Molecular Determinants of TRIF Proteolysis Mediated by the Hepatitis C Virus NS3/4A Protease*

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Persistent infections with hepatitis C virus (HCV) are a major cause of liver disease and reflect its ability to disrupt virus-induced signaling pathways activating cellular antiviral defenses. HCV evasion of double-stranded RNA signaling through Toll-like receptor 3 is mediated by the viral protease NS3/4A, which directs proteolysis of its proline-rich adaptor protein, Toll-IL-1 receptor domain containing adaptor-inducing interferon-β (TRIF). The TRIF cleavage site has remarkable homology with the viral NS4B/5A substrate, although an 8-residue polyproline track extends upstream from the Phe position in lieu of the acidic residue present in viral substrates. Circular dichroism (CD) spectroscopy confirmed that a substantial fraction of TRIF exists as polyproline II helices, and inclusion of the polyproline track increased affinity of P side TRIF peptides for the HCV-BK protease. A polyproline II peptide representing an SH3 binding motif (PPPPRRR, Sos) bound NS3 with moderate affinity, resulting in inhibition of proteolytic activity. Chemical shift perturbations in NMR spectra indicated that Sos binds a 3_10 helix close to the protease active site. Thus, a polyproline II interaction with the 3_10 helix likely facilitates NS3/4A recognition of TRIF, indicating a significant difference from NS3/4A recognition of viral substrates. Because SH3 binding motifs are also present in NS5A, a viral protein that interacts with NS3, we speculate that the NS3 3_10 helix may be a site of interaction with other viral proteins.

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis C, a globally distributed infection that affects more than 170 million persons worldwide and results in 8,000–10,000 deaths from liver disease annually in the United States alone (1, 2). Currently available therapeutic regimens include combination therapy with interferon and ribavirin, but these are limited in efficacy, frequently associated with adverse reactions, and costly (3). Thus, there is a compelling need for new antiviral drugs possessing greater efficacy against this virus.

HCV is a member of the family Flaviviridae, classified within the genus Hepacivirus. It has a relatively small, 9.7-kb positive-strand RNA genome, which contains a large open reading frame that spans most of the genomic RNA (4). Translation of genomic RNA results in the expression of a lengthy polypeptide that is co- and post-translationally processed into at least 10 functional proteins by both host and viral protease activities. The processing events that liberate the nonstructural HCV proteins required for viral RNA replication (NS3, NS4A, NS4B, NS5A, and NS5B) are directed either in cis or in trans by a serine protease formed by the noncovalent association of NS3 with a segment of NS4A (5, 6). Given the clinical success of inhibitors of the human immunodeficiency virus protease (7), the HCV NS3/4A protease has become a leading target for drug discovery efforts. Candidate NS3/4A protease inhibitors have entered clinical trials and have shown substantial promise (8). However, highly active NS3/4A inhibitors have proved to be quite challenging to develop because the active site of the viral protease is unusually featureless (9–11). The shallow contour and solvent-exposed nature of the NS3/4A substrate binding site lacks the pockets or crevices that would facilitate design of small molecule inhibitors with high affinity and binding specificity.

In addition to its critical role in processing the viral proteins that comprise the viral RNA replica, the NS3/4A protease disrupts innate intracellular antiviral defenses by blocking virus activation of interferon regulatory factor 3 (IRF-3) and NF-κB (12, 13). These are cellular transcription factors that induce the expression of a large number of cellular antiviral defense genes, including the type 1 α/β interferons and interferon-stimulated genes, as well as chemokines and proinflammatory cytokines (14, 15). Recent work indicates that the products of viral replication may lead to IRF-3 and NF-κB activation through two distinct and independent pathways, one involving engagement of Toll-like receptor 3 (TLR3) within endocytic vesicles by viral double-stranded RNA, and the other involving recognition of intracellular structured viral RNAs by the cellular DEkH/V RNA helicase, retinoic acid-inducible gene I (16, 17). Both pathways are specifically disrupted by the protease activity of the NS3/4A complex (13, 18). This disruption of antiviral signaling can be reversed by specific peptidomimetic inhibitors of the NS3/4A protease, suggesting that it targets for proteolysis one or more proteins involved in these signaling pathways. Although the cellular protein that resides within the retinoic acid-inducible gene I pathway and which is...
putatively cleaved by NS3/4A has yet to be identified, we have recently demonstrated that NS3/4A targets an essential protein within the TLR3 pathway for proteolysis: Toll-IL-1 receptor domain containing adaptor-inducing interferon-β (TRIF or TICAM-1) (13). TRIF is an essential adaptor protein that links TLR3 to downstream activation of IRF-3 and NF-κB (19, 20), and its cleavage by a viral protease would be expected to disrupt double-stranded RNA-induced signal transduction through the TLR3 pathway. Consistent with this, we have shown that NS3/4A blocks the activation of the interferon-β promoter normally induced by exposure to extracellular poly(I:C), a synthetic double-stranded RNA analog, both in osteosarcoma cells that conditionally express the protease and in HeLa cells supporting ongoing replication of subgenomic HCV RNA (13).

TRIF also supports MyD88-independent signaling after the engagement of TLR4 by pathogen-specific ligands (21). It is thought to interact with the TLRs through a Toll-IL-1 receptor (TIR) homology domain and to recruit multiple molecular signaling partners through specific domains in its N-terminal and C-terminal sequences (22). The signaling pathways it activates play critical roles in the response of cells to virus infection, and the ability of HCV to disrupt this signaling is likely to contribute substantially to its capacity for sustaining persistent infections in the face of both innate and adaptive immune responses (13). TRIF is cleaved proteolytically by the HCV protease between its Cys-372 and Ser-373 residues (13), effectively separating the TIR domain of the protein from an N-terminal TANK-binding kinase 1 interaction site required for IRF-3 phosphorylation, which is a prerequisite for dimerization, nuclear translocation, and activation of IRF-3 as a transcriptional factor (23).

