GB Virus B and Hepatitis C Virus NS3 Serine Proteases Share Substrate Specificity

ELISA SCARSELLI, ANDREA URBANI, ANDREA SBARDELLATI, LICIA TOMEI,
RAFFAELE DE FRANCESCO, AND CINZIA TRABONI*

Istituto di Ricerche di Biologia Molecolare P. Angeletti, 00040 Pomezia, Rome, Italy

Received 22 November 1996/Accepted 18 March 1997

GB virus B (GBV-B) is a recently discovered virus responsible for hepatitis in tamarins (*Saguinus* species). GBV-B belongs to the *Flaviviridae* family and is closely related to the human pathogen hepatitis C virus (HCV). Nonstructural protein 3 (NS3) of HCV has been shown to encompass a serine protease domain required for viral maturation. GBV-B and HCV share only about 30% of the amino acid sequence within the NS3 protease domain. The catalytic triad is conserved, and the residue Phe-154, presumed to be a crucial amino acid for determining the S_1 specificity pocket of the HCV NS3 protease, is also conserved. We have expressed a synthetic gene encoding the GBV-B NS3 protease domain in *Escherichia coli* and have characterized the purified recombinant protein for its activity on HCV substrates. We have shown that the NS3 region of the GBV-B genome actually encodes a serine protease that, despite the low sequence homology, shares substrate specificity with the HCV NS3 protease.

GB agent hepatitis was first described by Deinhardt and coworkers (4), who inoculated tamarins (*Saguinus* species) with a serum from a surgeon affected by acute hepatitis. This serum induced hepatitis in all inoculated tamarins and was passaged serially in these animals. Only recently has molecular characterization of this agent been achieved (14, 23), showing that two distinct RNA viruses, GB virus A (GBV-A) and GBV-B, were associated with GB agent hepatitis. Subsequently it was shown that the two viruses could be passaged separately in tamarins and that GBV-A does not replicate in the liver whereas GBV-B causes hepatitis (20, 21). Despite the human origin of the initial inoculum, it is not yet clear if GBV-A and GBV-B are indeed human pathogens. Even though immune reactivity against GBV-A- and GBV-B-derived peptides has been found in humans (16), attempts to detect viral sequences in the reactive plasma have been unsuccessful (22).

Sequence analysis of GBV-A and GBV-B genomes has suggested that they belong to the *Flaviviridae* family. They are closely related to hepatitis C virus (HCV): comparison of their entire open reading frames with those of different HCV strains shows from 26 to 33% identity at the amino acid level, but the comparison of the hydrophathy profiles suggests that despite the low overall amino acid sequence identity, these viruses are similar in protein structure (14, 15). Of the two, the one most closely related to HCV is GBV-B, not only at the level of sequence similarity but also because they are both hepatotropic viruses (30).

Nonstructural protein 3 (NS3) of HCV contains a zinc-binding serine protease domain that has been shown to be responsible for processing of the downstream nonstructural proteins (3, 10, 29). The S_1 specificity pocket was initially modelled on the basis of the known structure of trypsin-like serine proteases (17), and the model has recently been confirmed by X-ray crystallography (11, 13). The amino acid Phe-154 is one of the main determinants of the shape and the physicochemical char-

acteristics of the S_1 pocket. In fact, experimental investigations gave support to the notion that in the case of the HCV NS3 protease, Phe-154 determines the preference of the enzyme for cysteine in the P1 substrate position (8).

NS3 serine protease residues that are required for catalysis and for zinc binding (3, 11, 13) are conserved in all HCV genotypes, as well as in the homologous serine protease domains of both GBV-B and GBV-A. Interestingly, HCV and GBV-B also share the residue Phe-154, which, in contrast, is not present in GBV-A. This observation suggests that the GBV-B protease, despite the limited sequence similarity to HCV NS3, might show a preference for a cysteine residue in the P1 position of the substrate, as does the protease of HCV.

In this paper we report the assembly of a synthetic gene encoding the putative NS3 protease domain of GBV-B and its expression in *Escherichia coli*. We characterized the enzymatic activity of the purified GBV-B NS3 protein in vitro on HCV substrate sequences. We demonstrate that this enzyme is a serine protease, that it is able to cleave all of the substrates cleaved in *trans* by the HCV NS3 protease, and that cleavage indeed occurs after a cysteine residue.

