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Identification of Active-Site Residues in Protease 3C of Hepatitis A Virus by Site-Directed Mutagenesis

RAINER GOSERT,† GÜNTER DOLLENMAIER, AND MANFRED WEITZ*
Institute for Clinical Microbiology and Immunology, CH-9001 St. Gallen, Switzerland

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Picornavirus 3C proteases ($3C^{pro}$) are cysteine proteases related by amino acid sequence to trypsin-like serine proteases. Comparisons of $3C^{pro}$ of hepatitis A virus (HAV) to those of other picornaviruses have resulted in prediction of active-site residues: histidine at position 44 (H44), aspartic acid (D98), and cysteine (C172). To test whether these residues are key members of a putative catalytic triad, oligonucleotide-directed mutagenesis was targeted to $3C^{pro}$ in the context of natural polypeptide precursor P3. Autocatalytic processing of the polyprotein containing wild-type or variant $3C^{pro}$ was tested by in vivo expression of vaccinia virus-HAV chimeras in an animal cell-T7 hybrid system and by in vitro translation of corresponding RNAs. Comparison with proteins present in HAV-infected cells showed that both expression systems mimicked authentic polyprotein processing. Individual substitutions of H44 by tyrosine and of C172 by glycine or serine resulted in complete loss of the virus-specific proteolytic cascade. In contrast, a P3 polyprotein in which D98 was substituted by asparagine underwent only slightly delayed processing, while an additional substitution of valine (V47) by glycine within putative protein 3A caused a more pronounced loss of processing. Therefore, apparently H44 and C172 are active-site constituents whereas D98 is not. The results, furthermore, suggest that substitution of amino acid residues distant from polyprotein cleavage sites may reduce proteolytic activity, presumably by altering substrate conformation.

Hepatitis A virus (HAV) has been classified as the prototype virus of the genus *Hepatovirus* within the family *Picornaviridae* (36) and is the cause of acute hepatitis in humans and other primates (12). The virus is unique among the picornaviruses due to several distinct features, including its extreme physical stability, protracted replication in cell culture, and inability to shut off host cell macromolecular synthesis (30, 44, 46–49). Furthermore, its nucleotide and amino acid sequences are only distantly related to those of other picornaviruses (39, 50). The RNA genome of HAV is about 7,500 nucleotides long, is single stranded, and serves as a messenger for translation (9). A small virus-encoded protein, VPg, is covalently linked to the 5′ end of the RNA (51). The 3′ polyadenylated RNA contains a large 5′ nontranslated region (5′NTR) of 735 nucleotides that is involved in internal initiation of viral protein synthesis and a much shorter 3′NTR (6, 45, 50, 53). A single giant open reading frame encodes a 253-kDa polyprotein, which may be divided into structural region P1 (capsid proteins VP1 to VP4) and regions P2 and P3, which include at least seven nonstructural proteins, 2A to 2C and 3A to 3D, respectively (for a review, see references 23 and 52). The polyprotein is rapidly processed mainly by the virus 3C protease ($3C^{pro}$) to yield mature proteins (15, 18, 22, 25, 34). The activities of $3C^{pro}$ comprise autocatalytic cleavage in *cis* as well as cleavage in *trans*. For HAV, all polyprotein cleavages are accomplished by $3C^{pro}$, with the exception of the maturation cleavage (VP0 to VP4 and VP2), which in picornaviruses occurs following encapsidation of the viral RNA, and possibly also the VP1-2A cleavage, which remains poorly defined (3, 21).

Early amino acid sequence alignments of virus proteins indicated that a cysteine residue (e.g., C147 in poliovirus) is highly conserved among $3C^{pro}$s of picornaviruses and 3C-like proteases of some plant viruses (2, 13). More sophisticated comparisons based on structural predictions supported an evolutionary link between $3C^{pro}$, cellular serine, and cysteine proteases (5, 16, 17). Such analyses and investigations with specific inhibitors led to the concept that $3C^{pro}$s form a family of trypsin-like cysteine proteases that is unrelated by structure and function to papain-like cysteine proteases (16, 17, 38). The predicted functional importance of the respective cysteine residues in the picornavirus proteases was confirmed by site-directed mutagenesis for poliovirus, human rhinovirus, and foot-and-mouth disease virus (7, 19, 20, 24, 27). Furthermore, the close relationship of $3C^{pro}$ to serine proteases was experimentally substantiated by investigations with a mutated enzyme. Proteolytic activities were each modulated but not abolished when cysteine-147 of the poliovirus $3C^{pro}$ and the corresponding residues of coxsackievirus B3 and foot-and-mouth disease virus $3C^{pro}$s were conservatively replaced with serine (19, 29, 37). Hence, the active-site geometry of picornavirus $3C^{pro}$ may be similar to that of serine proteases. Predictions and experimental evidence called for H40 of poliovirus $3C^{pro}$ to serve as the general base in the process of catalysis (20, 24). The location of the third active-site residue in the putative catalytic triad is apparently not D85 as once predicted but, rather, glutamic acid-71 (5, 17, 20, 26).

