and that they remained intact after treatment *in vitro* with FXa or thrombin, the soluble extracts were analyzed by sucrose gradient centrifugation. In Fig. 5, the antigenicity and immunoblot analysis of gradient fractions are depicted for procapsids derived from coexpressions of P3 with either P1-2A(E/FXa/S) (Fig. 5*a*) or P1-2A(E-S) (Fig. 5*b*). Regardless of treatment with FXa, the majority of particles sedimented at 70 S, although some particles sedimented faster in the case of P1-2A(E/FXa/S)+P3 treated with FXa. The overall antigenicity was also unaffected, suggesting that FXa treatment did not destabilize HAV subviral particles or act on the conformation of the neutralizing epitope. The immunoblot of the gradient fractions shows that VP1-2A in monomeric and oligomeric forms (see below) were the prominent polypeptides in the untreated par-

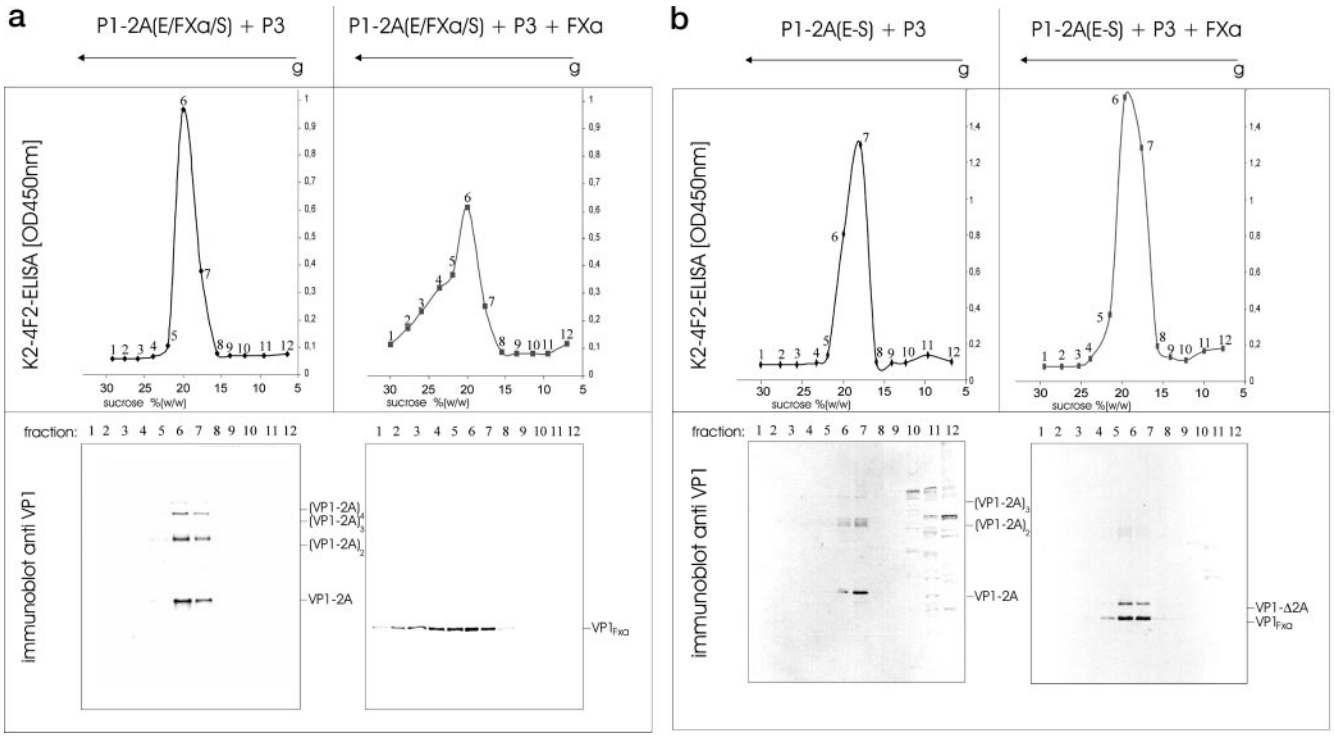

FIG. 5. **Rate zonal centrifugation and immunoblot analyses of subviral particles produced by recombinant coexpression of P1-2A(E/FXa/S) (*a*) and P1-2A(E-S) (*b*) together with P3.** After cDNA expression, the soluble extracts were incubated with or without FXa overnight at room temperature before they were subjected to rate zonal centrifugation. Gradient fractions were analyzed by ELISA and immunoblot to determine the formation of subviral particles and their protein pattern, respectively. The sucrose concentration of the fractions (5–30% w/w) is indicated. In *fractions 6* and *7*, $VP1_{FXa}$ as well as VP1-2A containing procapsids sedimented at 70 S.

ticles. After FXa treatment, the procapsids derived from P1-2A(E/FXa/S) contained exclusively $VP1_{FXa}$ (Fig. 5*a*, *right panel*), indicating the efficient cleavage of VP1-2A(E/FXa/S) exposed on the surface of the procapsid. Under the conditions of this experiment, FXa cleavage of VP1-2A(E-S) was incomplete as $VP1_{FXa}$ in addition to some VP1-Δ2A were produced (Fig. 5*b*, *lower right panel*). Based on its molecular mass and immunoreactivity, VP1-Δ2A contains the N-terminal portion of VP1 and a segment of 2A. Previously, low amounts of a polypeptide of similar size have been detected in HAV-infected cells (12, 15). The extracts of P1-2A(E-S)+P3-expressing cells contained small amounts of uncleaved P1-2A and other large precursors (Fig. 5*b*, *lower left panel*, *fractions 10–12*) that were virtually absent in the FXa-treated extract (*right panel*). It is likely that these proteins are constituents of 13 S pentamers that are composed of mostly uncleaved precursors and therefore not reactive with the neutralizing antibody used in the ELISA (6). Taken