Here, we describe studies of the molecular properties of TRIF which contribute to its ability to function as a substrate for the NS3/4A protease. We demonstrate that the amino acid residues of the protease which interact with TRIF to facilitate its proteolysis differ significantly from those interacting with the canonical viral substrates. The TRIF cleavage site lacks a conserved P3 acidic residue that has been shown in several studies to make a substantial contribution to viral substrate specificity and binding affinity (24, 25). This is replaced in TRIF with an 8-residue polyproline track for which we demonstrate a unique role in interactions with the protease.

**EXPERIMENTAL PROCEDURES**

**Peptides**—Synthetic peptides representing sequences spanning the NS3/4A cleavage sites at the viral NS5A/5B (E-HDVVoC/SMSSY-OH) (26), NS4A/4B (E-EFDMMEEC/ASHLPY-OH), and NS4B/5A (E-HECTP/SO6WLRD-OH) junctions, the NS3/4A cleavage site within TRIF (Ac-PSSTPC/SAHL-amide, Ac-PPPPPPPSSSTTPC/SAHL-amide) (13), their P side cleavage products (Ac-PSSTPC-OH, and Ac-PPPPPPP-SSSTPC-OH), and an HCV peptide suitable for use in a fluorescence resonance energy transfer (FRET) assay (Ac-EDANS/EDEAbuq [COO]ASK-[DABCYL]-am) (27) were purchased from AnaSpec and were >95% pure. SoS (Ac-PPVVPPRRR-amide) and SoY (Ac-PPVVPPR-RRKY-amide) (28) peptides were synthesized within the UTMB Peptide Synthesis Core Laboratory and kindly provided by Dr. Vincent Hilser with >95% purity. All peptides, except for SoY, were quantified by amino acid analysis. The concentration of SoS was measured by absorbance at 280 nm using Edelchow reagent (29).

**Protein Overexpression and Purification**—TRIF was cloned as a His tag fusion protein into pET21d vector (Novagen) and recovered from *Escherichia coli* grown in a 20-liter fermenter as follows. A single colony of *E. coli*, carrying the expression plasmid, was inoculated into a small flask (37 °C, overnight) and then transferred into 20 liters of LB broth supplemented with 100 μg/ml ampicillin. Cells were grown at 37 °C until the A600 reached 0.6–0.8, then induced by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside followed by further incubation at 37 °C for 4–5 h. Cells were harvested and stored at −80 °C prior to purification.

Immunoblots suggested that almost all of the expressed TRIF was present in inclusion bodies; therefore purification was carried under denaturing conditions. The cell pellet was dissolved in extraction buffer (6 M guanidine HCl, 100 mM sodium phosphate, 10 mM Tris, 2 mM β-mercaptoethanol, pH 8.5) supplemented with 50 μl of buffer/50 mg of cell pellet) for 24 h at 4 °C, with stirring. Cell debris was removed by ultracentrifugation, and the supernatant passed through a Ni²⁺-affinity fast protein liquid chromatography column. The column was rinsed with 10–15 volumes of wash buffer (8 M urea, 100 mM sodium phosphate, 10 mM Tris, 2 mM β-mercaptoethanol, pH 7.5) containing 50 mM imidazole. Bound proteins were eluted with wash buffer containing 500 mM imidazole. The initial fractions contained TRIF and other low molecular mass contaminants, whereas the final fractions contained mostly TRIF (>90% purity). Fractions with impurities were subjected to size exclusion chromatography (Superdex 200). TRIF was refolded by dialysis against 20 mM Tris, 2 mM DTT, pH 7.5, with the signature of the folded protein monitored by fluorescence and CD spectroscopy. From a 100-liter large scale purification, ~50 mg was obtained with purity of ~90%. The majority of the contaminants appeared to be TRIF fragments or degradation products. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (UTMB Protein Facility) confirmed that the molecular mass was ~77.2 kDa.

The HCV NS3 single-chain protease (scNS3) and a full-length single-chain NS3 (FL scNS3) containing the helicase domain were kindly provided by Bruce Malcolm (Schering-Plough Research Institute); both were derived from the BK strain of HCV (30).

**CD Spectroscopy**—Far-ultraviolet CD spectra of TRIF were recorded from 200 to 260 nm using an AVIV™ CD spectrometer with the temperature maintained at 25 °C and the bandwidth set at 1 nm with a 0.5-s averaging time. The protein concentration was ~0.4 mg/ml in 20 mM Tris, 2 mM DTT, pH 7.5. Buffer spectra were collected and subtracted from the protein spectra.

**Comparative Kinetic Analyses of NS3 Protease Activity with Different Viral and TRIF Substrates**—In vitro cleavage of the different peptide substrates (5A/5B, 4A/4B, 4B/5A, TRIF p372, and HCV-FRET) was monitored using an HPLC Shimadzu™ chromatograph, equipped with UV-visible detection. For the 5A/5B, 4A/4B, 4B/5A substrates, the peptide fragments were monitored at a 280 nm wavelength, versus 220 nm for the TRIF p372 substrate at 220 and 512 nm for the FRET peptide. The reaction mix consisted of varying concentrations of the peptide substrate and enzyme (scNS3, BK strain) in 25 mM Tris, 300 mM NaCl, 5 μM EDTA, 10% glycerol, 0.05% n-dodecyl-β-D-maltoside, 10 mM DTT, pH 7.5. Enzyme concentrations were 4.2 nM for the 5A/5B substrate, 85.7 nM for 4A/4B, 1 μM for 4B/5A, and 3.3 μM for TRIF p372 substrate, with substrate concentrations ranging from 15 to 70 μM for 5A/5B, 70 to 700 μM for 4A/4B, 100 to 1,800 μM for 4B/5A, and 100 to 3,500 μM for the TRIF p372 substrate. For most of the peptide substrates, such as 4B/5A and TRIF p372, the final peptide concentrations did not exceed the K_m because of poor solubility. Nonetheless, enzyme concentrations generally were at least 100-fold less than substrate concentrations. Preliminary experiments ensured linearity in the initial velocity under these conditions and demonstrated that the enzyme concentration was directly proportional to the initial velocity. Reactions were incubated at 30 °C for a period of time sufficient to achieve ~20% cleavage, then quenched with 1% trifluoroacetic acid. The products were analyzed by HPLC, with the fragments separated using a 10–50% acetonitrile gradient at 2.5 ml/min. Areas of product peaks were integrated and quantitated with calibration standards. Kinetic parameters (k_cat and K_m) were obtained by nonlinear fitting of the data (initial rates versus substrate concentration) with the Michaelis-Menten equation.