Comparisons of the HAV $3C^{pro}$ with those of other picornaviruses suggested that the catalytic triad may be composed of residues H44, D98, and C172. Crystallographic studies indicated that the structure of HAV $3C^{pro}$ resembles that of trypsin-like serine proteases but have been interpreted as suggesting that the catalytic site consists of only a dyad, C172 and H44, rather than a triad, as was found to be the case with human rhinovirus 14 (35). To determine the active-site residues of the HAV protease, individual single amino acid substitutions were introduced into $3C^{pro}$ at several sites. Vaccinia virus-HAV

* Corresponding author. Mailing address: Institute for Clinical Microbiology and Immunology, Frohbergstrasse 3, CH-9001 St. Gallen, Switzerland. Phone: 41-71-494-3700. Fax: 41-71-494-3705. E-mail: manfred.weitz@gd-ikmi.ktsg.ch.
† Present address: Division of Infectious Diseases, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

TABLE 1. Oligonucleotides used for mutagenesis of $3C^{pro}$

Oligonucleotide sequence (5′-3′)[c]	Location on HAV cDNA[a]	Amino acid substitution[b]
GCTTGTGCCTTCCTATGCTTATAAAT	5403–5428	His-44Tyr
TTCCTAAGTTTAGAAATATTACTCAGC	5564–5490	Asp-98Asn
CTTCCTGGAATGGGTGGTGGGGCCTT	5788–5813	Cys-172Gly
CTTCCTGGAATGAGTGGTGGGGCCTT	5788–5813	Cys-172Ser

[a] Nucleotide numbers according to those of HAV strain HM-175/7 MK5 (8).
[b] His-44Tyr, a change from histidine to tyrosine at position 44.
[c] Codons differing from wild-type sequence are underlined.

chimeras expressing wild-type and variant $3C^{pro}$s in the context of polyprotein P3 and in vitro transcription-translation (IVTT) of the corresponding HAV transcripts were used to investigate the effects of the substitutions on autoprocessing. Furthermore, analysis for products of polyprotein processing were extended to HAV-infected cells in culture. In summary, these functional analyses suggested that H44 and C172 are essential for polyprotein processing, whereas D98 is not.

MATERIALS AND METHODS

Plasmids. Restriction enzymes, ligase, DNA polymerase I (Klenow fragment), as well as alkaline phosphatase (calf intestinal) used in molecular cloning were purchased either from Boehringer Mannheim or New England Biolabs (Beverly, Mass.). Polynucleotide kinase T4 was obtained from Gibco/BRL (Gaithersburg, Md.). Oligonucleotides used for site-directed mutagenesis were synthesized by the phosphoramidite method on an Applied Biosystems DNA synthesizer 392 (Foster City, Calif.). DNA manipulations and phosphorylation of oligonucleotides were carried out by standard procedures (4, 43).

Plasmid pP3 contained sequences encoding the 19 carboxy-terminal residues of the 2C gene fused naturally to the P3 region of HAV, including the 3′NTR and poly(A) under transcriptional control of the T7 promoter. A *Bsp*HI/*Bal*I DNA fragment obtained from plasmid pHAV/7 was asymmetrically ligated into vector pRG-1 that had been linearized with *Sal*I blunt ended, and cleaved with *Nco*I, to yield plasmid p2C′ΔP3 (8, 18). To create pP3, the *Eco*RI/*Bam*HI fragment of p2C′ΔP3 was replaced with the *Eco*RI/*Bam*HI fragment of pHAV/7 containing P3. Individual variant 3C genes were exclusively generated in parent plasmid pP3.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis was performed with the Transformer kit (Clontech, Palo Alto, Calif.) based on the method described by Deng and Nickoloff (10). The oligonucleotides listed in Table 1 were used as primers to introduce single amino acid substitutions into the 3C gene of plasmid pP3. The mutations were confirmed by DNA sequence analysis of the complete HAV expression cassettes with the dideoxynucleotide chain termination method (4).

Viruses and cells. Wild-type strain WR25 of vaccinia virus, originally obtained from the American Type Culture Collection, was propagated in HeLa S3 suspension cell culture and purified according to standard procedures (4). CV-1 cells, BS-C-1 cells, and human thymidine kinase-negative (TK^-) 143 cells were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum (FBS) (42). HeLa S3 cells were grown in HAM F-12 medium containing 10% (vol/vol) FBS. Dulbecco's modified Eagle's medium with 10% (vol/vol) FBS and with 500 mg of geneticin per ml was used to propagate BT7-H cells (53), whereas MRC-5 cells were grown in MB medium with 10% (vol/vol) FBS (47).

Vaccinia virus-HAV chimeras. Vaccinia virus recombinants with P3 genes that contained the complete open reading frame of HAV were produced as has been described for vRGORF (18). Individual virus recombinants were isolated under selective pressure through three consecutive rounds of plaquing on 143 TK^- cells (4, 31, 32). Virus isolates were subsequently characterized by insert-specific PCR, propagated, and purified to high-titered stocks (4, 40).

