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Intermolecular Cleavage of Hepatitis A Virus (HAV) Precursor Protein P1-P2 by Recombinant HAV Proteinase 3C

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Active proteinase 3C of hepatitis A virus (HAV) was expressed in bacteria either as a mature enzyme or as a protein fused to the entire polymerase 3D or to a part of it, and their identities were shown by immunoblot analysis. Intermolecular cleavage activity was demonstrated by incubating in vitro-translated and radiolabeled HAV precursor protein P1-P2 with extracts of bacteria transformed with plasmids containing recombinant HAV 3C. Identification of cleavage products P1, VP1, and VP0-VP3 by immunoprecipitation clearly demonstrates that HAV 3C can cleave between P1 and P2 as well as within P1 and thus shows an activity profile similar to that of cardiovirus 3C.

Polyprotein processing is crucial for the assembly and maturation of viruses (9). In picornaviruses, virus-encoded proteinases L, 2A, and 3C are presumably responsible for all of the proteolytic cleavages within the polyprotein (5, 13). Since all proteinases are integrated parts of the primary translation product, it is generally assumed that the primary cleavage event within the polyprotein occurs in a monomolecular reaction. The precursor of the structural proteins is cotranslationally liberated from the nonstructural proteins, whereas further proteolytic processing is executed via a cascade of intermolecular cleavage steps (13).

Presumably, a similar scheme is followed during the formation of structural and functional proteins of hepatitis A virus (HAV), a member of the picornavirus family. Recently, HAV polyprotein processing was studied in two recombinant systems. The results indicated that no proteolytic activity is encoded within P2 of HAV, in contrast to the enteroviruses and rhinoviruses, and that polypeptide 3C, which is a cysteine-containing proteinase, is autocatalytically active within P3 (2, 3, 6, 7). Until now, neither the fusion protein nor the mature recombinant proteinase 3C of HAV has been shown to cleave in vitro-translated HAV precursor proteins in *trans*, as one might postulate for the formation of mature viral proteins (2, 6). Since we have recently observed that host cell factors do not modulate the intermolecular activity of HAV 3C, we made a new attempt to address this question by using more efficient expression systems which allow the production of substantial amounts of HAV 3C needed for a comprehensive analysis of the intermolecular cleavage products (2a).

To express mature HAV 3C, the *Nco*I-*Hin*dIII fragment encompassing the HAV 3C coding region, including a start and a stop codon, was cloned into pPROK-C (*Nco*I-*Hin*dIII; Clontech Laboratories). Because of the lack of appropriate cloning sites, the suggested N and C termini of HAV 3C (amino acids 1521 and 1736 of the polyprotein of strain MBB [15]) were generated by the polymerase chain reaction, using synthetic oligonucleotides and the HAV cDNA fragment (bp 3557 to 6149). The resulting construction (designated pPROK-3C) allows expression of the mature HAV 3C in *Escherichia coli* JM109 after 3 h of induction of the *tac* promoter with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) (Fig. 1). Furthermore, HAV 3C and 3CD were cloned into a pET vector which was used in another study to produce large amounts of recombinant 3C of poliovirus (12). For the construction of pET-3CΔD and pET-3CD, the *Hin*cII-*Hin*dIII and *Hin*cII-*Kpn*I cDNA fragments, respectively, of HAV strain HAS-15 were inserted into the *Bam*HI-restricted and blunt-ended vector pET (Fig. 1). Fusion proteins encoded by both recombinant pET constructs include 13 N-terminal amino acids of the bacterial gene 10 in addition to HAV domains 3CΔD and 3CD, respectively, and were expressed after transformation into competent cells of *E. coli* BL21(DE3) and induction with IPTG (0.5 mM) for 3 h (16). For all 3C expression products, a crude proteinase fraction was obtained by ultrasonication and extraction of bacteria with TET (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5% Triton X-100). As shown in Fig. 2, mature HAV 3C expressed from pPROK-3C is recognized by an antiserum directed against amino acids 131 to 144 of 3C (3) and migrates as a protein of 24 kDa (lane 4); as a result of the additional 13 vector-derived amino acids, HAV 3C expressed from pET-3CΔD and pET-3CD has an apparent molecular mass of 26 kDa and was therefore designated $3C^{26kd}$ (lanes 2 and 3, respectively). The full-length product of pET-3CD ($3C^{26kd}D$), which is autoproteolytically active, has a molecular mass of 78 kDa (lane 3).