\[
V_i = \frac{k_{\text{cat}}[E][S]}{K_m + [S]} + K_m
\]

(Eq. 1)

where \(V_i\) is the initial velocity, \(K_m\) is the total enzyme concentration, \(S\) is the substrate concentration, \(k_{\text{cat}}\) is the catalytic turnover, and \(K_m\) is the Michaelis constant.

**Full-length TRIF Cleavage by Full-length NS3 and scNS3**—For proteolytic cleavage of TRIF by the full-length NS3 (both protease and helicase domains), reactions contained 4 μM TRIF (20 mM Tris, 30 mM DTT, pH 7.5) and 2 μM NS3 or scNS3 (BK strain), with or without 10 μM SCH6, a ketoamide, peptidomimetic NS3/4A protease inhibitor (12). Reactions were incubated at 30 °C for 2 h.

**Estimated EC_50 for scNS3 Cleavage of Full-length TRIF**—To determine the enzyme concentration required to hydrolyze 50% of the full-length TRIF (EC_50), different reaction tubes were prepared with 4 μM TRIF and varying scNS3 concentrations (0.25, 0.5, 1, 2, 3, 5 μM) in 20 mM Tris, 30 mM DTT, pH 7.5. Reactions were incubated at 30 °C for 30 min, quenched with gel loading buffer, and frozen at −20 °C prior to SDS-PAGE analysis. The quantity of the remaining full-length TRIF was estimated by guest on July 23, 2018http://www.jbc.org/Downloaded from
species present in each reaction mix was estimated by densitometric analysis. The data (TRIF intensity versus enzyme concentration) was fitted in a nonlinear fashion to the following simplified four-parameter logistic function (27) derived from the Hill equation,

\[ y = \frac{100(1 + ((E/E_{50})^n))}{1 + ((E/E_{50})^n)} \]  

(Eq. 2)

where \( y \) is the substrate intensity (TRIF), normalized to 100% for no cleavage, \( E \) is the enzyme concentration, and \( n \) is the Hill slope.

Estimated IC_{50} for scNS3 Cleavage of the TRIF p372 Peptide—Reaction mixtures contained 4 \( \mu \)M TRIF p372 and varying scNS3 concentrations: 0, 1, 2, 5, 8.3, and 16.7 \( \mu \)M. The buffer conditions were identical to those for the full-length TRIF cleavage assay. The reactions were incubated at 30 °C for 30 min and quenched with an equal volume of 1% trifluoroacetic acid, prior to HPLC analysis. The decrease in the quantity of p372 was plotted against the enzyme concentration and fitted to Equation 2.

Fluorescence Spectroscopy—Binding of the scNS3 protease to the Sos peptide, and a short and long TRIF peptide (Ac-PSSTPC-OH and Ac-PPPPPPPPSSTPC-OH, respectively) were followed by fluorescence spectroscopy (SPEX FluoroMax™ spectrofluorometer). The excitation and emission wavelengths were fixed at 275 and 340 nm, respectively, to monitor tyrosine and tryptophan fluorescence changes. The observed decreases in the fluorescence intensity signal that occurred upon ligand binding were fitted to a single-site binding model equation,

\[ y = A(1 - \frac{1}{1 + ((E/E_{50})^n)}) \]  

(Eq. 3)

where \( y \) is the relative fluorescence intensity, \( x \) is the ligand concentration, \( A \) is the maximum fluorescence intensity, and \( K_0 \) is the dissociation constant. The experiments were performed at 25 °C and in 75 mM potassium phosphate buffer, 10 mM DTT, pH 6.5.

IC_{50} Determination of Sos Peptide Inhibition of NS3/4A Protease Activity—Protease activity was assayed using the FRET-based HCV peptide as substrate, monitored by HPLC at 512 nm. The concentration of the SosY peptide necessary to inhibit 50% of the reaction (IC_{50}) was determined from the decrease in the initial velocity of the reaction observed with increasing concentrations of the SosY peptide, fitted to the equation

\[ y = A(1 - \frac{1}{1 + ((E/E_{50})^n)}) \]  

(Eq. 4)

where \( y \) is the initial velocity, \( A \) is the maximum initial velocity, \( I \) is the inhibitor concentration, and \( n \) is the Hill slope. The buffer conditions were 50 mM Tris, 50% glycerol, 2% CHAPS, 30 mM DTT, pH 7.5.

NMR Spectroscopy—NMR spectra were collected at 25 °C using a Varian UnityPlus 750 MHz instrument equipped with a triple resonance probe and a pulsed field gradient. Sensitivity-enhanced 1H-15N HSQC spectra were recorded with identical acquisition parameters (15N-labeled scNS3 protein (75 mM potassium phosphate, 5% glycerol, 25 mM DTT, 0.015% NaN_3, 10% D_2O, pH 6.5) with different Sos peptide concentrations (0, 0.3, 1.3, 2.2 mM final). Data were processed and visualized on an SGI work station using Felix 98.0 software. To increase spectral resolution, time domain data were zero-filled twice, and 90° phase-shifted sine bell apodization functions were applied in both dimensions. The protein and chemical shift assignments (31) were kindly provided by Bruce Malcolm (Schering Plough Research Institute).