Expression of HAV proteins. Confluent monolayers of BT7-H cells in T25 culture flasks were infected with individual vaccinia virus isolates each at a multiplicity of infection of 10 PFU per cell. Metabolic radiolabelling was achieved by supplementing originally methionine-free culture medium with 100 μCi of [^{35}S]methionine (>1,000 Ci per mmol; NEN) per ml. At various times postinfection (p.i.), as indicated in the figure legends, harvested cells were lysed, and HAV-specific products were isolated by immunoprecipitation with rabbit anti-3C antibody (14, 18). The antibody had been raised against a peptide that represented the 10 carboxy-terminal amino acid residues of $3C^{pro}$ of HAV (51). Finally, standard procedures of polyacrylamide gel electrophoresis (PAGE) and enhanced radiofluorography served to characterize individual HAV $3C^{pro}$ antigens specifically (28). Specific conditions of propagation of HAV in MRC-5 cells and analysis of radiolabelled antigens have been described earlier (18).

In vitro translation. Synthesis of radiolabelled HAV antigens was achieved by coupled IVTT of individual plasmid DNAs in the TNT system according to the manufacturer's protocol (Promega, Madison, Wis.). Hence, 50-μl reaction mixtures were programmed each with 1 μg of DNA and incubated at 30°C. At various time points, as indicated in the figure legends, aliquots of 7 μl were removed and subjected to analysis by immunoprecipitation with anti-3C antibody as previously described (18).

RESULTS

Recombinant vaccinia virus-HAV chimeric viruses. Various transfer vector plasmid recombinants were used to insert HAV genes into vaccinia virus by homologous recombination (Fig. 1). Plasmid pP3 contained cDNA sequences encoding 19 amino acid carboxy-terminal residues of 2C fused naturally to the P3 region of HAV. To generate $3C^{pro}$ variants, single amino acid substitutions of putative active-site residues were introduced into parent plasmid pP3 by oligonucleotide-directed mutagenesis (Table 1). Analysis by nucleic acid sequencing confirmed the desired changes of histidine-44 to tyrosine (pH44Y), of aspartic acid-98 to asparagine (pD98N), of cysteine-172 to glycine (pC172G), and of cysteine-172 to serine (pC172S). In addition to these plasmids with variant 3C, a double-mutant pV47G/D98N was isolated. The sequence of the P3 open reading frame in this construct differed from the wild-type by an exchange for a glycine of valine-47 of the putative 3A protein in addition to the D98N substitution in $3C^{pro}$. This variant was one among numerous isolates that contained, in addition to the targeted substitution, a second site mutation. Although use of this isolate would not be expected to add to the characterization of the active site of 3C, it served to correlate effects of mutation within 3C with effects of mutation outside of 3C.

To direct effective initiation of translation to an AUG codon at position 4939 of the HAV genome (nucleotide numbering according to the system of Cohen et al. [8]), a synthetic Kozak consensus sequence was inserted upstream of truncated gene 2C in the parent plasmid pP3. Comparison of the sequences with those previously published for wild-type HAV strain HM-175/MK-5 showed a silent mutation to be present in all plasmid recombinants, in region 3D at nucleotide position 6211 (T to C). This mutation was also present in plasmid pP3 and the progenitor plasmid pHAV/7. Hence, they were not inadvertently introduced by the site-directed mutagenesis procedure.

Vaccinia virus recombinants were generated by homologous recombination in cells that had been infected with wild-type vaccinia virus WR25 and that had been transfected individually with the plasmid recombinants described above. The respective vaccinia virus chimeras, vP3, vH44Y, vD98N, vC172G, vC172S, and vV47G/D98N, were each identified by insert-specific PCR analysis on infected-transfected cell lysates (data not shown). Subsequent isolation and purification of individual chimeras resulted in high-titered stocks (10^{10} to 10^{11} PFU/ml) that were used for expression of HAV proteins in BT7-H cells.

HAV wild-type polyprotein processing in the vaccinia virus expression system. HAV-specific proteolytic cleavage of P3 polyprotein should result in production of mature $3C^{pro}$, 3A, 3B, 3D, and precursor peptides as intermediates of processing. Expression of HAV P3-specific polypeptides by vaccinia virus chimeras was monitored by immunoprecipitation with anti-3C antibody. When the complete giant open reading frame of HAV was expressed with vRGORF in BT7-H cells, the 3C-specific peptide antigens were effectively produced (Fig. 2). Polypeptides from the P3 polyprotein region were detected at various times p.i. The products of expression and proteolytic processing were 3ABC (36.5 kDa), $3C^{pro}$ (24.7 kDa), and extremely small amounts of 3BC (27.2 kDa, visible in original film exposure). By pulse-labelling of the peptide, 3ABC was