together, the data on the FXa cleavage pattern of mutated and native particles combined with the sedimentation analyses unequivocally demonstrate that the VP1/2A cleavage region is exposed on the surface of HAV procapsids and accessible to a host proteinase. Moreover, FXa-mediated removal of 2A from procapsids derived from P1-2A(E-S) neither significantly destabilized the particle nor altered its sedimentation property. By exogenous treatment with host proteinases, homogenous engineered particles can be produced that comprise VP1, the mature form of the major structural protein.

VP1-2A Multimerizes through Its 2A Domain—In this and previous studies, genetic evidence was presented that the 2A domain of P1-2A is the primary signal for HAV particle assembly, which might map to the 5-fold axis of the HAV procapsid (1, 11). Immunoblot analysis of the HAV procapsid (70 S) proteins (Fig. 5, *a* and *b*) had shown additional polypeptides with VP1 immunoreactivity and lower electrophoretic mobility. To test whether these polypeptides represented oligomers of VP1-2A and whether oligomerization depended on the presence of 2A, 70 S particles were analyzed by immunoblot with anti-VP1, anti-2A, and anti-VP0, after protein separation by SDS-PAGE. As shown in Fig. 6, multimeric forms of VP1-2A were found in HAV particles produced by transient expression of P1-2A (E/FXa/S) with P3. Not only the putative VP1-2A pentamer, but also VP1-2A oligomeric intermediates (dimer, trimer, and tetramer), were distinguishable after electrophoretic separation. These oligomers carried specific VP1 and 2A (*lanes 1* and *3*) but no VP0 antigenicity (*lane 5*). The oligomers were not found in other gradient fractions or control extracts (not shown). After FXa treatment (see the *scheme* above the *lanes*), VP1-2A oligomers were no longer detectable (*lanes 2* and *4*), indicating that removal of 2A abrogates oligomerization. The oligomeric forms of VP1-2A were also identified in particles derived from the expression of P1-2A(E-S)+P3 (Fig. 5*b*). The resistance of the 2A-containing oligomers to the denaturing and reducing gel conditions is striking and points to strong non-covalent interactions. In fact, SDS-stable interactions have recently been described for some hydrophobic proteins (24, 25). These results provide strong evidence in support of our model that 2A mediates pentamerization of P1-2A as the primary step of particle assembly (1). The 2A domain of VP1-2A might function as a clamp on the 5-fold axes of the HAV procapids and stabilize this intermediate particle during its protracted process of maturation.