MATERIALS AND METHODS

Gene assembly, amplification, and cloning of the GBV-B serine protease domain. Nine oligonucleotides, each 60 nucleotides (nt) in length, overlapping for 20 nt, and collectively encoding both strands of the GBV-B putative protease domain (nt 3266 to 3814 [accession number HGU22304]) have been synthesized. Single-step gene assembly (26) was performed as follows. A mixture of the nine overlapping oligonucleotides was added at a final concentration of 20 nM each to 50 μ l of a PCR mixture containing 0.2 mM each deoxynucleoside triphosphate (dNTP), PCR buffer (Boehringer), 1 U of *Taq* polymerase (Boehringer), and 0.02 U of *Pfu* polymerase (Stratagene). The PCR program consisted of 55 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. The assembly reaction mix was then diluted 40-fold in a new reaction tube containing 1 $\times$ PCR buffer, 0.2 mM each dNTP, 2.5 U of *Taq* polymerase, and 1 μ M each of two most external primers (GB⁺ [5'-CATACATATGGCACCTTTACGCTGCAGTG] and GB⁻ [5'-GCGAATCCGAATCCGCTAGCCTGCTGTGTGTA CTGGGGATGG]). The PCR program consisted of 23 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Five units of Klenow enzyme (Boehringer) and 0.2 mM dNTPs were then added to the reaction tube and incubated for 1 h at room temperature. Amplified DNA was then digested with *Nde*I and purified by gel electrophoresis. The pT7-7 vector (27) was prepared by *Nde*I and *Sma*I digestion, and ligation was performed with T4 DNA ligase. The insert coding for the GBV-B protease domain was sequenced by the dideoxy chain termination method (18).

* Corresponding author. Mailing address: Istituto di Ricerche di Biologia Molecolare P. Angeletti (IRBM), Via Pontina, Km 30.600, 00040 Pomezia, Roma, Italy. Phone: (39.6)91093241. Fax: (39.6)91093225. E-mail: traboni@irbm.it.

Protein expression and purification. *E. coli* B834(DE3) transformed by the recombinant pT7-7 plasmid encoding the GBV-B putative protease domain was inoculated in Luria broth containing 100 µg of ampicillin per ml and grown at 37°C to an optical density of 0.6 to 0.8 at 600 nm. After induction of protein expression by addition of 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside), bacterial cultures were incubated for additional 4 h at room temperature prior to harvesting by centrifugation. It was empirically observed that lowering the temperature resulted in an increased expression of soluble recombinant protein. The bacterial cell pellet was resuspended in 25 mM sodium phosphate (pH 7.5)–500 mM NaCl–10% glycerol–6 mM dithiothreitol (DTT) and lysed through two passages in a French pressure cell. The homogenate was clarified by centrifugation for 30 min at 70,000 × g, and the soluble fraction was dialyzed against a buffer containing 25 mM sodium phosphate (pH 7.5), 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 10% glycerol, 50 mM NaCl, and 3 mM DTT.

The supernatant was applied to a Resource S 6-ml column (Pharmacia) equilibrated with 50 mM sodium phosphate (pH 7.5)–0.1% CHAPS–3 mM DTT and operating at the flow rate of 2 ml/min. The protease was eluted with a linear gradient from 0 to 1 M NaCl in the same buffer. Fractions containing the protein, identified by use of Coomassie blue-stained polyacrylamide gels, were pooled, concentrated to 3 ml by using an Amicon stirred ultrafiltration cell equipped with a YM10 membrane, and chromatographed on an HR26/60 Superdex 75 column (Pharmacia) equilibrated with 50 mM sodium phosphate (pH 7.5)–5% glycerol–3 mM DTT–0.1% CHAPS and operating at 1 ml/min. The purity of the enzyme was checked on silver-stained sodium dodecyl sulfate (SDS)–polyacrylamide gels run under denaturing conditions. The protein concentration was determined by the Bradford protein assay (Bio-Rad) with bovine serum albumin as a standard and was confirmed by quantitative amino acid analysis performed on HCl-hydrolyzed samples. N-terminal analysis was carried out by Edman degradation on an Applied Biosystems model 470A gas phase sequencer.