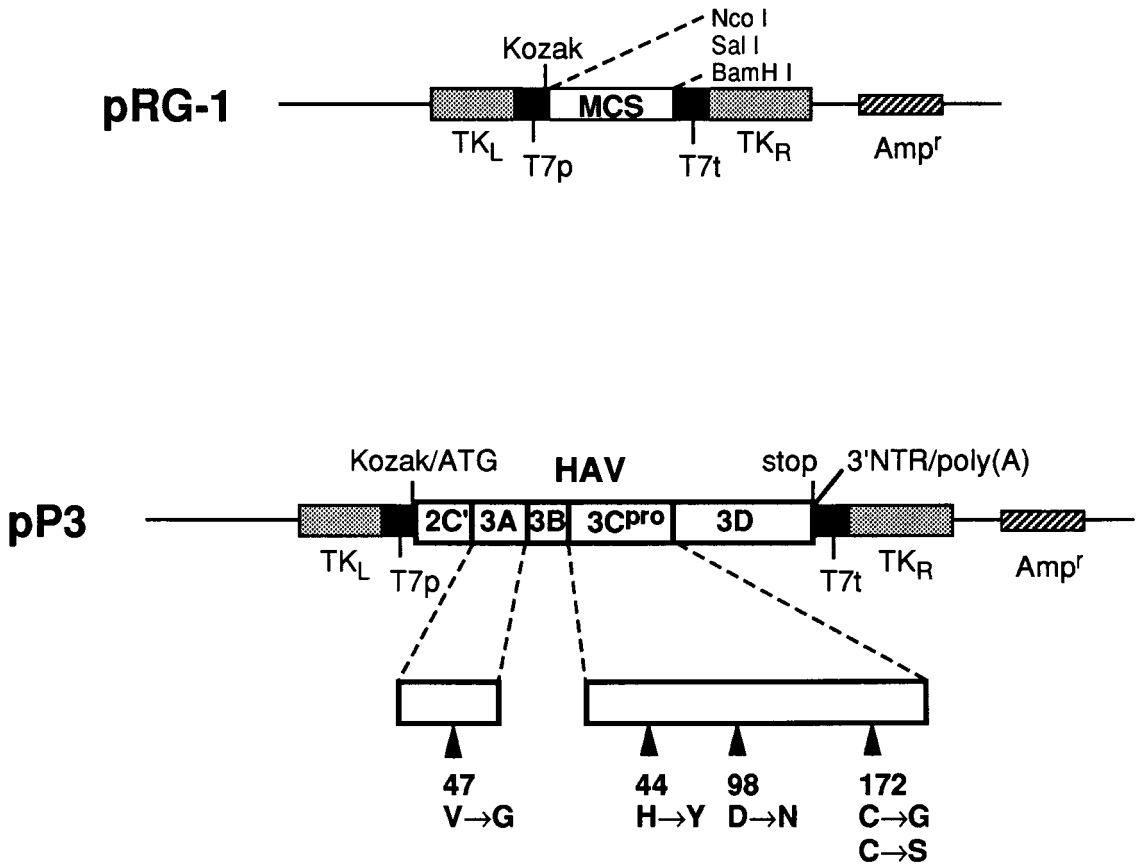

FIG. 1. Plasmid recombinants and location of targeted mutations. Transfer vector pRG-1, into which the HAV region P3 was inserted to yield pP3, is shown. Plasmid pP3 was used as the template for targeted mutations. This parent and derivatives with variant P3 served to generate vaccinia virus chimeras and to program IVTT. TK_L and TK_R, left and right portions, respectively, of vaccinia virus TK gene; T7p and T7t, promoter and terminator, respectively, of bakteriophage T7; Amp^r, ampicillin resistance gene for selective propagation of plasmids; Kozak and Kozak/ATG, artificial consensus sequence to direct effective translation; genes 3A, 3B, $3C^{pro}$, and 3D, constituents of region P3 of HAV and 2C′ residual gene 2C; stop, authentic termination codons of HAV RNA; 3′NTR/poly (A) complete nontranslated end of HAV RNA; MCS, multiple cloning site; open boxes with numbering locations of artificial substitutions within protein 3A and $3C^{pro}$.

unequivocally established as a precursor to 3C. Thus, peptide $3C^{pro}$ was the prominent end product, whereas 3ABC represented the most stable intermediate of processing. In contrast, none of the various putative peptide precursors, such as P3 polyprotein or larger molecules including P2 and/or P1 in addition to P3, were detected at any time in the course of expression (data not shown for times earlier than 8 h p.i.). Likewise, no evidence for a peptide 3CD was found. The identities of the observed HAV peptides were confirmed by their respective absence in mock- or WR25-infected cells (data not shown). Monitoring of cells infected with vP3 resulted in a pattern of expression and a course of proteolytic processing that were indistinguishable from those produced by infection with vRGORF. Also, pulse-labelling of peptides in the vP3 system confirmed the apparent precursor-product relationship between 3ABC and $3C^{pro}$ (Fig. 2).

HAV replication and P3 polyprotein processing. To assess the relevance of the observed processing events, investigations were extended to infection of MRC-5 cells with HAV (Fig. 3). Analysis by immunoprecipitation of infected cell lysates with anti-3C antibody revealed three HAV-specific peptides corresponding to 3ABC (35.0 kDa), discernible as the lower of a doublet signal; 3BC (27.2 kDa); and $3C^{pro}$ (24.7 kDa) (Fig. 3, lane 2). Furthermore, as indicated by the different amounts of the peptides, 3ABC and mature $3C^{pro}$ appeared to be the most prominent products of proteolytic processing, whereas peptide