Visualizations—The Molmol program (32) was used to generate ribbon models of the NS3 protease (PDB entry 1NS3) (see Fig. 7) and the ball-and-stick model of the Sos peptide (see Fig. 4B), adopted from the co-crystal structure of the SEM-5 C-terminal SH3 domain with the Sos peptide (PDB entry 1SEM, chain C) (33).

RESULTS

TRIF Is a Novel Host Substrate for the HCV NS3/4A Protease—NS3 is a bifunctional protein, with a serine protease domain located within its N-terminal third, and ATPase/RNA helicase activity located within its C-terminal two-thirds (34). The NS3/4A protease is responsible for directing cleavage within the HCV polyprotein at the NS3/4A, 4A/4B, 4B/5A, and 5A/5B junctions. NS4A remains noncovalently associated with NS3 after scission at the NS3–4A junction, and it is a necessary cofactor for the full expression of NS3 protease activity (6, 35, 36). In the studies presented here, we used a single-chain protease, scNS3, in which residues 21–32 of NS4A are fused to the N terminus of the protease domain of NS3 (30, 37). This construction allows the NS4A cofactor peptide sequence to fold properly to form a mature NS3/4A protease possessing activity that is very similar if not identical to that of the noncovalent NS3/4A complex. In previous demonstrations of TRIF cleavage by NS3/4A, we used both an scNS3 protein and a bacterially expressed product representing the NS3 protease domain complexed with an NS4A peptide cofactor, both derived from the HCV-N strain of HCV (13). In the experiments described here, we used proteases expressed from the HCV-BK strain because we encountered less aggregation at higher concentrations of these proteins. Use of a protease from an additional HCV strain also allowed us to demonstrate that TRIF hydrolysis is not limited to a single strain of HCV.

Given the bifunctional nature of NS3/4A, we first set out to establish whether there is any difference between the ability of a scNS3 protein representing only the protease domain of NS3 and a full-length scNS3 (FL scNS3) containing both the protease and helicase domains, to cleave TRIF. As shown in Fig. 1A, purified bacterially expressed TRIF was cleaved at the same position by both scNS3 (protease domain only) and the FL scNS3 (complete NS3 protein containing both protease and helicase domains, lanes 2 and 6, respectively). These reactions yielded two fragments, Δ C340 and Δ N372, representing the N-terminal (P side) and C-terminal (P’ side) fragments, respectively (Fig. 1A) (13). The addition of SCH6, a specific peptidomimetic ketoamide inhibitor of the HCV protease, abolished the cleavage (Fig. 1A, lanes 3 and 7). These results confirm that TRIF proteolysis is mediated by the NS3/4A protease, as shown before (13), regardless of the strain or type of construct.

To characterize further TRIF proteolysis by the viral prote-
ase, a 10-residue peptide substrate (spanning residues P$_6$–P$_4$) was designed based on the amino acid sequence of the viral cleavage site (TRIF p372, PSSTPC/SAHL). This length is the minimum generally required for efficient NS3/4A proteolysis of viral substrates and includes the acidic residue conserved at the P$_6$ position of the viral polyprotein trans-cleavage substrate sites. This acidic residue contributes to catalytic efficiency through electrostatic interactions with the protease (24, 25). On the other hand, longer peptides representing the viral substrate generally do not show appreciably greater substrate activity. Fig. 1B shows a three-dimensional representation of an HPLC chromatogram of the peptide cleavage reaction where the substrate 10-mer peptide (TRIF p372, PSSTPC/SAHL) decreases in abundance in concert with increases in the peptide products as a function of reaction time. The products increase in a hyperbolic fashion characteristic of a Michaelis-Menten enzymatic reaction.

**Kinetics of Proteolysis of TRIF versus Viral Substrates**—The sequence of TRIF p372 on the P side of the Cys-Ser cleavage site is quite similar to that at the NS4B/5A junction, except for the notable difference in the P$_6$ position which is a conserved acidic residue in the viral substrate, as noted above (Table I, bold) (13). The role of these conserved, negatively charged residues in the viral cleavage sites has been investigated by Koch et al. (24), who have shown that peptides representing the NS5A/5B cleavage site with substitutions resulting in neutral charges at the P$_6$ and P$_5$ position possess as much as a 55-fold difference in $K_m$ while exhibiting similar $k_{cat}$ values. The P’ side of TRIF p372 is quite similar to the NS4A/4B site, except that the Ser and Ala residues at the P’1 and P’2 positions are reversed (Table I).

We compared the differences in proteolysis between the TRIF peptide and peptide substrates based on the viral cleavage sequences using the HCV-BK scNS3 protease in HPLC-based cleavage assays (for details, see “Experimental Procedures”). Table II summarizes the kinetic parameters for viral substrates based on the NS5A/5B, NS4A/4B, NS4B/5A junctions within the viral polyprotein (Table I), as well as for the cellular substrate TRIF p372 peptide. The affinity of the protease for the TRIF p372 peptide was relatively weak ($K_m = 10$ mM), with a $k_{cat}$ of $14.5$ min$^{-1}$ and $k_{cat}/K_m$ of $23$ m$^{-1}$ s$^{-1}$ (Table II). Proteolysis of the TRIF p372 peptide proceeded more slowly than with the viral substrate peptides, with the following rank order for cleavage efficiency: NS5A/5B > NS4A/4B > NS4B/5A > TRIF p372 (see Table II). The rank order of the viral substrates was similar to that observed in previous studies (10). From Table II, it can be seen that the difference in proteolysis of the TRIF p372 and viral substrates, 4A/4B and 4B/5A, is due largely to differences in $K_m$ (typically interpreted as the affinity for the substrate). The $k_{cat}$ values were comparable and within the range of error in the assay. Nonetheless, the $k_{cat}/K_m$ for the TRIF p372 peptide was approximately half that observed for the most slowly cleaved 4B/5A peptide.