The purification of the NS3 protease domain of HCV was performed as described previously (25).

In vitro translation of HCV substrates and determination of GBV-B protease cleavage activity. Plasmids pCiteNS4A-NS4B, pCiteNS4B-NS5AΔC216, and pCiteNS5A-NS5BΔC51 (6, 25), containing DNA fragments derived from the HCV BK strain (28), were transcribed in vitro by using T7 RNA polymerase. In vitro translation was performed for 1 h at 30°C with an RNA-dependent rabbit reticulocyte lysate (Promega) in the presence of [³⁵S]methionine. Purified GBV-B NS3 protease or purified HCV NS3 protease was added to the in vitro-translated substrates and incubated for 2 h at 30°C. The final concentration of GBV-B or HCV protease was 100 nM for the experiments with NS4A-NS4B and NS5A-NS5BΔC51 substrates; for the NS4B-NS5A substrate the concentration used was 5 µM. The cleavage activities of the two proteases were also analyzed in the presence of a 10-fold molar excess of NS4A peptide Pep4A21-34 (GSVVIVGRILSGR), derived from the HCV BK sequence and described previously (12, 24). Cleavage of labelled precursors was assessed by SDS–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) and visualized by autoradiography.

Inhibition studies. GBV-B recombinant protease (1 µM in 50 mM sodium phosphate buffer [pH 7.5]–50% glycerol–2% CHAPS, 2 mM DTT) was incubated with various known protease inhibitors at 22°C for 1 h. The NS5A-NS5BΔC51 substrate was produced as described above. In vitro translation was stopped by addition of cycloheximide to a final concentration of 2.5 mM. Five microliters of labelled substrate was incubated with 5 µl of inhibitor-treated protease for 2 h at room temperature. The inhibitors used and their final concentration were as follows: phenylmethylsulfonyl fluoride (PMSF), 1 mM; tosyl-phenylalanyl chloromethyl ketone (TPCK), 1 mM; N^α-p-tosyl-L-lysine chloromethyl ketone (TLCK), 0.5 mM; aprotinin, 0.5 mg/ml; chymostatin, 0.5 mg/ml; *ortho*-phenanthroline, 2 mM; EDTA, 2 mM; and leupeptin, 0.25 mg/ml. Samples were subjected to SDS–12.5% PAGE. Data were analyzed on a PhosphorImager and quantified by volumetric integration with ImageQuant software.

HPLC assays. The peptides PEP4A/4B derived from the HCV BK strain (DEMEECASHL) and PEP4B/5A (DCSTPCSGSW) were synthesized by standard solid-phase methods based on 9-fluorenylmethoxycarbonyl chemistry. They were purified to 95% homogeneity by high-pressure liquid chromatography (HPLC). Identities were checked by mass spectrometry. The peptides were solubilized in dimethyl sulfoxide, and concentrations of stock solutions were determined by quantitative amino acid analysis performed on HCl-hydrolyzed samples. The cleavage assay was performed with 500 nM enzyme in 30 µl of a buffer containing 50 mM Tris (pH 7.5), 50% glycerol, 2% CHAPS, 30 mM DTT, and the appropriate amounts of substrate. The reaction was stopped after 1 h of incubation at 30°C with 70 µl of H₂O containing 0.1% trifluoroacetic acid. Cleavage was determined by HPLC with a Merck-Hitachi chromatograph equipped with an autosampler. Ninety-microliter samples were injected on a Beckman Ultrasphere octyldecyl silane reversed-phase column (4.6 mm by 25 cm), and peptides were separated by using a 5 to 40% acetonitrile gradient at 0.8%/min. Peak detection was accomplished by monitoring absorbance at 220 nm and/or fluorescence (excitation, 280 nm; emission, 350 nm). The cleavage product was quantified by integration of chromatograms with respect to the appropriate standard peptide (ASHL or SGWS). All experiments were run in duplicate. The initial rate of cleavage was determined on less than 10% conversion. Kinetic

FIG. 1. Comparison of amino acid sequences encoded by GBV-B and HCV NS3 protease domains, showing catalytic residues (*), zinc-binding residues (+), and the conserved phenylalanine in the putative specificity pocket (•).

parameters were calculated from a least-squares fit of the initial rate as a function of substrate concentration, assuming Michaelis-Menten kinetics. The NaCl titration experiment was performed by using the peptide PEP4A/4B (DE MEECASHLPYK) (25), to allow quantitation of the cleavage product by monitoring fluorescence (excitation, 260 nm; emission, 305 nm).