DISCUSSION

Particle assembly and maturation are the final steps in the viral life cycle that are generally regulated by distinct morphogenic factors and may occur at different sites within or outside the infected cell. During maturation of most picornaviral capsids, non-infectious particles are rendered infectious following cleavage of VP0 on the inside of the viral particle by an as yet unexplained mechanism. Maturation cleavage is accompanied

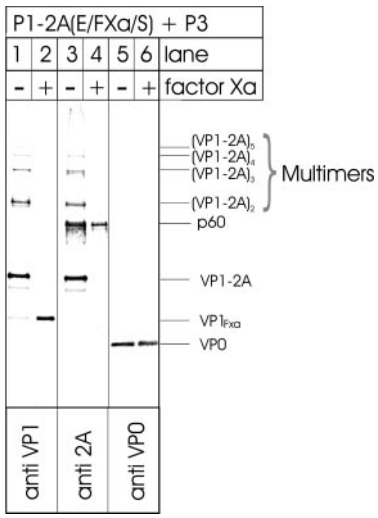

FIG. 6. **Oligomerization of VP1-2A found in 70 S procapsids.** 70 S viral procapsids produced by coexpression of P1-2A(E/FXa/S) with P3 and separated by rate zonal centrifugation were treated with FXa as indicated *above* the *lanes* and analyzed by immunoblot with anti-VP1, anti-VP0, and anti-2A. The immunologically identified proteins are marked on the *right*. The anti-2A-reactive polypeptide p60 of 60 kDa that is resistant to FXa digestion was unspecifically stained with this antiserum.

by a conformational change of the particle (6, 26, 27). Since immature HAV particles differ from mature virions not only in their VP0 but also in their VP1-2A content (6–9, 14), HAV maturation apparently involves the removal of 2A from the procapsids as an additional processing step. As recently proposed, domain 2A of P1-2A, the precursor of the structural proteins, functions as the primary assembly signal that is removed from procapsids in a later step of the infectious cycle (1, 11). We now show that FXa is able to specifically remove the assembly signal 2A from VP1-2A in procapsids, thereby generating VP1-containing particles. Based on their comigration, VP1 liberated by FXa from recombinant HAV procapsids is identical to VP1 of infectious virions and to an engineered control protein that has arginine 278 as its C-terminal amino acid residue. Our data raise the possibility that 2A removal by FXa (or a host proteinase with similar specificity) is a step in maturation of infectious HAV particles. Viral proteinase 3C can also remove 2A, most likely from its non-assembled precursor P1-2A, yet the liberated VP1 polypeptide (termed $VP1_{3C}$ here) is shorter than that found in infectious virions.

The biochemical evidence provided here and the immunological data presented elsewhere (23) imply that domain 2A and the region spanning the VP1/2A cleavage site are exposed at the surface of HAV procapsids and accessible to host proteinases. Here, we show that mutant precursor polypeptides carrying surrogate consensus cleavage sites of up to 5 amino acid residues were able to assemble into procapsids, indicating that the region N-terminal to the proposed FXa-mediated cleavage site is not only surface-exposed but also spatially flexible. The ability of these mutants to assemble into procapsids was sharply contrasted by mutant P1-2A-R278M that carries a single amino acid change at the proposed FXa cleavage site. As assembly was completely abolished in this mutant, it is tempting to speculate that residue 278 is both involved in oligomerization of P1-2A and involved in maturation of procapsids. In line with these observations, the crucial role of the N-terminal half of 2A for assembly has been shown in a recent study (11). Overall, the functional involvement of the region spanning the C terminus of VP1 and the N-terminal half of 2A both in assembly and as a substrate for host and viral proteinases is intriguing and unique to HAV. Further genetic analyses will be necessary to map this domain in more detail and to define the amino acid residues that mediate intermolecular interactions.