The substrate for recombinant HAV proteinases was produced by translating a synthetic transcript encoding the HAV P1-P2 genomic region (plasmid pHAV/7 linearized with *Eco*RI) for 60 min in rabbit reticulocyte lysate (7). Polypeptides migrating faster than the HAV P1-P2 precursor protein arise by internal initiation of translation (Fig. 3, lane 1) (7). Proteinase assays were performed by incubating 10 μl of the P1-P2 translation mixture with 30 μl of soluble bacterial extracts (corresponding to 3×10^7 bacteria) for 7 h at 30°C prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The amount of the largest polypeptide (150 kDa) and of p68 decreased, and additional polypeptides of 88 and 55 kDa appeared after incubation with extracts of BL21(DE3) transformed with pET-3CΔD and pET-3CD, respectively, (lanes 3 and 4). No *trans*

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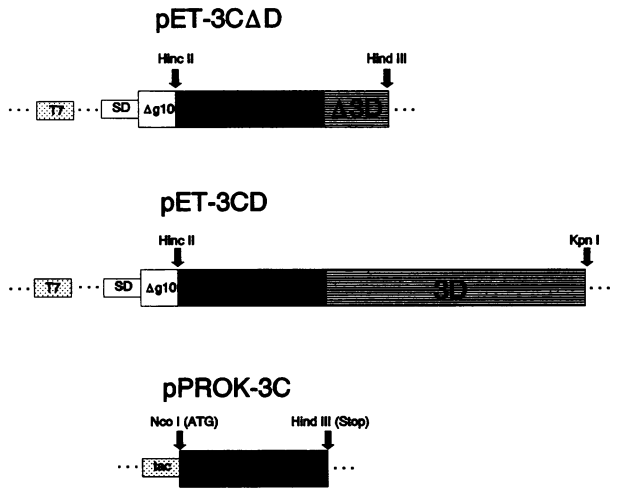

FIG. 1. 3C expression vectors encoding mature 3C, 3CΔD, and 3CD, respectively. T7, promoter of the T7 RNA polymerase; SD, Shine-Dalgarno sequence; Δg10, fusion part of bacterial gene 10 (13 amino acids).

activity was observed with extract of bacteria transformed with pET alone, indicating that even during long incubation periods, no unspecific degradation occurs (lane 2). Incubation periods shorter than 2 h did not result in significant cleavage by recombinant enzymes (not shown). HAV 3C derived from pPROK-3C cleaved P1-P2 more efficiently and produced polypeptides of 88, 50, and 33 kDa (lane 5).

To identify the polypeptides produced by processing with mature 3C, immunoprecipitation was performed, using specific sera directed to viral proteins VP1 and VP0 and recombinant protein 2CΔ3A (4). As shown in Fig. 3, the precursor polypeptide P1-P2 was precipitated by anti-2CΔ3A (lane 6), anti-VP0 (lane 7), and anti-VP1 (lane 8), whereas the 88-kDa protein was recognized only by anti-VP0 (lane 7) and anti-VP1 (lane 8), confirming that p88 contains P1. In addition, anti-2CΔ3A precipitated a 65-kDa polypeptide which might be the truncated P2 precursor. Anti-VP1 also detected

