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Hepatitis C NS3 Protease: Restoration of NS4A Cofactor Activity by N-Biotinylation of Mutated NS4A Using Synthetic Peptides

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The NS3 serine protase of Hepatitis C virus (HCV) requires NS4A protein as a cofactor for efficient cleavage at four sites in the nonstructural region. The cofactor activity has been mapped to the central hydrophobic region (aa 22–34) of this 54-amino-acid NS4A protein, and site-directed mutagenesis has identified alternating hydrophobic amino acids, particularly Ile25 and Ile29, as critically important. A double mutant of NS4A cofactor peptide, I25A/I29A, completely abolished the cofactor activity. We now report that the cofactor peptide activity in the I25A/I29A double mutant can be restored specifically by introducing a biotin–aminohexanoic acid fusion at the N-terminus. In addition, a similar N-terminal fusion of biotin–aminohexanoic acid with the wild-type 4A peptide significantly enhanced cofactor activity. Our data corroborate the crystal structure-based hypothesis of hydrophobic interaction between the N-terminus of NS4A and the N-terminal α_0 helix of NS3 protease.

Hepatitis C virus, the major etiological agent of post-transfusion non-A, non-B hepatitis, contains a single-stranded positive-sense RNA genome of about ~9.6 kb which encodes a polyprotein of ~3000 amino acids. The polyprotein is proteolytically processed by host signalase in the structural region and by two HCV encoded proteases in the nonstructural region to produce mature viral proteins. NS2/3, a Zn^{2+}-dependent metalloprotease, is responsible for cleavage at the NS2/3 junction (1, 2). The other four cleavages in the nonstructural region, between NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B, are catalyzed by NS3 protease, a chymotrypsin-like serine protease encoded within the N-terminal portion of the NS3 protein (2, 3). NS4A, a 54-amino-acid protein, is a cofactor for NS3 and is required for efficient cleavage by the protease (4, 5). This cofactor activity has been mapped to a central region of approximately 12 amino acids (aa 22–33) in NS4A, within which the hydrophobic amino acids Ile25 and Ile29 have been identified as determinants of the cofactor activity (6–8). Confirming the importance of Ile25 and Ile29 is the finding that substitutions of these two amino acids by alanine residues destroys the ability of the cofactor peptide to form a complex with the NS3 protease (8). Crystal structures of the NS3/NS4A peptide complex have further elucidated the nature of the interaction of protein and cofactor, revealing that the NS4A cofactor peptide intercalates with the enzyme and becomes an integral part of the enzyme core (9, 10). No structure has been published, however, for NS3 complexed with the entire 54 amino acid NS4A protein.

During our investigation of the NS3/NS4A complex formation, we made the observation that an N-terminally fused biotin-aminohexanoic acid moiety restored the cofactor activity of the mutant NS4A peptide. In this report we describe the novel role of this biotin moiety in the NS4A peptide-mediated cofactor activity. In addition to reactivating the mutant peptide, a similar N-terminal fusion of biotin to the wild-type peptide cofactor significantly enhanced cofactor activity.

MATERIALS AND METHODS

Expression and purification of NS3 protease. Cloning of NS3 protease H77 strain catalytic domain with a T7 tag at the N-terminus and a polyhistidine (His-) tag at the C-terminus has been described previously, as has the expression and purification of this construct (9). Purified protease was characterized using electrospray ionization mass spectrometry (ESI-MS), N-terminal sequence analysis and SDS–polyacrylamide gel electrophoresis. Purity was determined to be approximately 95% based on amino acid sequence analysis.

Cloning and expression of in vitro translated substrate. The cloning of HCV NS4A/NS4B polyprotein substrate (pNB NAE) into cell-