**Kinetics of Cleavage of Full-length TRIF versus the TRIF Peptide**—From the kinetic studies above, we concluded that proteolysis of the TRIF peptide is less efficient than the viral substrates, although still within the range of what might be expected for a biologically relevant cleavage. One possible explanation for the reduced cleavage observed with the TRIF peptide is the absence of an acidic residue at the P$_6$ position which has been shown to contribute to enhancement in $K_m$ values with viral substrates. In the TRIF sequence, the P$_6$ residue is proline, and it is preceded by a string of 7 other proline residues. Polyproline sequences are ubiquitous in cell signaling molecules, and they have been shown to play a role in the regulation of cellular processes by binding to SH3, WW, and EVH domains (38, 39). Consistent with its role as a signaling molecule, TRIF is highly proline-rich, and we reasoned that this might play a role in its recognition by the HCV protease.

To test the hypothesis that there might be other determinants beyond the 10 residues flanking the cleavage site which could enhance its affinity for NS3/4A and facilitate subsequent proteolysis, we compared the kinetics of scNS3-mediated cleavage of full-length, bacterially expressed TRIF protein and the TRIF p372 peptide. Because it was difficult to achieve a concentration of the full-length TRIF sufficient for determination of the $k_{cat}/K_m$ of this cleavage reaction, we estimated the difference in the enzyme concentrations ($E_{cat}$) required to achieve 50% cleavage of the peptide and full-length protein from the Hill equation, keeping other conditions constant (for details, see “Experimental Procedures”). Fig. 2 shows that there was complete cleavage of the full-length TRIF substrate at a scNS3 concentration of 5 µM (Fig. 2A), whereas only 10% of the peptide substrate was hydrolyzed at this enzyme concentration (Fig. 2B). A quantitative analysis suggested that the $E_{cat}$ for full-length TRIF is 0.69 ± 0.04 µM, compared with 27 ± 3 µM for TRIF p372 (Fig. 2C), indicating a 40-fold difference in the rate of proteolysis. These results thus clearly indicate a substantial difference between the two substrates, leading us to conclude that there are other interaction sites beyond the p372 peptide sequence which contribute to the recognition of TRIF by NS3/4A and which enhance the efficiency of proteolysis.

**TRIF Is a Proline-rich Protein**—As indicated previously, the TRIF sequence contains abundant proline residues (13.6%, 97 of 712 residues). This is consistent with its aberrant migration in SDS-PAGE, where it appears to have an apparent molecular mass of >94 kDa under denaturing conditions (Figs. 1A and 2A), even though mass spectral analysis confirmed that the purified recombinant TRIF possessed the expected mass of 77.2 kDa. This anomalous migration is most likely attributable to polyproline kinks in the molecule, as observed in other proline-rich proteins (40). Importantly, the NS3/4A cleavage of TRIF between Cys-372 and Ser-373 (as demonstrated previously) (13) should result in two fragments ($\Delta$ C340 and $\Delta$ N372) of almost equal molecular mass (39 and 38 kDa for the N-terminal and C-terminal fragments, respectively). However, the two cleavage products are clearly separated in SDS-PAGE, with the N-terminal fragment (which contains 59 of the 96 proline residues) showing the characteristic anomalous migration (>43 kDa, see Figs. 1A and 2A). There are numerous polyproline stretches in the protein, including notably, the 8-residue polyproline track within the N-terminal cleavage fragment, just proximal to the cleavage site (Fig. 3A).
Proline-rich sequences are recognized by a wide variety of signaling molecules that contain SH3, WW, EVH1, and GYF domains (see Refs. 38 and 39 and references therein). Typical signaling molecules contain more than one recognition module. An example is the cellular Grb2 protein that has two SH3 domains and one SH2 domain. SH3 domains recognize proline-rich motifs with moderate to weak affinities, usually in the $\mu$M to mM range (39, 41). These interactions, although not very strong, involve specific hydrogen bond interactions and rigid intercalation of aromatic side chains (33). As illustrated by the Grb2 protein, these proline-rich sequence binding motifs are generally part of larger recognition domains that are capable of interacting with multiple domains of the binding partner, such that the weak interactions result in enhanced specificity and affinity. A characteristic feature of these proline-rich sequences is that they form a left-handed polyproline II (PPII) helix, which is characterized by proline residues in the trans position. To determine whether TRIF possesses such a structure, we analyzed the CD spectra of the purified full-length TRIF. As shown in Fig. 3B, we observed a substantial dip in the ellipticity at $\sim 205$ nm, which is indicative of the presence of PPII helices (42, 43).

These observations led us to speculate that the polyproline stretch just upstream from the cleavage site could play an important role in enhancing the affinity of TRIF for the NS3 protease. Based on knowledge of the structural basis of the interaction between SH3 domains and proline-rich polypeptides (33, 44), we looked for a potential binding site within the NS3/4A protease. Interestingly, we identified a $3_{10}$ helix, composed of hydrophobic residues (Ile-132, Tyr-134, Leu-135), in close proximity to the protease active site. We reasoned that this could act as a possible site for interaction with the polyproline track in TRIF.

The NS5A protein is a nonstructural protein expressed by HCV which has no precise function identified as yet in viral RNA replication (4), but it appears to contribute to viral disruption of cellular antiviral defenses through an interaction with protein kinase R (for review, see Ref. 45 and references therein). NS5A is also a proline-rich protein, containing 11% prolines and encoding two PXPF motifs such as are capable of interacting with SH3 domains (46). In the genotype 1a H77c strain of HCV, one of these proline-rich motifs has a sequence that is identical to the PXXP motif within the murine Sos homolog protein (PPVPPRR, see Fig. 4B), whereas in the genotype 1b BK strain this sequence is closely homologous (possessing a substitution of the central hydrophobic Val residue in Sos with Ile). The peptide representing the Sos domain (PPVPPRR, Fig. 4A) has been shown to interact with the SH3 domain of Grb2 involved in the Ras-ERK pathway (47, 48). Not surprisingly, therefore, the viral NS5A protein has also been shown to interact with Grb2 and perturb mitogenic signaling (46, 49). The Sos peptide is a model PPII helix (Fig. 4B) and is thus potentially representative of many of the proline-rich stretches in TRIF.

