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Substrate Specificity of HCV NS3 Protease Analyzed With Modified Peptide Substrates. M. Tsiang, J.J. Toole, D.W. Lee, A.K. Jain, S. Xiong, C. Flores, J.L. Smith and C.S. Gibbs. Gilead Sciences Inc., Foster City, CA, USA.

Hepatitis C virus (HCV) is the major cause of non-A non-B hepatitis. The HCV polyprotein is processed into at least 9 different proteins: C, E1, E2, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The N-terminal domain of NS3 encodes a serine protease required for the processing of the non-structural proteins and therefore constitutes an important therapeutic target. We have cloned the NS3 protease from an HCV isolate belonging to subtype 1a. The protease domain encoded by the first 181 amino acids of the NS3 protein was expressed in E. coli with a 9 amino acid affinity tag fused to the N-terminus and purified by immunoaffinity chromatography. The protease activity towards peptide substrates was determined by analyzing the cleavage products on reverse-phase HPLC. A synthetic 19 amino acid peptide derived from the NS5A/NS5B junction (5A/5B19-tide) was an efficient substrate with K_m = 104 ± 17 μM and k_{cat} = 1.8 ± 0.1 min^{-1}. An NS4A cofactor peptide had a minimal effect on activity under our assay conditions. A series of N-terminal and C-terminal deletions of the 5A/5B19-tide were used to delineate a minimal peptide substrate of 7 amino acids (P5-P2'). Alanine scanning substitutions of the minimal sequence indicated that positions P4, P3 and P1 were important for recognition. The natural specificity is for Cys at P1 of the trans-cleavage sites (4A/4B, 4B/5A, and 5A/5B), or for Thr at P1 of the cis-cleavage site (3/4A). Various P1 substitutions in 5A/5B19-tide were tested. Eleven substitutions, including Thr and the cysteine isostere, 2-aminobutanoic could not substitute for Cys. However, 4 amino acids could replace Cys without complete loss of activity (S-methyl-cysteine > Norvaline > Leu > Met). Definition of the substrate specificity may contribute to the discovery of potent inhibitors of HCV protease.

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Purification and Characterization of Hepatitis C Virus NS3 Helicase Activity. R. Chase[1], C.-G. Lee[3], E. Ferrari[1], J. Wright-Minogue[1], J. Chen[1], C. Risano[1], D. Sali[2], Z. Hong, and A.D. Kwong. Antiviral Chemotherapy[1] and Structural Chemistry[2], Schering-Plough Research Institute, Kenilworth, NJ; Memorial Sloan-Kettering[3], New York, NY.

The HCV NS3 protein is a multi-functional enzyme with three activities: a N-terminal serine protease activity and a C-terminal RNA-stimulated NTPase activity and RNA helicase activity. NS3 proteolytic activity is stimulated by the NS4A cofactor. These studies were designed to investigate the role of NS4A and the N-terminal protease domain on NS3 RNA helicase activity. RNA helicase activity was optimized and RNA and DNA substrate specificieties were compared using different NS3 proteins containing the RNA helicase domain ($NS3_{631}$/4A, His-$NS3_{631}$/4A, His-$NS3_{631}$ and His-$NS3_{468}$) which were purified to homogeneity from either insect cells or bacterial cells. We find that: 1) Under optimized conditions, all of the RNA helicase constructs have similar activities, but full length NS3 constructs are more stable than those with the C-terminal helicase domain alone. 2) There is a narrower pH range for the RNA helicase activity than for the RNA-stimulated ATPase activity. 3) The RNA helicase is most active at 37°C-42°C (similar to the RNA-stimulated ATPase activity), whereas the protease is more active at lower temperatures (15°C-25°C). 4) The optimum temperature for helicase activity is higher for $NS3_{631}$ than for $NS3_{468}$. 5) ATP complexed with Mg^{++} or Mn^{++} is required for helicase activity, but free Mg^{++} or Mn^{++} is inhibitory. 6) The NS3 RNA helicase unwinds 3'-tailed DNA/DNA duplexes with ~2 to 4-fold less efficiency than 3'-tailed RNA/RNA, RNA/DNA, and DNA/RNA duplexes, suggesting that the typical B-form DNA helical structure may impede strand displacement.