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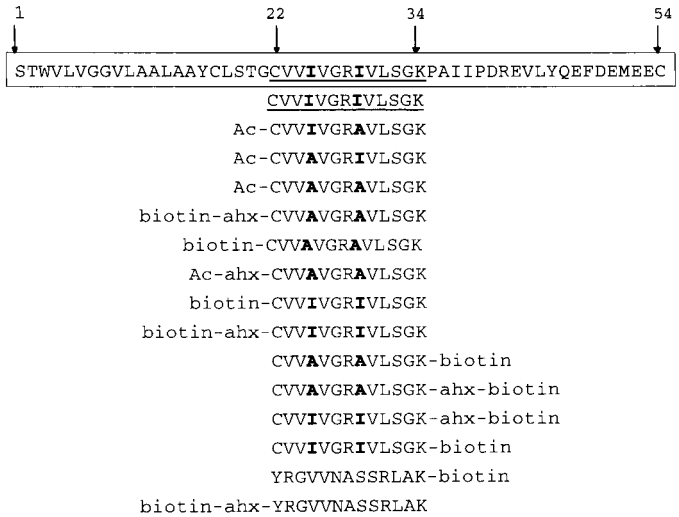

FIG. 1. Sequence of NS4A peptides used in these studies. The cofactor region (aa 22–34) derived from HCV H77 isolate (HCV 1a) is underlined. Amino acid residues 25 and 29, which are critical for the NS4A cofactor activity, are shown in bold type. The last two sequences in the series are non-4A peptides. For comparison, the entire 54-amino-acid sequence of NS4A (HCV 1a) is shown in the top box. The following terminology has been used: Ac, *N*-acetyl; ahx, 6-aminohexanoyl; K-biotin, Lys [N^{ϵ}-biotinyl]; K-ahx-biotin, Lys [N^{ϵ}-(N^{6}-biotinyl)aminohexanoyl].

free expression plasmid pBD7 has been previously described (8). The *in vitro* transcribed RNA produced from the plasmid DNA was translated in rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine (Amersham) according to the manufacturer's recommendation.

Peptide synthesis. Peptides containing NS4A sequence and its derivatives were synthesized in house and by AnaSpec (San Jose, CA). The peptides were cleaved off the resin and deprotected following a standard TFA cleavage protocol. Peptides were purified to >95% purity on reverse phase HPLC and confirmed by mass spectrometry analysis.

NS3 protease translation assay and analysis. HCV protease translation assays were initiated with the addition of 2 μl [^{35}S]-labeled translated substrate to a reaction mix of 20 μl containing 2.5 nM purified protease, 100 μM NS4A peptide, 50 mM Tris, pH 7.5, 0.3 M NaCl, 5 mM DTT, 0.1% NP-40, and 10% glycerol, followed by a 30 min incubation at 30°C. Reactions were stopped by the addition of an equal volume of 2× Laemmli sample buffer and boiling for 3 min. Samples were analyzed by SDS–15% PAGE and autoradiography. Data was quantitated and compared by volumetric integration using a PhosphorImager and ImageQuant software (Molecular Dynamics).

RESULTS AND DISCUSSION

For these studies we used an HCV polyprotein substrate containing the NS4A/4B cleavage site produced by cell-free transcription-translation systems. This substrate, designated Δ4A/Δ4B, contains amino acids 36 through 54 of NS4A and the N-terminal 192 amino acids of NS4B. It does not contain the NS4A cofactor sequence (aa 22–33), and, as we have previously shown (8), cleavage of this polypeptide is strictly dependent on the addition of NS4A cofactor peptide. Various NS4A cofactor peptides used in these studies are shown in Fig. 1. Our experiments utilized the HCV protease N-terminal catalytic domain (181 amino acids) to study the role of various NS4 cofactor peptides on NS3-mediated *trans*-cleavage activity. Cloning, expression, and purification of the protease has been previously described (9). We first examined the effect of alanine substitutions at Ile25 and Ile29 in the 13-mer peptide from the cofactor region (aa 22–34). An alanine substitution at Ile25 showed a reduced cofactor activity to about 40% of wildtype levels (see Fig. 2, lane 5). A similar mutation at Ile29 had a more dramatic effect, reducing activity to only 8% of wildtype, while a double mutant containing both of these substitutions showed a similar profile (lanes 4 and 6, respectively). When we looked at a peptide containing the biotin-aminohexanoyl (ahx) moiety fused N-terminal to this double mutant cofactor peptide, we observed an almost complete restoration of activity to control levels (lane 7). This reactivation was specific to the NS4A cofactor sequence, since fusion of biotin-ahx to a non-4A sequence (biotin-ahx-YRGVVNASSRLAK in lane 8) showed no cofactor activity. We next examined the effects of modifying this N-terminal fusion. Only biotin with the ahx spacer fused to NS4A mutant cofactor peptide showed restoration of cofactor activity (Fig. 3A, lane 5). Neither biotin nor ahx alone fused to the 13-mer mutant cofactor (lanes 6 and 7, respectively), restored activity. Biotin itself, either with or without the mutant NS4A