Our finding that FXa was able to specifically remove 2A from the surface of recombinant HAV procapsids was appealing in the light of the liver-specific replication of HAV *in vivo*. PreproFX is produced in the liver and converted to the active proteinase FXa by extracellular cleavage mediated by proteinase factor IX or VIIa. Substantial amounts of FXa, its precursor, and its activators are present in serum and ensure its function in blood clotting. For paramyxoviruses and orthomyxoviruses, it was shown that FXa modified viral envelope proteins in such a way that they were able to fuse with the host cell membrane, allowing their penetration into the cell (28–30). Our finding that FXa removes 2A from native HAV procapsids fosters the hypothesis that this host proteinase might be involved in liver-specific HAV maturation and argues for an extracellular site of viral maturation that makes HAV different from other picornaviruses. Dependent on its adaptation to cell culture, HAV replicates in cells of hepatic and non-hepatic origin. Little is known about the expression of FXa or proteinases with similar specificity in non-hepatic cells. At present, we cannot rule out the possibility that *in vivo*, a host proteinase other than FXa might remove 2A from HAV procapsids, and thus, studies with other proteinases are in progress. Our observation that the primary assembly signal (2A) can be removed by a host proteinase during a late step in the HAV life cycle might indicate that HAV capsid egress and maturation need to be regulated independently both in space and time. In this context, it has been speculated that the 2A domain might be required for the efficient viral egress from the cell, a process that is not understood for HAV (31).

After attempts to map mature HAV VP1 in the viral polyprotein had failed, the C-terminal amino acid sequence was directly determined for the polypeptide contained in purified viral particles (14). Amino acids Thr-272, Glu-273, and Ser-274, located 6–4 amino acid residues N-terminal to the proposed site of FXa cleavage, were determined as C-terminal residues. As these residues neither match the 3C nor the FXa consensus sequences, it seems unlikely that these proteinases alone generated the VP1 C terminus formed under the cell culture conditions used. Since the VP1/2A cleavage site seems to be exposed to the surface of the procapsid, it is reasonable to speculate that the VP1 C terminus liberated by FXa or another host proteinase is subsequently targeted by carboxypeptidase(s). Interestingly, heterogeneous C-terminal amino acid residues were found for VP1 of mengovirus, a member of the cardiovirus genus in the picornavirus family (32). In line with its proteolytic trimming, the crystal structure of this virus had shown that the C terminus of mengovirus VP1 is located on the surface of the particle (33).

To our knowledge, the insertion of cleavage consensus sequences with the aim to render the picornavirus polypeptide susceptible to selective proteolysis is shown here for the first time. This strategy might be useful for structural analysis of HAV particles as well as for producing viral vectors suited for gene therapy and prophylactics. Viral particles produced in infected cell cultures are usually heterogeneous as they contain variable amounts of VP2 due to the protracted maturation cleavage. In addition, they might contain both VP1-2A and VP1 with variable C termini (14). It is possible that this heterogeneity has hampered HAV crystallization and structure determination. P1-2A mutants with engineered cleavage sequences for host proteinases can be used to produce homogeneous, noninfectious HAV procapids with either VP1-2A or VP1, dependent on the *in vitro* cleavage by FXa or thrombin. Both kinds of

particles are vaccine candidates that might have different immunogenic and biophysical properties. In addition, these particles might be better suited for crystallization and three-dimensional structure determination than particles purified from infected cell cultures. Finally, the ability of the insertion mutants to assemble implies that the C terminus of VP1 is structurally flexible and might yet be another target for the insertion of antigenic epitopes (23).

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Homogenous Hepatitis A Virus Particles: PROTEOLYTIC RELEASE OF THE ASSEMBLY SIGNAL 2A FROM PROCAPSIDS BY FACTOR Xa

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