RESULTS AND DISCUSSION

Gene assembly, expression in *E. coli*, and purification of the GBV-B NS3 protease domain. The gene coding for the amino-terminal part of NS3, the putative serine protease domain of GBV-B (Fig. 1), was produced by single-step assembly from a mixture of oligonucleotides which collectively encode both strands of the sequence from nt 3266 to 3814 of the viral genome. This technology was used in place of conventional cDNA amplification from viral RNA because of the unavailability of a virus source. We designed the oligonucleotides according to the published GBV-B sequence (23). A methionine residue was added to the amino terminus of the original sequence. Upon induction of *E. coli* cells harboring the NS3 construct, we obtained a protein detectable as a prominent band on a Coomassie blue-stained SDS–12.5% polyacrylamide gel (Fig. 2, lane 2) running at a position corresponding to an apparent molecular mass of 21.5 kDa, in good agreement with the molecular mass of 19.4 kDa deduced from the cDNA sequence data. After cell homogenization, most of the protein was in the soluble fraction and was then subjected to chromatography on a cation-exchange column followed by gel filtration chromatography. We purified the GBV-B protein in the presence of the zwitterionic detergent CHAPS and 10% glycerol under reducing conditions by following a procedure similar to that successfully used for the purification of an active HCV NS3 protease domain from a bacterial lysate (25). The recombinant protein obtained appeared as a single band in a Coomassie blue-stained SDS–12.5% polyacrylamide gel (Fig. 2, lane 3) and was more than 95% pure as judged by silver staining (Fig. 2, lane 5). The final yield ranged between 2 and 4 mg of purified protein per liter of bacterial culture. Experimental sequence analysis revealed that 75% of the purified protein had the expected N-terminal sequence MAPFTLQ CLSE, whereas removal of the N-terminal methionine residue occurred in the remaining 25%.

Activity on in vitro-translated HCV substrates. The NS3-dependent cleavage sites within the GBV-B polypeptide have not yet been experimentally determined. The observation that the residue Phe-154, located in the S₁ specificity pocket of HCV NS3 protease, is conserved in the GBV-B protease domain has led us to hypothesize that the two proteases might display overlapping sequence specificities. In order to assess whether the GBV-B NS3 protease could recognize the HCV

FIG. 2. Purification of GBV-B protease domain, showing Coomassie blue staining (lanes 1 to 4) and silver staining (lane 5) after SDS-12.5% PAGE. Lane M, molecular mass standards; lane 1, bacterial lysate before induction of GBV-B protease expression; lane 2, bacterial lysate after induction of GBV-B protease expression; lanes 3 and 5, purified GBV-B protease (2 µg); lane 4, purified HCV protease (2 µg).

NS3-dependent cleavage sites, the activity of the purified GBV-B NS3 fragment on HCV polyprotein precursors was analyzed. Three different ^{35}S -labelled substrates were synthesized by in vitro transcription and translation, the first including the NS4A-NS4B cleavage site (Fig. 3A), the second including the NS4B-NS5A site (Fig. 3B), and the third including the NS5A-NS5B site (Fig. 3C). We have excluded the HCV NS3-NS4A cleavage site from this investigation since it is cleaved only in *cis* (10, 29).