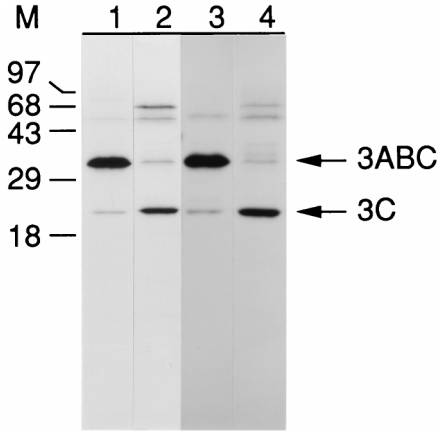

FIG. 2. Standard processing of polyprotein P3 in vaccinia virus-HAV chimeras. BT7-H cells in culture were infected under standard conditions with vRGORF (lanes 1 and 2) and with vP3 (lanes 3 and 4). Proteins were metabolically pulse-labelled in the presence of [^{35}S]methionine from 5 to 8 h (lane 1) and 2 to 10 h (lane 3) p.i. Subsequently, protein synthesis was extended with unlabelled methionine until 24 h (lane 2) and 26 h (lane 4). Immunoprecipitation with anti-3C antibody served to identify HAV proteins by radiofluorography for 61 h (vRGORF) and 48 h (vP3) after separation by sodium dodecyl sulfate (SDS)–12% PAGE. M, positions of peptide molecular mass markers (in kilodaltons).

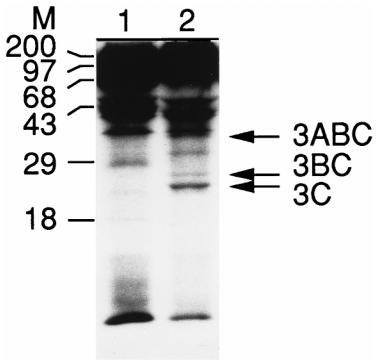

FIG. 3. Replication of HAV and polyprotein processing. Proteins in MRC-5 cells were metabolically radiolabelled with [^{35}S]methionine (100 μCi per ml) for an extended period of 8 days. Subsequent analysis by immunoprecipitation with anti-3C antiserum and SDS-PAGE was done as described in the legend to Fig. 2. Lane 1, mock-infected cells; lane 2, HAV-infected cells. Autoradiofluorographic detection was performed over 21 days at −70°C. M, positions of peptide molecular mass markers (in kilodaltons).

3BC was detected only in traces. Due to the extreme conditions of radiolabelling and detection, other areas of the gel were heavily overexposed by unspecific compounds. Therefore, inspection for putative polyprotein precursors was futile. Hence, overall, both standard heterologous expression with vRGORF or vP3 and HAV replication itself resulted in a similar pattern of P3 polyprotein processing products (3ABC, 3BC, and 3C).

Effects of mutations on HAV polyprotein processing in the vaccinia virus expression system. Because wild-type polyprotein processing events observed with vRGORF and vP3 were indistinguishable, functional analysis with variant 3C^{pro} was confined to expression with vP3 chimeras (Fig. 4). The proteolytic products that were obtained by infection of BT7-H cells individually with vP3, with variant vD98N (data not shown), and with vV47G/D98N (Fig. 4, lanes 3 and 5) were similar to each other. However, processing of 3BC appeared to be delayed for both the single and the double mutant, since the ratio

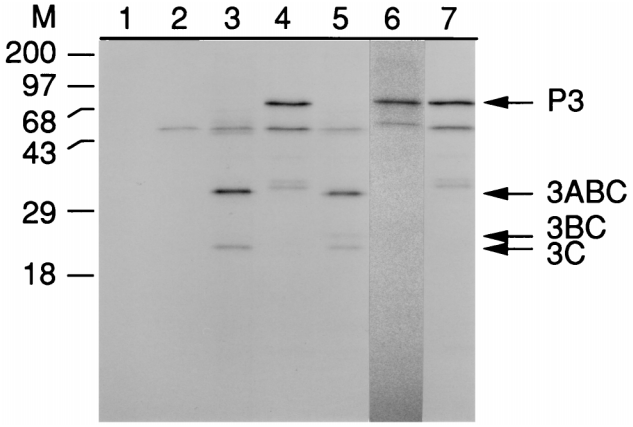

FIG. 4. Processing of polyprotein P3 with variant 3C^{pro} in vaccinia virus-HAV chimeras. BT7-H cells were infected under standard conditions with individual chimeras. Except for continuous metabolic labelling with [^{35}S]methionine, analysis of antigen was done as described in the legend to Fig. 2. Lane 3, vP3; lane 4, vH44Y; lane 5, vV47G/D98N; lane 6, vC172G; lane 7, vC172S. Mock infection and infection with vaccinia virus strain WR25 are shown in lanes 1 and 2, respectively. Identification and detection of HAV proteins were done as described in the legend to Fig. 2 and by 16 h of film exposure. The panel is a composite of two electrophoresis gels. M, positions of peptide molecular mass markers (in kilodaltons).

of 3BC to 3C peptide was significantly higher than with wild-type 3C^{pro}. Thus, neither the targeted substitution of aspartic acid-98 by asparagine nor that of valine-47 of 3A by glycine abolished processing of P3 polyprotein to 3ABC, 3BC, and 3C^{pro}.