Sos Peptide Interacts with scNS3—To determine whether the polyproline-rich Sos peptide is capable of interacting specifically with the scNS3 molecule, we assessed binding using fluorescence spectroscopy and demonstrated a signal decrease in fluorescence intensity upon addition of the peptide. The data were fitted to a single-site binding model equation (see “Experimental Procedures”), resulting in a dissociation constant of $0.53 \pm 0.13$ mM. The affinity of Sos for scNS3, albeit within the $\mu$M range, was thus measurable and characteristic of proline interactions.

If the Sos peptide does interact with the protease near the active site, such as at the $3_{10}$ helix, it could compete with substrate for binding to the scNS3 protease. We assessed this possibility using a FRET-based peptide as substrate and detecting proteolysis by HPLC at 512 nm. The concentration of a SosY peptide (VPPPVPPRRRY) necessary to inhibit 50% of the cleavage reaction ($IC_{50}$) was determined from the decrease in the initial velocity of the reaction as a function of increasing SosY peptide concentration and fitted to Equation 4 (Fig. 5B).

These results indicate that the SosY peptide is capable of inhibiting the scNS3 protease activity with an $IC_{50}$ of $2.7 \pm 0.2$ mM. The derived value of $n = 1.3 \pm 0.1$ is consistent with single-site binding. Inhibition was also observed in a similar experiment carried out with scNS3 derived from the HCV-N strain of HCV (data not shown).

Localization of the Sos Peptide Interaction with NS3/4A—To determine the specific site of interaction of the Sos peptide with scNS3, we used NMR to determine chemical shift changes occurring in a purified $^{15}$N-labeled single-chain protease in
response to the binding of the peptide. Fig. 6A shows representative segments of the $^1$H-$^{15}$N HSQC spectra of the unbound (red) and bound (blue) protease. The residues that comprise the catalytic triad which are responsible for the proteolytic reaction are His-57, Asp-81, and Ser-139. Upon binding of the Sos peptide, there was a significant chemical shift change in the Ser-139 residue (Fig. 6A, left panel), whereas in contrast Arg-123, for example, did not demonstrate a shift (Fig. 6A, right panel). Fig. 6B shows the magnitude of the chemical shift differences between the unbound and bound protease at each residue. The greatest differences occurred within residues in the $\beta$C2 and $\beta$F2 strand and certain residues in $\alpha$C2 and $\alpha$F2. Contrary to what we observed for Sos peptide binding, its binding was associated with large shifts in residues in $\beta$C2 and $\beta$F2. In the case of Sos, although many scNS3 residues, including Ser-139, Ser-138, Lys-136, Ala-157, and His-57 demonstrated significant chemical shift changes (Fig. 6B), most of the residues contributing to $\beta$C2 and $\beta$F2 did not shift significantly (e.g., Arg-123, Ala-45, Asp-168) (Fig. 6A). These data thus suggest that Sos binds primarily to the $\beta$C2 helix side, possibly affecting some residues in $\beta$E2 because of proximity. Although the Sos peptide lacks a reactive Cys, it is nonetheless capable of perturbing the catalytic site residues, Ser-139, His-57, Asp-81 (Fig. 6B), which is consistent with both the binding and inhibition data (Fig. 6). Because the sequence of the Sos SH3 binding domain is present within NS5A (Fig. 4A), the demonstration of a Sos interaction domain on NS3 raises the possibility that NS3 may interact with NS5A in a similar fashion during assembly of the viral RNA replicase (see “Discussion”). In the absence of the lengthy polyproline track present in TRIF, the P6–P1 residues of the TRIF p372 peptide (PSSTPCHQA-GL) are likely to bind to the $\beta$E2 strand of NS3 in a manner similar to that observed in studies of viral substrate-based product inhibitors. However, the NMR data suggest that the upstream polyproline segment in TRIF (Fig. 3A), by interacting with the $\beta$C2 helix, may enhance the affinity of TRIF for the NS3/4A protease, possibly explaining the enhanced proteolytic rate we observed for the full-length TRIF compared with the TRIF p372 peptide (Fig. 2). To assess the impact of the polyproline sequence on cleavage of a peptide substrate, we compared the rates of cleavage of the TRIF p372 peptide with a peptide containing the upstream polyproline sequence (PPPPPPP-
The low solubility of this extended peptide precluded a complete determination of the cleavage kinetics. However, at equivalent substrate concentrations, the proteolytic cleavage of the extended peptide substrate proceeded at a slower rate than the TRIF p372 peptide (Fig. 8A). This was not unexpected because the P side product of the cleavage of the extended peptide is likely to dissociate more slowly from NS3 following cleavage than the TRIF p372 peptide P side product and to possess significant protease inhibitory activity as shown for the Sos peptide in Fig. 5B.

To assess this polyproline “anchoring” hypothesis directly we used fluorescence spectroscopy to determine the difference in the NS3 binding affinity of short and long TRIF P side oligopeptides that either did or did not include the upstream polyproline segment. These experiments demonstrated that the extended TRIF peptide containing the upstream polyproline segment possessed ~10-fold greater affinity for the protease than a short peptide lacking the polyproline sequence (0.15 ± 0.03 and 1.2 ± 0.1 mM for PPPPPPPPSSTPC and PSSTPC TRIF peptides, respectively) (Fig. 8B).