The precursors were incubated with the putative GBV-B protease and, as a control, with the recombinant HCV serine protease (Fig. 2, lane 4) purified as described previously (25). The GBV-B NS3 protease was able to cleave all three substrates (Fig. 3). It is worth pointing out that the NS4B-NS5A site, which was not cleaved by HCV protease (Fig. 3B, lane 2), was instead efficiently cleaved by equal amounts of the GBV-B NS3 protease (Fig. 3B, lanes 4 and 5). This site is in fact cleaved by the HCV protease only in the presence of the NS4A cofactor (7) (Fig. 3B, lane 3). We investigated whether the HCV NS4A cofactor could further activate the GBV-B NS3 protease. To this end, we used a peptide encompassing residues 21 to 34 of the central region of NS4A. The same peptide was previously shown to efficiently mimic the effect of NS4A when added to HCV protease (25). Under the same experimental conditions in which activation of the HCV protease was observed (Fig. 3, lanes 3), it was not possible to detect any activation of the GBV-B protease by the HCV NS4A peptide (Fig. 3, lanes 5). Thus, the proteolytic activity of the GBV-B NS3 serine protease measured on the three HCV cleavage sites was independent of the HCV NS4A cofactor. This finding is not surprising, since the HCV NS4A sequence shows no obvious homology with that of the presumptive GBV-B NS4A protein. Attempts to identify alternative regions of the GBV-B polyprotein involved in the interaction with GBV-B NS3 on the basis of the crystal structure data for the HCV NS3-NS4A complex (11) have not led to conclusive results (data not shown). Since no reliable predictions can be made, more experimental work will be necessary to experimentally identify a potential cofactor of the GBV-B NS3 serine protease.

We tested the inhibitory activities of a series of known protease inhibitors on the cleavage of in vitro-translated NS5A-NS5B substrate. Obvious inhibition of the activity was detectable in the presence of some classical serine protease inhibitors, such as aprotinin and chymostatin, whereas for others, like TPCK, the inhibitory activity was less evident, even at a high concentration of inhibitor (Fig. 4). The inhibition of the

enzymatic activity elicited by some of the classical serine protease inhibitors confirms the identification of the GBV-B protease as a serine protease. Similar results have already been reported for HCV NS3 serine protease (24).

Activity on peptide substrates. Recombinant NS3 HCV protease has been shown to be able to cleave 10-amino-acid synthetic peptides corresponding to the P6-P4' substrate sequence. Similarly, we analyzed the cleavage activity of the GBV-B serine protease on decamer peptides corresponding to the substrate sequences of HCV NS4A-NS4B and NS4B-NS5A, in order to evaluate the kinetic parameters for the enzymatic reaction. We used the peptide sequences of NS4A-NS4B and NS4B-NS5A of HCV since the peptide correspond-

FIG. 3. Activity of purified GBV-B protease on in vitro-translated HCV substrates. ^{35}S -labelled HCV precursor proteins NS4A-NS4B (A), NS4B-NS5A Δ C216 (B), and NS5A-NS5B Δ C51 (C) were incubated with purified recombinant GBV-B or HCV protease. Samples were subjected to SDS-12.5% PAGE, and proteins were detected by autoradiography. Lane M, molecular mass standards; lane 1, substrates incubated with protease buffer; lane 2, substrates incubated with HCV NS3 protease; lane 3, substrates incubated with HCV NS3 protease in the presence of HCV cofactor NS4A; lane 4, substrates incubated with GBV-B NS3 protease; lane 5, substrates incubated with NS3 GBV-B protease in the presence of HCV cofactor NS4A.

FIG. 4. Effect of protease inhibitors on GBV-B protease activity. ^{35}S -labelled HCV precursor protein NS5A-NS5B Δ C51 was incubated with purified GBV-B protease in the presence of different classical protease inhibitors. The extent of cleavage in the presence of the inhibitor was determined by densitometric analysis, and the residual activity was expressed as a percentage. PMSF, phenylmethylsulfonyl fluoride; OP, *ortho*-phenanthroline.

ing to NS5A-NS5B site is more difficult to handle due to its hydrophobicity and tendency to oxidize (24). Proteolytic cleavage was detected by HPLC as described in Materials and Methods. Cleavage of HCV peptide substrates occurred upon incubation with the GBV-B NS3 protease domain, after the P1 cysteine residue, as revealed by mass spectrometry performed on the purified reaction products (data not shown). The kinetic parameters of the enzymatic reaction are shown in Table 1. The specific enzymatic activity on the synthetic heterologous substrates was rather low but was similar to that determined for the HCV NS3 protease under the same experimental conditions (24, 25) and was of the same order of magnitude as that found for other viral proteases (2, 5, 19).