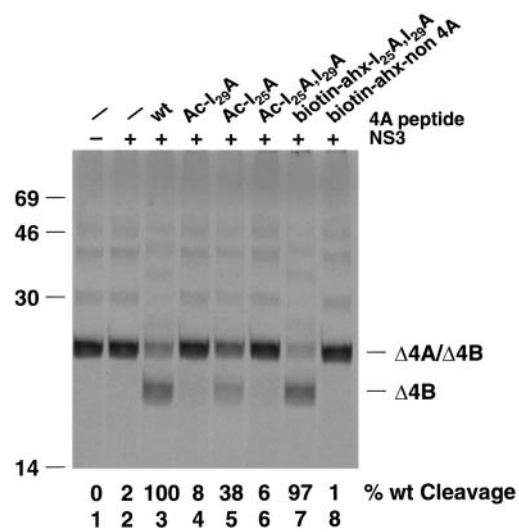

FIG. 2. HCV NS3 protease activity abolished by NS4A I25A and I29A mutations can be restored by introducing a biotin–aminohexanoic acid (ahx) fusion at the N-terminus of 4A peptide. *trans*-cleavage reaction mixtures containing [^{35}S]-labeled substrate designated Δ4A/Δ4B was mixed with 20 nM purified protease in the presence of 100 μM synthetic peptides from either wildtype NS4A or alanine-substituted NS4A derived from the 13-mer peptide (aa 22–34). Modification of the wildtype peptide tested is shown at the top of each lane and this corresponds to the sequence identification described in the legend to Fig. 1.

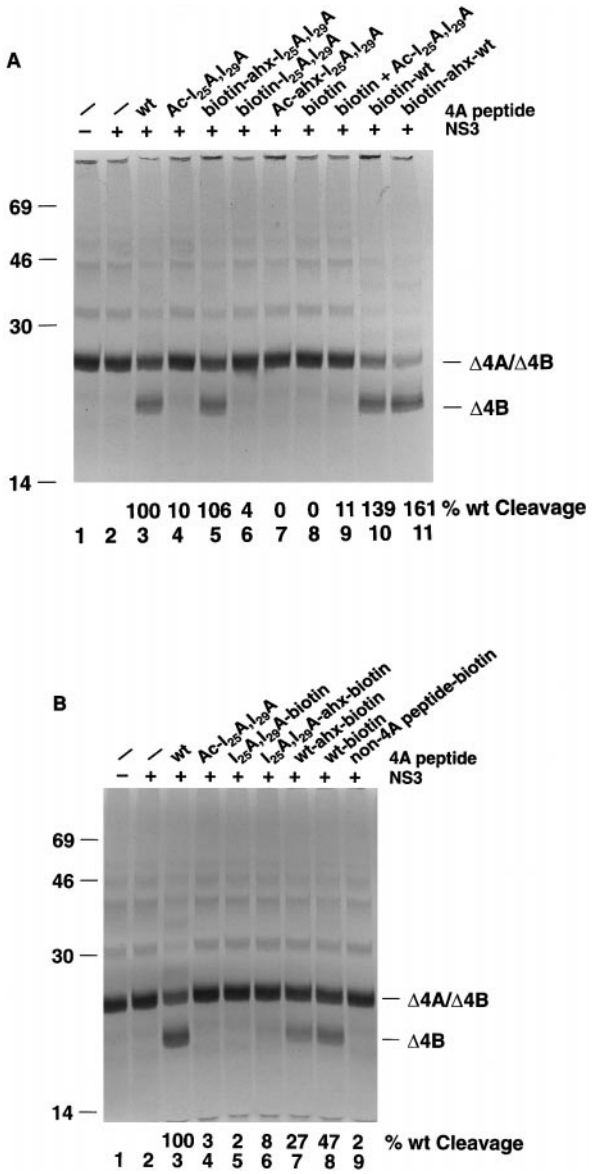

FIG. 3. (A) Reactivation of I25A/I29A mutant NS4A and enhancement of wildtype cofactor peptide is specific to biotin–ahx fusion at the N-terminus. (B) Effects of C-terminal biotin–ahx fusions on I25A/I29A double mutant 4A peptide and on the wildtype 4A cofactor peptide. Experimental conditions are as described in the legend to Fig. 2. The NS4A cofactor peptide tested is shown at the top of each lane and this corresponds to the sequence identification described in the legend to Fig. 1.