DISCUSSION

We have shown here that there is a significant difference (~40-fold) in the rate of NS3 cleavage of full-length TRIF and the short TRIF p372 peptide. It is likely that this difference is caused by features of the TRIF molecule which direct its binding to NS3 outside the protease active site, including the polyproline track immediately upstream of the NS3 cleavage at Cys-372 of TRIF. The NMR studies (Fig. 6) and increased affinity of TRIF peptides containing the upstream polyproline segment possessed ~10-fold greater affinity for the protease than a short peptide lacking the polyproline sequence (0.15 ± 0.03 and 1.2 ± 0.1 mM for PPPPPPPPSSTPC and PSSTPC TRIF peptides, respectively) (Fig. 8B).
PPII motifs are known to play an essential role in cellular signaling (38, 39). Furthermore, these motifs may also be involved in viral regulatory processes. For instance, the human immunodeficiency virus type 1 Nef protein contains proline-rich motifs that bind to the Src family SH3 domain promoting enhanced replication of the virus (51). There are several potential mechanisms by which proline binding domains may enhance the specificity of intermolecular interactions (39). These include the extension of the specificity surface beyond the active site as well as the provision of a separate, independent recognition surface. Both seem likely for the TRIF-NS3/4A interaction. The interaction with the protease active site appears to be effectively extended to include the $3_{10}$ helix of the protease, which intercalates with the PPII helix of TRIF. Thus, the lack of a P$_6$ acidic residue in TRIF may be compensated by the presence of a “PPII anchor” that extends beyond the substrate site, resulting in enhanced affinity and specificity. Other PPII segments in TRIF could also interact with the protease at sites remote from the catalytic triad, a possibility that is not excluded by our NMR studies.

Previous studies have shown that the nonstructural proteins spanning the NS3–NS5B segment of the HCV polyprotein are required for RNA replication and are likely to contribute to the assembly of a large, macromolecular viral replicase (52, 53). Indeed, yeast two-hybrid, glutathione S-transferase pull-down, and coimmunoprecipitation assays suggest that NS3 interacts with NS5A (54) and may influence the phosphorylation status of NS5A (55). Such interactions might be moderate to weak, yet still necessary for the transient and dynamic interactions that are likely to be involved in assembly of the macromolecular HCV RNA replicase complex. The presence of the Sos peptide sequence within NS5A (Fig. 4), coupled with a demonstration of the affinity of Sos for NS3 (Fig. 5), suggests the possibility that the Sos domain of NS5A could interact similarly with the $3_{10}$ helix of NS3. If so, the putative polyproline interaction between NS5A and NS3 could be strengthened by the interaction of NS3 with its NS4A cofactor sequence because NS4A also interacts with NS5A (45, 56) (see Fig. 4A). There may also be additional, yet uncharacterized interaction sites elsewhere.