We then investigated the dependence of the proteolysis on the ionic strength of the activity buffer with the NS4A-NS4B peptide. An NaCl titration experiment (Fig. 5) showed that the GBV-B protease is inhibited by increasing the ionic strength. The inhibition was detectable at an NaCl concentration of as low as 50 mM. Interestingly, the inhibition of the enzymatic activity observed upon addition of NaCl was found to be purely competitive, as previously described for the HCV protease (25), indicating that electrostatic interactions may play a major role in enzyme-substrate complex formation.

The sequences of the natural cleavage sites of the GBV-B NS3 protease are not yet known. Based on the results presented in this paper, we expect that they should resemble the sequences cleaved by HCV NS3 within the HCV polyprotein. We thus performed alignments of the complete HCV polyprotein sequence, as well as the individual HCV cleavage site

FIG. 5. Effect of ionic strength on GBV-B protease activity, showing Lineweaver-Burk plot kinetics obtained at NaCl concentrations of 0 (□), 50 (■), 100 (○), and 150 (●) mM. The NS4A-NS4B cleavage site peptide was used as a substrate.

sequences, with the GBV-B polyprotein sequence. Tentative NS3-dependent cleavage sites within the GBV-B polyprotein, similar to those already proposed (14), are displayed in Fig. 6. Although the homology is evident for the NS4A-B site, in the case of the NS4B-5A site a very weak similarity can be found only by introducing a gap. The homology is very poor in terms of both residue identity and presence of specific features known to be important in HCV substrates, such as an acidic residue in the P6 position that is missing in the GBV-B sequence. The putative NS5A-5B site is strictly conserved in the P' portion, but, strikingly, a lysine residue is present at the P6 position. It has been suggested that in the case of the HCV NS3 protease, Arg-161 and Lys-165 could interact with the conserved acidic P6 substrate residue (13). The respective residues in the GBV-B protease are a Gly and a Ser. This could account for the apparent lack of conservation of the P6 residue

NS4A/NS4B

DEMEEC	ASHL	HCV-BK
:	:	
EIVEEC	ASFI	GBV-B

NS4B/NS5A

DCSTPC	S-GSW	HCV-BK
:	: :	
PTEDDC	GLIAW	GBV-B

NS5A/NS5B

EDVVCC	SMSY	HCV-BK
:		
KSEFSC	SMSY	GBV-B

FIG. 6. Putative cleavage sites of GBV-B obtained by sequence alignment of HCV and GBV-B polyproteins. The alignment was obtained by comparing the complete HCV and GBV-B polyproteins by running the FASTA program of the Genetics Computer Group, Madison, Wis. Only the regions corresponding to the P6-P4' residues of HCV NS3 serine protease cleavage sites are shown. The space in the peptide sequences represents the cleavage point. The HCV sequences shown correspond to those for HCV strain BK (accession number M58335). Vertical lines indicate identity; colons show conservative replacements.

TABLE 1. GBV-B and HCV protease activities on HCV peptide substrates^a

Peptide	K_m (μM)		k_{cat} (min^{-1})		k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)	
	GBV-B	HCV	GBV-B	HCV	GBV-B	HCV
NS4A-NS4B	129	100	3.0	0.3	387	47
NS4B-NS5A	>4,000	2,100	ND ^b	0.05	0.8	0.4

^a Data for HCV protease activity are from reference (25).

^b ND, not determined (k_{cat}/K_m could not be measured because K_m exceeded the limits of solubility of the peptide).

in the natural substrates of GBV-B NS3 proteases. An unambiguous identification of GBV-B cleavage sites will require more experimental work on the characterization of in vivo GBV-B polyprotein processing.

In summary, we have expressed the recombinant NS3 serine protease domain of GBV-B in *E. coli*. The purified recombinant enzyme has shown proteolytic activity on polyprotein and peptide substrates containing HCV NS3-dependent cleavage sites, suggesting that at least some features of the substrates are critical for recognition by both the HCV and the GBV-B enzymes.