cofactor, had no cofactor activity (see lanes 8 and 9). These observations led us to investigate the effect of N-terminal fusion of biotin-ahx to the wildtype cofactor sequence. Interestingly, this fusion enhanced the cofactor activity over wildtype control by 160% (lane 11). Biotin fused alone also showed some enhancement (lane 10), with nearly 140% of control cleavage activity.

These data, showing enhancement of mutant and wildtype cofactor activity, seem to indicate a favorite interaction between biotin, with an appropriate ahx spacer fused specifically at the N-terminus of NS4A cofactor peptide, and the NS3 protease domain. Historically the protein-binding properties of biotin have been well-studied (14–17). X-ray structures of the biotin-binding proteins avidin and streptavidin, for example, indicate an array of hydrophobic and polar interactions resulting in extremely tight and specific binding of biotin and protein. The bicyclic ring system of the biotin molecule is lured by a hydrophobic region formed by aromatic residues in these proteins (18). In the NS3/4A complex, crystal structure studies show that a hydrophobic patch is created on the surface by hydrophobic amino acids on the α_0 helix of NS3 located at the N-terminus (Fig. 4A). The α_0 helix itself is stabilized by the local hydrophobic environment, which in one way can be supplied by crystal packing (10). *In vivo,* the hydrophobic stability observed in the crystal structures (9, 10) could mirror the stabilization induced by the membrane-bound hydrophobic N-terminus of NS4A protein in the native protein-to-protein interactions of NS3 and NS4A. Such a functional role for the N-terminal 20 amino acids of NS4A is suggested by Roth *et al.* through their prediction that the N-terminal 20 amino acids of NS4A forms a hydrophobic *trans*-membrane helix (16), and this is further supported by data which suggest that the NS4A protein itself is membrane-associated (2, 17). One aspect of NS4A function may be to serve as an anchor to recruit NS3 and other nonstructural proteins to the membranes of the endoplasmic reticulum (ER). More recently, this idea has been reinforced by the findings of Moradpour *et al.* who demonstrated co-localization of C, E1, E2, NS3, NS4, and NS5A with the ER in a human cell line inducibly expressing the entire HCV open reading frame (18). It seems likely that the specific N-terminal fusion of biotin and ahx spacer to NS4A cofactor peptide mimics a similar hydrophobic environment which stabilizes the NS3/4A complex. Confirmation of this stabilization by comparing such a fusion to the native NS4A N-terminal sequence fused to the cofactor peptide is technically difficult because of solubility and aggregation issues at the concentrations necessary to saturate the system. It should be noted that the K_d for the complex formed between a catalytic domain of NS3 protease and NS4A cofactor peptide is in the low micromolar range, while that of NS3 and full-length NS4A protein is more than a thousand fold less (19–21). A proposed model for ahx-biotin-interaction with NS3 is shown in Fig. 4. Here ahx, covalently attached to the aliphatic side chain of biotin, is shown fused to the N-terminus of the NS4A cofactor peptide (aa 22–34). Although alternative interaction sites in the NS3 protease cannot be ruled out, this effectively positions the bicyclic ring of biotin to the hydrophobic surface area of the NS3 protease N-terminal region, potentially promoting stabilization of the α_0 helix. Such a model was based on crystal structures