In contrast to standard products of proteolytic processing of P3 with virus vP3, vD98N, or vV47G/D98N, complete inhibition of the proteolytic cascade was observed with vH44Y and vC172G, because the only HAV peptide produced was polyprotein P3 (91 kDa) (cf. lanes 4 and 6 in Fig. 4). Likewise, the overwhelming HAV-specific product of expression by vC172S was polyprotein P3. However, minute amounts of additional anti-3C reactive peptides with apparent molecular masses of 36.5 and 37.5 kDa were sometimes detected (Fig. 4, lanes 4 and 7). Similar peptides were variably observed with all of the individual vaccinia virus recombinants, including those with wild-type 3C^{pro}.

In vitro translation and P3 polyprotein processing. To further establish the significance of observations made in the vaccinia virus system and to exclude the possibility that observed effects of amino acid substitutions on polyprotein processing were dependent on the choice of the expression system, coupled IVTT in rabbit reticulocyte lysates was used as an alternative approach. The plasmid recombinants that had been used to generate vaccinia virus-HAV chimeras and that, therefore, individually contained the identical wild-type and mutated P3 genes were subjected to IVTT. HAV-specific peptide products of translation were identified by immunoprecipitation with anti-3C antibody.

For IVTT of pP3, the analysis resulted in a peptide pattern that contained the same peptides as the standard pattern of expression with vaccinia virus recombinants vRGORF and vP3 (Fig. 5, lanes WT). Peptides 3ABC, 3BC, and 3C^{pro} were the major components of the pattern. In contrast to vaccinia virus expression, however, IVTT produced large amounts of peptide precursor P3. Furthermore, products of internal initiation that are typically observed with in vitro translation of HAV RNA were observed in the molecular mass range of 45 to 68 kDa. The latter were apparently of no significance for P3 polyprotein processing. The time courses of peptide synthesis and of the disappearance of P3, 3ABC, 3BC, and 3C^{pro} were consistent with a proteolytic cascade by which P3 polyprotein was primarily converted to 3ABC and by which subsequently increasing amounts of 3C^{pro} were produced at the expense of P3 and 3ABC. Also, during the final 3-h interval of IVTT, peptide 3BC accumulated. Taking this pattern of peptide synthesis and proteolytic processing as the standard for events in IVTT of P3 polyprotein, by comparison mutant P3 proteins showed a significantly different behavior (Fig. 5, lanes V47G/D98N and D98N). For the sake of standardization of reaction conditions, the respective IVTTs were programmed with identical amounts of input plasmid DNAs and analyzed at identical time points under standard conditions. As a result, therefore, overall peptide synthesis occurred at the same levels irrespective of programming by wild-type or variant plasmid. However, although IVTT of mutant plasmid pD98N resulted in the standard pattern of peptides, conversion of peptide precursors to mature 3C^{pro} occurred more slowly than with the wild-type 3C^{pro} (Fig. 5, lanes D98N). This effect of slowing down polyprotein processing was even more pronounced when valine-47 of peptide 3A was substituted by glycine in addition to the D98N mutation in 3C^{pro} (Fig. 5, lanes V47G/D98N). The double mutant was still able to direct synthesis of P3 effectively, and the proteolytic cascade produced 3ABC, 3BC, and 3C^{pro} (the latter two were clearly visible at a comparable ratio in the original radioautograph), but HAV-specific proteolysis pro-

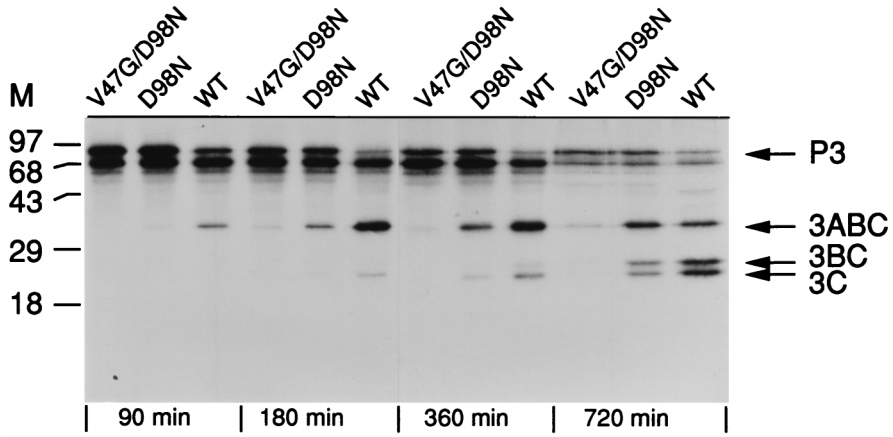

FIG. 5. Dependence of processing of in vitro-translated P3 polyprotein on variants of $3C^{pro}$. Shown are results of analysis by immunoprecipitation with anti-3C antibody and SDS-PAGE as described in the legend to Fig. 2 of products of coupled transcription-translation of wild-type and variant polyprotein genes in plasmid recombinants. Individually programmed reaction mixtures were monitored for their contents of HAV proteins after the indicated periods. Plasmids are represented by WT for authentic P3 genes and by the respective substitution of amino acid residues in HAV proteins 3A and/or $3C^{pro}$. Autoradiofluorographic exposure was for 94 h. M, apparent peptide molecular mass markers (in kilodaltons).

ceeded at an extremely low rate. In contrast, the standard proteolytic cascade operating on P3 polyprotein was completely inhibited by the H44Y, the C172G, and the C172S substitutions (Fig. 6). In each case, the variably programmed IVTTs produced P3 polyprotein effectively. However, all other peptides (i.e., 3ABC, 3BC, and $3C^{pro}$) of the standard processing pattern were absent even after prolonged times of reaction (i.e., 12 h).