Polyprotein processing is a crucial step for virus maturation, and the HCV NS3 protease is considered an important target for development of anti-HCV therapeutic agents. Unfortunately, a cell culture system for HCV is still very problematic, and HCV infection is reproducible only in chimpanzees (1, 9). Our results suggest that GBV-B and HCV proteases have overlapping substrate specificities, and they encourage us to believe that GBV-B, a naturally infectious agent for *Saguinus* species, may be proposed as an alternative model for evaluating HCV protease inhibitors in vivo.

ACKNOWLEDGMENTS

We are grateful to R. Cortese, A. Tramontano, C. Steinkühler, and M. Sollazzo for helpful discussions. We thank P. Pucci for mass spectrometry analysis. We also thank S. Acali for peptide synthesis, P. Neuner for oligonucleotide synthesis, M. C. Nardi for help in protein purification, and M. Emili for artwork.

REFERENCES

1. Abe, K., G. Inchauspe, T. Shikata, and A. M. Prince. 1992. Three different patterns of hepatitis C virus infection in chimpanzees. *Hepatology* **15**:690–695.
2. Darke, P. L., E. Chen, D. L. Hall, K. M. Sardana, C. A. Veloski, L. R. Lafemina, J. A. Shafer, and L. C. Kuo. 1994. Purification of active herpes simplex virus-1 protease expressed in *Escherichia coli*. *J. Biol. Chem.* **269**:18708–18711.
3. De Francesco, R., A. Urbani, M. C. Nardi, L. Tomei, C. Steinkühler, and A. Tramontano. 1996. A zinc binding site in viral serine proteinases. *Biochemistry* **35**:13282–13287.
4. Deinhardt, F., A. W. Holmes, R. B. Capps, and H. Popper. 1967. Studies on the transmission of human viral hepatitis to marmoset monkeys. Transmission of disease, serial passages, and description of liver lesion. *J. Exp. Med.* **125**:673–687.
5. Dilanni, C. L., C. Mapelli, D. A. Drier, J. Tsao, S. Natarajan, D. Riexinger, S. M. Festin, M. Bolgar, G. Yamanaka, S. P. Weinheimer, C. A. Meyers, R. J. Colonna, and M. G. Cordingley. 1993. In vitro activity of the herpes virus type 1 protease with peptide substrates. *J. Biol. Chem.* **268**:25449–25454.
6. Failla, C., L. Tomei, and R. De Francesco. 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* **68**:3753–3760.
7. Failla, C., L. Tomei, and R. De Francesco. 1995. An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. *J. Virol.* **69**:1769–1777.
8. Failla, C. M., E. Pizzi, R. De Francesco, and A. Tramontano. 1996. Redesigning the substrate specificity of the hepatitis C virus NS3 protease. *Folding Design* **1**:35–42.
9. Farci, P., T. W. London, D. C. Wong, J. G. Dawson, S. D. Vallari, R. Engle, and R. H. Purcell. 1992. The natural history of infection with hepatitis C virus (HCV) in chimpanzees: comparison of serologic response measured with first- and second-generation assays and relationship to HCV viremia. *J. Infect. Dis.* **165**:1006–1011.
10. Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**:2832–2843.
11. Kim, J. L., K. A. Morgenstern, C. Lin, T. Fox, M. D. Dwyer, J. A. Landro, S. P. Chambers, W. Markland, C. A. Lepre, E. T. O'Malley, S. L. Harbeson, C. M. Rice, M. A. Murcko, P. R. Caron, and J. A. Thomson. 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**:343–355.
12. Lin, C., J. A. Thomson, and C. Rice. 1995. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro. *J. Virol.* **69**:4373–4380.
13. Love, R. A., H. E. Parge, J. A. Wickersham, Z. Hostomsky, N. Habuka, E. W. Moomaw, T. Adachi, and Z. Hostomska. 1996. The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell* **87**:331–342.