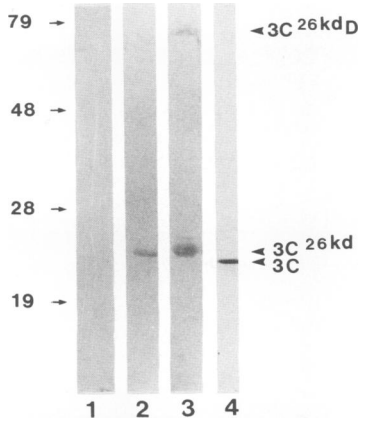

FIG. 2. Immunoblot analysis of recombinant HAV 3C. Proteins of the soluble extracts of bacteria transformed with pET (lane 1), pET-3CΔD (lane 2), pET-3CD (lane 3), and pPROK-3C (lane 4) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-3C directed to a synthetic peptide (3). Positions (in thousands) of molecular weight markers are shown on the left.

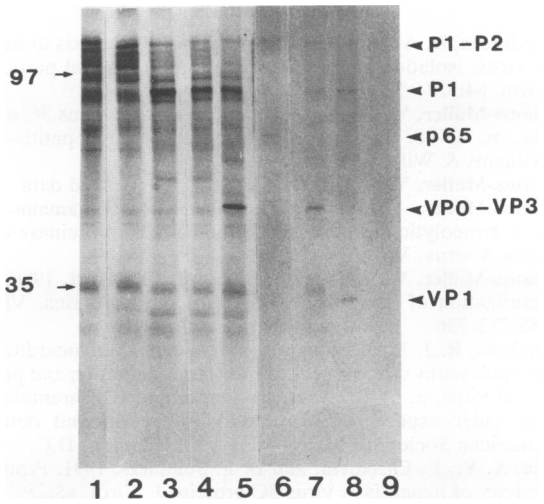

FIG. 3. *trans* cleavage by recombinant HAV 3C, using in vitro-translated P1-P2 as the substrate, and identification of the resulting products by immunoprecipitation. To produce the P1-P2 substrate, the RNA transcript derived from *Eco*RI-linearized pHAV/7 was translated in rabbit reticulocyte lysate for 60 min (lane 1) and subsequently incubated for 7 h with extracts of bacteria transformed with pET (lane 2), pET-3CΔD (lane 3), pET-3CD (lane 4), and pPROK-3C (lane 5). For immunoprecipitation of processing products of lane 5, anti-2CΔ3A (lane 6), anti-VP0 (lane 7), anti-VP1 (lane 8), and a preimmune serum (lane 9) were used, and the fluorograph was exposed for 25 days. Among the products shown in lane 5, VP1 is not detectable since the fluorograph of lanes 1 to 5 was exposed for 3 days only. Translation and processing products were analyzed by SDS-PAGE. Positions of marker proteins are indicated in kilodaltons on the left; positions of HAV proteins are indicated on the right.

small amounts of p55 and p33, suggesting that these proteins represent VP3-VP1 and VP1, respectively (lane 8). The main processing product of 50 kDa was exclusively recognized by anti-VP0, suggesting that mature 3C is able to cleave efficiently at the VP3-VP1 junction, thus producing predominantly VP0-VP3 and VP1 (lane 7).

These results show that HAV 3C can cleave intermolecularly between P1 and P2 as well as within P1. Recent data suggested alternative cleavage sites for liberation of the precursor of the structural proteins, resulting in polypeptides larger than VP1 termed PX and VP1-2A, respectively (1, 10, 11). The difference of specificity and the reduced activity of $3C^{26kD}$ compared with mature 3C might be explained by the elongated N terminus of the recombinant proteinase. Since the enzymes expressed from pET-3CΔD and pET-3CD both produced P1 and p55, their cleavage specificities seem to be similar. In poliovirus, however, proteinase 3C cleaves within P1 efficiently only if it is present in large molar excess, whereas 3CD is the active proteinase for processing within P1 (17). The difference in substrate specificity of poliovirus 3C and 3CD exerts a regulatory effect on the production of structural and nonstructural proteins (8). Our results are in accordance with those obtained for cardiovirus 3C, which cleaves not only at the P1-P2 junction but also within P1 as well as within P2 and P3 (14).

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