Cleavage Specificity of Coxsackievirus 3C Proteinase for Peptide Substrate (2): Importance of the P2 and P4 Residues

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Coxsackievirus 3C proteinase (3Cpro) cleaves between Gln and Gly, but additional amino acids are required to constitute a cleavage site. To investigate the additional sequence requirements, cleavages of the peptide substrate, and its derivatives were examined. Substitutions of each residue from the P2 to P5 positions showed the importance of the P2 Phe and P4 Ala for recognition by 3Cpro.

Key words: cleavage specificity; coxsackievirus; peptide substrate; proteinase 3C; substrate requirements

Proteolytic processing plays an essential role in the replication of picornaviruses, which include a large number of human pathogens such as enteroviruses (e.g., polioviruses, coxsackieviruses), human rhinoviruses, and hepatitis A virus. These viruses translate their single-stranded, positive-sense RNA into a single large polyprotein, from which all the viral proteins (structural and nonstructural) are generated by a series of controlled proteolytic cleavages.

Virus-encoded 3C proteinase (3Cpro) is responsible for most of these processing events and is important in the virus replication cycle. The viral proteinase, a neutral cysteine proteinase (~20kDa) with a trypsin-like structure, cleaves viral polyprotein between Gln-Gly pairs or related amino acid pairs. In enteroviruses and rhinoviruses, most of the 3Cpro-mediated cleavages occur at Gln-Gly pairs, but the presence of a Gln-Gly pair is not sufficient for cleavage by 3Cpro, suggesting the importance of other amino acid residues flanking the scissile bond. To find the additional sequence determinants of 3Cpro specificity, investigations using synthetic peptides as substrates for purified recombinant 3Cpro have been done. Those studies have suggested that the P4 and P2 positions are important to constitute a cleavage site for 3Cpro of poliovirus and human rhinovirus 14 (HRV14) and that the P2 Prol is essential for cleavage by HRV14 3Cpro.

In our preceding paper, we examined the substrate requirements of coxsackievirus 3Cpro on the C-terminal side of the scissile bond and showed that the C-terminal amino acids from the scissile bond were not essential for cleavage by coxsackievirus 3Cpro, differing from HRV14 3Cpro. The peptides used in those experiments had 5 residues on the N-terminal side of the scissile bond. Therefore, it was considered that in addition to the P1 Gln, other important residues for substrate recognition must be present from the P2 to P5 positions. To find which residues are important, we replaced each amino acid from the P2 to P5 positions of the peptide substrate Ac-EALFQGPPV with Gly and examined cleavages of the resultant peptides Ac-EALFQGPPV (P2-G), Ac-EAGFQGPPV (P3-G), Ac-EGLFQGPPV (P4-G), and Ac-GALFQGPPV (P5-G) by purified recombinant 3Cpro of coxsackievirus B3 (CVB3), which showed a single band on SDS-PAGE.

The peptides were synthesized by solid phase Fmoc chemistry and purified by reverse-phase HPLC as described previously. The composition of the purified peptide was confirmed by amino acid analysis. Two hundred μmol of the peptide was incubated with 1 μmol of CVB3 3Cpro in reaction buffer (100 mM HEPES, pH 7.5, containing 100 mM dithiobreatrole, 1 mM EDTA) at 30 °C and cleavage was measured with TNBS as described previously. The results are shown in Fig. 1. Cleavage of P4-G was greatly reduced and almost no cleavage of P2-G was observed. These results indicate that the residues in the P2 and P4 positions of the substrate are important for cleavage by CVB3 3Cpro. The importance of the P4 residue was already shown by using 3Cpro of poliovirus9 and HRV14. The results described here are in agreement with the earlier findings. However, the importance of the P2 residue has not been demonstrated so far. Therefore, we then investigated which amino acid is preferred in the P2 position.

A series of peptides were synthesized that had amino acid substitutions in the P2 position of Ac-EALFQGPPV: the P2 Phe was replaced by Tyr, Leu, His, Trp, Arg, Ala, and Glu. Cleavages

Fig. 1. Importance of the P2 and P4 Residues of the Peptide Substrate on Cleavage by CVB3 3Cpro.