14. Muerhoff, A. S., T. P. Leary, J. N. Simons, T. J. Pilot-Matias, G. J. Dawson, J. C. Erker, M. L. Chalmers, G. G. Schlauder, S. M. Desai, and I. K. Mushahwar. 1995. Genomic organization of GB viruses A and B: two new members of the *Flaviviridae* associated with GB agent hepatitis. *J. Virol.* **69**:5621–5630.
15. Ohba, K., M. Mizokami, J. Y. N. Lau, E. Orito, K. Ikeo, and T. Gojobori. 1996. Evolutionary relationship of hepatitis C, pesti-, flavi-, plantiviruses, and newly discovered GB hepatitis agent. *FEBS Lett.* **378**:232–234.
16. Pilot-Matias, T. J., A. S. Muerhoff, J. N. Simons, T. P. Leary, S. L. Buijk, M. L. Chalmers, J. C. Erker, G. J. Dawson, S. M. Desai, and I. K. Mushahwar. 1996. Identification of antigenic regions in the GB hepatitis viruses GBV-A, GBV-B, and GBV-C. *J. Med. Virol.* **48**:329–338.
17. Pizzi, E., A. Tramontano, L. Tomei, N. La Monica, C. Failla, M. Sardana, T. Wood, and R. De Francesco. 1994. Molecular model of the specificity pocket of the hepatitis C virus protease: implication for substrate recognition. *Proc. Natl. Acad. Sci. USA* **91**:888–892.
18. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
19. Sardana, V. V., J. A. Wolfgang, C. A. Veloski, W. J. Long, K. LeGrow, B. Wolansky, E. A. Emini, and R. L. LaFemina. 1994. Peptide substrate cleavage specificity of the human cytomegalovirus protease. *J. Biol. Chem.* **269**:14337–14340.
20. Schlauder, G. G., G. J. Dawson, J. N. Simons, T. J. Pilot-Matias, R. A. Gutierrez, C. A. Heynen, M. F. Knigge, G. S. Kurpiewsky, S. L. Buijk, T. P. Leary, A. S. Muerhoff, S. M. Desai, and I. K. Mushahwar. 1995. Molecular and serologic analysis in the transmission of the GB hepatitis agents. *J. Med. Virol.* **46**:81–90.
21. Schlauder, G. G., T. J. Pilot-Matias, G. S. Gabriel, J. N. Simons, A. S. Muerhoff, G. J. Dawson, and I. K. Mushahwar. 1995. Origin of GB-hepatitis viruses. *Lancet* **346**:447–448.
22. Simons, J. N., T. P. Leary, G. J. Dawson, T. J. Pilot-Matias, A. S. Muerhoff, G. G. Schlauder, S. M. Desai, and I. K. Mushahwar. 1995. Isolation of novel virus-like sequences associated with human hepatitis. *Nat. Med.* **1**:564–569.
23. Simons, J. N., T. J. Pilot-Matias, T. P. Leary, G. J. Dawson, S. M. Desai, G. G. Schlauder, A. Scott Muerhoff, J. C. Erker, S. L. Buijk, M. L. Chalmers, C. L. Van Sant, and I. K. Mushahwar. 1995. Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc. Natl. Acad. Sci. USA* **92**:3401–3405.
24. Steinkühler, C., L. Tomei, and R. De Francesco. 1996. In vitro activity of hepatitis C virus protease NS3 purified from recombinant baculovirus-infected Sf9 cells. *J. Biol. Chem.* **271**:6367–6373.
25. Steinkühler, C., A. Urbani, L. Tomei, G. Biasiol, M. Sardana, E. Bianchi, A. Pessi, and R. De Francesco. 1996. Activity of purified hepatitis C virus protease NS3 on peptide substrates. *J. Virol.* **70**:6694–6700.
26. Stemmer, W. P. C., A. Cramer, K. D. Ha, T. M. Brennan, and H. L. Heyneker. 1995. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxynucleotides. *Gene* **164**:49–53.
27. Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82**:1074–1078.
28. Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama. 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol.* **65**:1105–1113.
29. Tomei, L., C. Failla, E. Santolini, R. De Francesco, and N. La Monica. 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.* **67**:4017–4026.
30. Zuckerman, A. J. 1995. The new GB hepatitis viruses. *Lancet* **345**:1453–1454.