Our findings may have significance for drug discovery efforts. Because the HCV NS3 substrate binding site is rather shallow and featureless, the design of specific small molecule
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2. Alter, M. J., Mast, E. E., Moyer, L. A., and Margolis, H. S. (1998) Infect. Dis. Clin. North Am. 12, 13–26.
3. McHutchinson, J. G., and Fried, M. W. (2003) Clin. Liver Dis. 7, 149–161.
4. Reed, K. E. F., and Rice, C. M. (2000) Curr. Top. Microbiol. Immunol. 242, 55–84.
5. Grakoui, A., McComb, D. W., Wychowski, C., Feinstone, S. M., and Rice, C. M. (1993) J. Virol. 67, 2832–2843.
6. Bartenschlager, R., Lohmann, V., Wilkinson, T., and Koch, J. O. (1995) J. Virol. 69, 7519–7528.
7. Randolph, J. T., and De Goeij, D. A. (2004) Curr. Top. Med. Chem. 4, 1079–1095.
8. Lamarre, D., Anderson, P. C., Bailey, M., Beaulieu, P., Bolger, G., Bonneau, P., Bos, M., Cameron, D. R., Cartier, M., Cordingly, M. G., Faucher, A. M., Goudreau, N., Huard, S. H., Kukolj, G., Lagace, L., LaPlante, S. R., Narjes, H., Poupart, M. A., Rancourt, J., Sentjens, R. E., George, R., Simonneau, B., Steinmann, G., Thibeault, D., Tsantrizos, Y. S., Weldon, S. M., Yong, C. L., and Llinas-BruNet, M. (2003) Nature 426, 186–189.
9. Love, B. A., Parge, H. E., Wickersham, J. A., Hostomsky, Z., Habu, N., Moomaw, R. W., Adachi, T., and Hostomsky, Z. (1996) Cell 87, 331–342.
10. Kim, J. L., Morgenstern, K. A., Lin, C., Fox, T., Dwyer, M. D., Landro, J. A., Catter, C. S., Markland, W., Lepre, C. A., O'Malley, E. T., Harbous, S. L., Rice, C. M., Murck, M. A., Caron, P. R., and Thomson, J. A. (1996) Cell 87, 343–355.
11. De Francesco, R., and Steinkuhler, C. (2000) Curr. Top. Microbiol. Immunol. 243, 149–169.
12. Foy, E., Li, K., Wang, C., Sumter, R., Ikeda, M., Lemon, S. M., and Gale, M., Jr. (2003) Science 300, 1145–1148.
13. Li, K., Foy, E., Ferreon, J. C., Nakamura, M., Ferreon, A. C. M., Ikeda, M., Ray, S. C., Gale, M. Jr., and Lemon, S. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2992–2997.
14. Hiscott, J., Pitha, P., Genin, P., Nguyen, H., Heylbroeck, C., Mamane, Y., Alagarte, M., and Lin, B. H. (1999) J. Interferon Cytokine Res. 19, 1–13.
15. Santoro, M. G., Rossi, A., and Amici, C. (2003) EMBO J. 22, 2552–2560.
16. Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imazumi, T., Miyagishi, M., Taira, K., Akira, S., and Fujita, T. (2004) Nature 435, 730–737.
17. Alexopoulos, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) Nature 413, 732–738.
18. Foy, E., Li, K., Sumpter, J. R., Luo, M. Y., Johnson, C., Wang, C., Fish, P., Yoneyama, M., Fujita, M., Lemon, S. M., and Gale, M., Jr. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 2986–2991.
19. Oshimi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003) Nat. Immunol. 4, 161–167.
20. Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takada, K., and Akira, S. (2003) J. Immunol. 171, 4304–4310.
21. Fitzgerald, K. A., Rowe, D. B., Barnes, J. B., Caffrey, D. R., Visintin, A., Latz, E., Monk, D., Pita, P. M., and Golenbock, D. T. (2003) J. Exp. Med. 198, 1043–1055.
22. Akira, S. (2003) J. Biol. Chem. 278, 38105–38108.
23. Servant, M., Grandvaux, N., and Hiscott, J. (2002) Biochem. Pharmacol. 64, 985–992.
24. Koch, U., Biasiol, G., Brunetti, M., Fattori, D., Pallaro, M., and Steinkuhler, C. (2001) Biochemistry 40, 631–640.
25. Urban, C., Bianchi, E., Narjes, F., Tramontano, A., De Francesco, R., Steinkuhler, C., and Pessi, A. (1997) J. Biol. Chem. 272, 9204–9209.
26. Landro, J. A., Raybuck, S. A., Luong, Y. P., O'Malley, E. T., Harbous, S. L., Morgenstern, K. A., Rao, G., and Livingston, D. J. (1997) Biochemistry 36, 9340–9348.
27. Talanian, R. N., Buchvarov, P., Natsukawa, T., Urban, C., Steinkuhler, C., and De Francesco, R., Pessi, A. (1996) Anal. Biochem. 240, 60–67.
28. Ferreon, J. C., and Hilser, V. J. (2003) Protein Sci. 12, 447–457.
29. Edelhoch, H. (1967) Biochemical 6, 1948–1954.
30. Howe, A. Y., Chae, R., Tareni, S. S., Risano, C., Beyer, B., Malcolm, B., and Lau, J. Y. (1999) Protein Sci. 8, 1332–1341.
31. McCoy, M. A., Senior, M. M., Gesell, J. J., Ramanathan, L., and Wyss, D. F. (2001) J. Mol. Biol. 305, 1099–1110.
32. Koredi, R., Biller, M., and Wuthrich, K. (1996) J. Mol. Graph. 14, 29–32, 1–15, 55–58.
33. Lim, W. A., Richards, F. M., and Fox, R. O. (1994) Nature 372, 375–379.
34. Yao, N., Reichert, P., Tareni, S. S., Proside, W. W., and Weber, P. C. (1999) Struct. Fold. Des. 7, 1353–1363.
35. Gallinarini, P., Paulini, C., Breznan, D., Nardi, C., Steinkuhler, C., and De Francesco, R. (1999) Biochemistry 38, 5620–5632.
36. Lin, C., Thomson, J. A., and Rice, C. M. (1995) J. Virol. 69, 4373–4380.
37. Tareni, S. S., Beyer, B., Mahler, M., Yeo, N., Proside, W. W., and Weber, P. C. (1999) Protein Sci. 8, 1431–1439.
38. Kay, B. K., Williamson, M. P., and Sudol, M. (2000) FASEB J. 14, 231–241.
39. Zarrinpar, A., Bhattacharyya, R. P., and Lim, W. A. (2003) Sci. STKE April 2003, 8.
40. Proft, T., Hilbert, H., Plagens, H., and Herrmann, R. (1996) Gene (Amst.) 171, 79–82.
41. Reed, K. E. F., Rice, C. M., and De Francesco, R. (2000) Curr. Top. Microbiol. Immunol. 242, 55–84.
42. Reed, K. E. F., Rice, C. M., and De Francesco, R. (2000) Curr. Top. Microbiol. Immunol. 242, 55–84.
43. Reed, K. E. F., Rice, C. M., and De Francesco, R. (2000) Curr. Top. Microbiol. Immunol. 242, 55–84.
44. Reed, K. E. F., Rice, C. M., and De Francesco, R. (2000) Curr. Top. Microbiol. Immunol. 242, 55–84.
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Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992) *Cell* **70**, 431–442

49. He, Y., Nakao, H., Tan, S. L., Neddermann, P., Vijaysetti, S., Jacobs, B. L., and Katze, M. G. (2002) *J. Virol.* **76**, 9207–9217

50. Cicero, D. O., Barbato, G., Koch, U., Ingallinella, P., Bianchi, E., Nardi, M. C., Steinkuhler, C., Cortese, R., Matassa, V., De Francesco, R., Pessi, A., and Bazzo, R. (1999) *J. Mol. Biol.* **289**, 385–396

51. Lee, C. H., Saksela, K., Mirza, U. A., Chait, B. T., and Kuriyan, J. (1996) *Cell* **85**, 931–942

52. Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999) *Science* **285**, 110–113

53. Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H. E., Bienz, K., and Moradpour, D. (2003) *J. Virol.* **77**, 5487–5492

54. Dimitrova, M., Imbert, I., Kieny, M. P., and Schuster, C. (2003) *J. Virol.* **77**, 5401–5414

55. Neddermann, P., Clementi, A., and De Francesco, R. (1999) *J. Virol.* **73**, 9984–9991

56. Asehe, S. I., Tanji, Y., Satoh, S., Kaneko, T., Kimura, K., and Shimotohno, K. (1997) *J. Virol.* **71**, 790–796

57. Ferguson, M. R., Pan, X., Mukherjee, M., Luo, J., Khan, R., Ferreon, J. C., Hilser, V. J., Shope, R. E., and Fox, R. O. (2004) *Protein Sci.* **13**, 626–632

58. Archer, S. J., Camac, D. M., Wu, Z. J., Farrow, N. A., Domaille, P. J., Wasser, Z. R., Bukhtiyarova, M., Rizzo, C., Jagannathan, S., Mersinger, L. J., and Kettner, C. A. (2002) *Chem. Biol.* **9**, 79–92