In summary, by comparison, all expression systems—the vaccinia virus system, IVTT, and replication of HAV itself—generated the same standard pattern of P3 polyprotein processing. The recombinant systems demonstrated gradually increasing inhibition of the proteolytic cascade by the D98N substitution in $3C^{pro}$ and the additional V47G substitution in 3A. Furthermore, both recombinant approaches demonstrated consistently complete inhibition of the standard processing of P3 by individual substitutions of histidine-44 and cysteine-172. However, P3 processing apparently was less effective in IVTT than in the vaccinia virus expression system, because in the former, unprocessed P3 polyprotein was constantly observed already at very early times, whereas in chimeric expression, peptide precursors larger than 3ABC were absent.

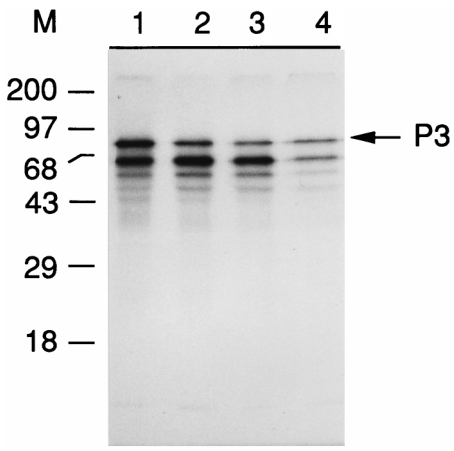

FIG. 6. Inhibition of polyprotein processing. Conditions of IVTT and analysis were identical to those described for Fig. 5, except that the reaction was programmed with pC172G. The patterns of anti-3C reactive antigens are shown in lanes 1 to 4, individually representing reaction times of 90, 180, 360, and 720 min, respectively. Autoradiofluorographic detection of peptides was achieved by exposure of film for 120 h. M, positions of peptide molecular mass markers (in kilodaltons).

DISCUSSION

Investigation of the functional role of single amino acid residues of $3C^{pro}$ of HAV with wild-type and variant enzymes supported earlier theoretical considerations that amino acid residues cysteine-172 and histidine-44 would be essential for the proteolytic activity of $3C^{pro}$. In contrast, aspartic acid-98, which had been proposed to serve as the third member of the classical catalytic triad, showed only marginal involvement. Moreover, the apparent effect of a substitution in the virus protein 3A (valine-47) on polyprotein processing may indicate that amino acid residues distant from the virus protease and from natural cleavage sites may participate in the control of virus-specific proteolytic processing.

Transient expression of the complete open reading frame of HAV or of region P3 resulted in effective production of polyprotein and cleavage through a cascade, with 3ABC as the most prominent peptide precursor, of $3C^{pro}$ and of 3BC. This proteolytic cascade was also active on P3 polyprotein when synthesized by in vitro translation. The relevance of these observations in heterologous systems was confirmed by identification of peptide species colinear with the recombinant peptides in lysates of HAV-infected cells. Hence, the standard proteolytic cascade observed in heterologous and in vitro expressions of the complete polyprotein and of the P3 part of polyprotein reflected authentic effects of HAV replication. Interestingly, the evidence supports fast cleavage of large peptide precursors such as polyprotein P3 or of such precursors that would include additional regions upstream of P3, because with wild-type $3C^{pro}$, the prominent intermediately stable precursor molecule was 3ABC. These findings are corroborated by investigations on the expression and processing of polyprotein to protein 2B, for which also, even at early times of expression, large precursors were absent (18). The dominance of 3ABC may also imply that scission at the 3C-3D junction occurred at a comparably high rate. This would be in contrast with the typical finding with poliovirus and may indicate that different mechanisms are in control of virus replication (54).

Individual members of the putative active site were identified by complete inhibition of polyprotein processing by variant $3C^{pro}$ both in the vaccinia virus expression system and in IVTT. Hence, both histidine-44 and cysteine-172 were indispensable for the functional integrity of the enzyme. These results are in agreement with the earlier proposal that histidine-44 may serve as a catalytic base in the proteolytic reaction (5, 17). They also corroborated deductions made from the analysis of the atomic structure of variant $3C^{pro}$, which included the observation of a spatial relationship of histidine-44 and cysteine-172 compatible with the formation of a catalytic site. Furthermore, the key role of histidine-44 correlates well with that suggested for the corresponding histidine residue at amino acid position 40 of both enterovirus and human rhinovirus $3C^{pro}$s (7, 20, 37).