Each of the peptides Ac-EALFQGPPV (■), P2-G ( ), P3-G ( ), P4-G ( ), and P5-G ( ) was incubated with CVB3 3Cpro at 30 °C. At indicated times, samples were removed from the reaction mixture and the amino groups released by hydrolysis of the peptide bond were measured using TNBS as described previously. Cleavage (V1) = concentration of amino groups × 100 initial concentration of the substrate peptide (200 μM).

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Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonic acid; Fmoc, 9-fluorenylmethyloxycarbonyl.
Fig. 2. Cleavage of Peptide Substrates Varying in the P2 Position by CVB3 3Cpro.

The P2 Phe of the peptide substrate Ac-EALFGQPPV (■) was replaced by either Tyr (▲), Leu (▲), His (□), Trp (▲), Arg (□), Ala (△), or Glu (●). Each peptide was incubated with 3Cpro and cleavage was measured as described in Fig. 1.

Fig. 3. Cleavage of Peptide Substrates Varying in the P4 Position by CVB3 3Cpro.

The P4 Ala of the peptide substrate Ac-EALFGQPPV (■) was replaced by either Val (▲), Thr (▲), Gly (▲), Asn (□), or Leu (●). Each peptide was incubated with 3Cpro and cleavage was measured as described in Fig. 1.

of these peptides are shown in Fig. 2. Cleavage efficiency varied widely with the amino acid in the P2 position. The best substrate was the parental peptide Ac-EALFGQPPV, indicating a preference for Phe in the P2 position. Hydrophobic interaction between the side chain of the P2 residue and 3Cpro appears to be important because replacement of the P2 Phe with His considerably decreased cleavage efficacy in spite of its steric resemblance. Replacement of the P2 Phe with charged amino acids (Glu and Arg) greatly reduced cleavage, but the reduction was small in the case of replacement with Leu. These results also support the importance of hydrophobicity of the P2 residue.

The amino acid preferred in the P4 position was also examined. Alanine and amino acids with noncharged short side chains have been shown to be preferred in the P4 position of the poliovirus and HRV14 3Cpro, respectively. Therefore, the P4 Ala of the substrate peptide Ac-EALFGQPPV was replaced with other aliphatic noncharged amino acids, Val, Leu, Thr, Gly, and Asn. Cleavages of these peptides were examined. In these experiments, 10% DMSO was included in the reaction buffer because of the low solubility of the substituted peptides (the parental peptide was also cleaved in the buffer containing 10% DMSO). The results showed that every substitution of the P4 Ala resulted in a large decrease in cleavage (Fig. 3), suggesting that Ala is the most preferred amino acid in this position.

The side chains of the P2 Phe and P4 Ala thus appear to be important in the enzyme-substrate interaction. Recently, the three-dimensional structure of HRV14 3Cpro was identified by X-ray crystallography, and a model for substrate binding to 3Cpro was also presented. The model showed the presence of a spacious cavity and a shallow hydrophobic pocket that accommodate the side chains of the P2 and P4 residues, respectively. Our results strongly suggest that CVB3 3Cpro also has similar S2 and S4 pockets that interact with the side chains of the P2 Phe and P4 Ala, respectively. Of these 2 important residues for enzyme-substrate interaction, the P4 Ala is conserved in 6 of the 8 putative 3Cpro-mediated cleavage sites of CVB3 polyprotein. In contrast, the P2 is poorly conserved and various amino acids are present in this position. These facts appear to be somewhat inconsistent with the results obtained here, but in the virus-infected cells, cleavage efficiency of each site is not similar. It is well known that cleavage of the 3C:3D site is inefficient. The P2 position in the 3C:3D site is occupied by the unpreferred residue Glu, and it is likely that the P2 Glu might be one of the major factors for low cleavage efficiency. Picornaviruses are unable to regulate gene expression at the transcriptional level, and all gene products are synthesized in an equimolar ratio. Their gene expression is considered to be regulated at the stage of polyprotein processing. In this regulation, the P2 amino acid of the cleavage site might have a major role although cleavage must also be affected by other factors, such as conformation of the cleavage site and other amino acids surrounding the cleavage site.

In this report, we have shown that coxsackievirus 3Cpro recognizes the side chains of the P2 and P4 residues in addition to the P1 Gln. These findings would contribute to understanding the key step of picornavirus replication and to designing specific inhibitors for this virus-specific proteinase.

References