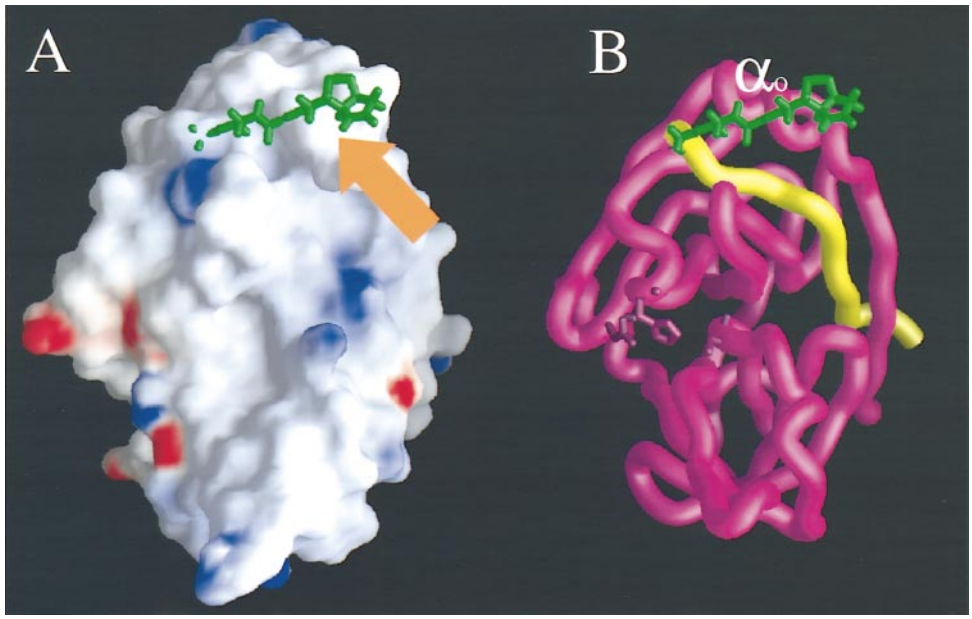

FIG. 4. (A) Surface representation of the crystal structure of HCV protease domain showing the color-coded electrostatic potential distributions on the protease surface. Color-coding is as follows: blue, positive; red, negative; white, neutral and hydrophobic. The *N*-(biotinyl)-aminohexanoyl group is shown in green covalently fused to the NS4A cofactor peptide. An arrow indicates the N-terminal hydrophobic region of NS3 protease domain. (B) Ribbon representation of the NS3 protease domain. NS3 protease is shown in magenta, while the intercalating NS4A cofactor peptide (aa 22–34) is shown in yellow. Stick figures in magenta position the catalytic triad of His-57, Asp-81, and Ser-139 in NS3 protease. The *N*-(biotinyl) aminohexanoic acid is again shown in green, fused to the N-terminal cysteine of NS4A peptide as described in A. The α-helix (α_0) is indicated at the top of the figure.

of HCV NS3 protease/NS4A cofactor complexes (9, 10), as well as recent modeling predictions proposed for NS3 and NS4A protein (22). Interestingly, if the ahx moiety is removed, leaving biotin fused directly to N-terminal NA4A cofactor peptide, the bicyclic ring has limited access to the hydrophobic domains of NS3. Such limited access could explain failure of N-terminal biotin alone to reactivate the I25A/I29A mutant cofactor peptide (Fig. 3A, lane 6).

An additional piece of evidence pointing to a potential structure-stabilizing role for the biotin-ahx fusion moiety would be the site specificity. We therefore constructed fusions at the C-terminus of both the I25A/I29A mutant and the wildtype cofactor peptides. As shown in Fig. 3B (lanes 5 and 6), C-terminal fusions to the double mutant of either biotin alone or biotin-ahx showed virtually no restorative effect on cofactor activity. Also, as expected, biotin fused C-terminal to a non-4A peptide (YRGVVNASSRLAK-biotin, lane 9) had no cofactor activity. Looking at effects on the wildtype cofactor peptide, C-terminal fusion of biotin alone resulted in an inhibition of activity, with about half the control cleavage levels (lane 8), while an ahx-biotin fusion resulted in an even more dramatic reduction to only 27% of the control (lane 7). This is in direct contrast to the enhancing effects observed with the N-terminal fusions of these moieties. Structural predictions show that such a C-terminal fusion would put the ahx-biotin moiety in close proximity to the P3′ and P4′ amino acids in the substrate binding pocket, perhaps explaining the negative impact of this fusion.

In summary, we have reengineered an NS4A cofactor peptide, by fusing biotin with ahx spacer to the N-terminus of NS4A aa 22–34 resulting in enhanced cofactor activity. This enhanced activity is demonstrated by our observations of reactivation of mutant cofactor peptide disabled by alanine substitutions for critical hydrophobic isoleucine residues, as well as enhancement of native (wild type) cofactor peptide activity. The results point to the biological importance of the NS4A N-terminus, in fully potentiating NS3 enzymatic activity.

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