The D98N variant of $3C^{pro}$ retained substantial proteolytic activity. The only effect observed was a moderately impaired efficiency of the standard proteolytic cascade for the asparagine variant. This result indicates that a negative charge at the respective position is not absolutely required for proteolytic activity. Therefore, aspartic acid-98 is apparently not essential for the active site, and another amino acid residue may be the third constituent of the putative catalytic triad, if the latter actually exists. The minimal effect of amino acid substitution at aspartic acid-98 is consistent with the suggestion, based on the structure of $3C^{pro}$ deduced by X-ray crystallography, that this residue is not part of the active site (1). Aspartic acid-98 and various other contiguous aspartic acid residues were too distant from both histidine-44 and cysteine-172 to directly participate in acceptor site hydrolysis. However, instead of direct participation in scission of amide bonds, aspartic acid-98 may be involved in the binding of the substrate. This hypothesis was substantiated by alignments of amino acid sequences of picornavirus $3C^{pro}$s and cellular serine proteases which indicated that certain amino acids may serve this function. This would explain the modest reduction in proteolytic activity observed with substitution of aspartic acid-98. In this respect, aspartic acid-98 of $3C^{pro}$ may then resemble aspartic acid-189 of trypsin.

Our findings that aspartic acid-98 was only marginally involved in the control of the proteolytic cascade contrast with the effect of substitution by asparagine of the corresponding aspartic acid-85 of poliovirus (20). In the case of poliovirus, a D85N variant showed severe inhibition of autocatalytic processing in *cis* of P3 polyprotein. However, the amino acid exchange caused only a slight reduction of *trans* proteolytic activity of $3C^{pro}$. Interestingly, a similar D85N variant of coxsackievirus B3 showed pronounced inhibition of *cis* cleavage as well (37).

In the case of HAV, a more severe influence on proteolytic processing was caused by a secondary-site amino acid exchange (V47G in 3A) in addition to the D98N substitution. The former site was not only outside of $3C^{pro}$ but also located 27 amino acid residues upstream of the 3A-3B junction (51). Hence, this substitution may have had an influence on processing by affecting polyprotein cleavage sites instead of having a direct effect on $3C^{pro}$. This hypothesis may include a mechanism of altered folding of the peptide acting against effective cleavage. Such dependence of efficiency of polyprotein cleavage on conformation has been elegantly demonstrated by replacement of authentic $3C^{pro}$ by that of rhinovirus 14 or coxsackievirus B3 in chimeric poliovirus (11). Similar interactions have been observed with poliovirus (41). In the latter case, substitution of isoleucine-25 of protein 2C caused processing defects that led to the nearly complete loss of production of proteins 2C and 3AB.

On the basis of similarities in amino acid sequence with poliovirus $3C^{pro}$, HAV cysteine-172 was predicted to be the active-site nucleophile of HAV $3C^{pro}$ (24). This hypothesis was corroborated by a complete absence of the standard proteolytic cascade when cysteine-172 was either replaced by glycine or conservatively exchanged with serine. These results, furthermore, confirmed a published finding that hydrolytic activity of recombinant $3C^{pro}$ on synthetic peptide substrates was eliminated by substitution of alanine for cysteine-172 (33).

The total inactivation of the $3C^{pro}$-specific cascade by replacement of cysteine-172 with serine implies a strict requirement for the thiol group as the catalytic nucleophile for processing of contiguous 3ABCD peptides in the P3 polyprotein. In contrast, when the equivalent cysteine residues of poliovirus (cysteine-147), coxsackievirus B3 (cysteine-147), and foot-and-mouth disease virus (cysteine-163) had been conservatively substituted by serine, reduced but significant levels of protease activity were retained (19, 29, 37). In the latter cases, the serine hydroxyl group was apparently sufficient for the nucleophilic attack on the appropriate amide bonds in virus polyprotein cleavage sites. With acidic residues present in the respective deficient (serine) variant catalytic sites, hydrolysis may be driven with the aid of carboxylate residues, whereas in $3C^{pro}$ of HAV the lack of acidic residues within the active site may explain the inability of the serine variant to cleave authentic polyprotein.

These considerations are supported by the chemical nature of the involved residues (cysteine-SH and serine-OH). Oxygen and sulfur both belong to the 6A chemical group of periodic elements and posses similar chemical properties. As is evident from the respective pK_a values (8.3 for R-SH and >15 for R-OH), however, removal of a proton from a sulfhydryl group to create a nucleophilic thiolate anion is more favorable than removal of a proton from a hydroxyl group. Hence, in contrast to abstraction of a proton from serine, the catalytic cysteine of HAV $3C^{pro}$ may not require attractive electrochemical support by neighboring carboxylate residues that were proposed to function as the third member of a catalytic triad (5, 17). The observation with $3C^{pro}$ of HAV, therefore, suggests that the microenvironment in its active site differs considerably from those of other picornavirus trypsin-like cysteine proteases.

Picornavirus $3C^{pro}$s are structurally related to cellular serine proteases (16). They are thought to contain a catalytic triad reminiscent of trypsin in which phylogenetically an active-site serine residue (hydroxyl) has been replaced by cysteine (sulfhydryl) (5, 17). At present, evidence supports the presence of a catalytic dyad in HAV $3C^{pro}$, but it is not entirely clear whether histidine-44 and cysteine-172 of HAV $3C^{pro}$ are the only active-site residues essential for the proteolytic cascade of P3 polyprotein. Additional functional analysis as presented here will help to shed more light on the mechanisms driving HAV polyprotein processing. And certainly, resolution of the atomic structure of wild-type HAV $3C^{pro}$ will add to our understanding of the catalytic process.

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