Note

Mutational Analysis of the Putative Substrate-binding Site of 3C Proteinase of Coxsackievirus B3

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Single amino acid substitutions were introduced into the putative substrate-binding site of 3C proteinase (3Cpro) of coxsackievirus B3, a member of the picornavirus family. Mutations at either Thr142, His161, Gly164, Gly169, or Ala172 severely impaired or abolished the proteolytic activity except that a conservative Thr142 to Ser mutant had detectable activity. These results, which have shown the participation of the 5 residues in 3Cpro activity, are consistent with the earlier predictions that these residues might be involved in substrate binding.

Picornaviruses translate their single-stranded, positive-sense RNA into a single, large polyprotein, which is cleaved by a series of proteolytic events to yield the mature viral proteins. The virally encoded 3C proteinase (3Cpro) plays a major role in these proteolytic events. Although 3Cpro has been classified as a cysteine proteinase, it shows no sequence similarity with the papain family. Computer-assisted sequence analyses have shown that 3Cpro is related to trypsin-like (chymotrypsin-like) serine proteinases and two models of 3Cpro have been presented independently. The validity of these models has been supported by recent mutational analyses. For the preceding report, we introduced single amino acid substitutions into the putative active site residues of 3Cpro of coxsackievirus B3 (CBV3), a member of the picornavirus family, and indicated that the residue Cys147 is a nucleophile corresponding to Ser195 (chymotrypsin numbering system) of trypsin-like serine proteinases.

3Cpro specifically cleaves the peptide bond between Gln and residues with small side chains. Bazan and Fletterick suggested that Thr142 and His161, highly conserved among picornavirus 3Cpro, could form hydrogen bonds with an enzyme-bound Gln side chain and might be the primary determinants of the 3Cpro cleavage specificity. In addition, the sequence alignments showed that Gly164, a highly conserved residue of 3Cpro, is equivalent to Gly216 of chymotrypsin. Two sequence alignments differ in the designation of the 3Cpro residue equivalent to Gly226 of chymotrypsin: they assign Gly169 and Ala172, respectively (Fig. 1).

In this report, we introduced single amino acid substitutions into Thr142, His161, Gly164, Gly169, and Ala172 of CBV3 3Cpro, which might be involved in substrate binding, and examined the effects of these mutations on the autocatalytic cleavage activity of this proteinase in Escherichia coli. We also mutated the residue Gly163, adjacent to Gly164, and partially conserved among picornaviruses.

Construction of the 3Cpro expression plasmid pCXB117T was described previously. This plasmid encodes a fusion protein of 196 amino acids, a precursor of mature CBV3 3Cpro. It consists of 7 amino acids derived from the lacZ gene of pUC118.

Table: Oligonucleotides Used as the Mutagenic Primers

<table>
<thead>
<tr>
<th>Sequence of oligonucleotide 5'-3'</th>
<th>Location on CBV3 cDNA</th>
<th>Amino acid substitution</th>
<th>New restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCCGTGCCTAGGGGAAGGTT</td>
<td>5776–5797</td>
<td>Thr142→Ser (T142S)</td>
<td>XbaI</td>
</tr>
<tr>
<td>CCTGCTCTTAAAGGGGAAGTT</td>
<td>5776–5796</td>
<td>Thr142→Leu (T142L)</td>
<td>AffI</td>
</tr>
<tr>
<td>TTCCACACCCGGGATACCCAGT</td>
<td>5832–5854</td>
<td>His161→Arg (H161R)</td>
<td>NcoI</td>
</tr>
<tr>
<td>TTCCACACATAGATACCCAGT</td>
<td>5832–5854</td>
<td>His161→Tyr (H161Y)</td>
<td>--</td>
</tr>
<tr>
<td>ATTCGCCATGGCGCCACACTATAGG</td>
<td>5840–5862</td>
<td>Gly163→Ala (G163A)</td>
<td>NaeI</td>
</tr>
<tr>
<td>ATTCGCCATGGCGCCAACATAGG</td>
<td>5841–5862</td>
<td>Gly164→Ala (G164A)</td>
<td>NarI</td>
</tr>
<tr>
<td>GCTGAGAAGACGCCTAGTGCC</td>
<td>5858–5876</td>
<td>Gly169→Ala (G169A)</td>
<td>SnaI</td>
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<tr>
<td>TGCTGAGAACACGCTGATGCC</td>
<td>5857–5877</td>
<td>Gly169→Leu (G169L)</td>
<td>PnuI</td>
</tr>
<tr>
<td>GAGGAGGTCTGAGCGAGACCCCT</td>
<td>5864–5886</td>
<td>Ala172→Leu (A172L)</td>
<td>NheI</td>
</tr>
</tbody>
</table>

* Nucleotide number shown is based on the CBV3 sequence reported by Klump et al.14

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside.
C-terminal 6 amino acids of viral 3B peptide, and the entire 3Cpro sequence (183 amino acids). Mature 3Cpro is produced from this precursor protein by autocatalytic cleavage at its own N-terminus (3B/3C site) in E. coli. Mutations were introduced into pCXBl177 using Transformer site-directed mutagenesis kit (Clontech) based on the method of Deng and Nickoloff.14 The mutagenic oligonucleotides used are listed in the Table. The mutations introduced were confirmed by dye terminator cycle sequencing using a DNA sequencer (ABI 373A). E. coli JM109 cells containing wild type or mutated pCXBl177 were incubated and the expression of the 3Cpro precursor protein was induced with 1 mM IPTG as described previously.11,13 Whole cell proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with anti-3Cpro antisera (Fig. 2). The precursor protein was efficiently cleaved and only the mature 3Cpro form was detected (Fig. 2, lane 1).

The putative cleavage specificity determinants, Thr142 and His161, were mutated to generate 4 mutants, Thr142 to Ser (T142S), Thr142 to Leu (T142L), His161 to Arg (H161R), and His161 to Tyr (H161Y). The autocleavage activity of 3Cpro was lost and the precursor protein was not cleaved in these mutants (Fig. 2, lanes 3–5) except for the T142S mutant, indicating the importance of these residues for autocatalytic cleavage of Gln-Gly bond at the 3B/3C site. In T142S mutant, 3Cpro activity was retained to some extent and similar amounts of the precursor and mature 3Cpro were detected (Fig. 2, lane 2). This result is in agreement with the prediction that Thr142 could form a hydrophobic bond with the side chain of Gln. A similar result was obtained with 3Cpro of human rhinovirus.9

The replacement of the residue on the wall of the substrate-binding pocket with a larger amino acid was expected to interfere with the interaction between the enzyme and the substrate. In fact, the activity of trypsin was impaired by the replacement of Gly216 or Gly226 by Ala.10 When Gly164 of 3Cpro was mutated to Ala, the activity was severely inhibited and almost no mature form was produced (Fig. 2, lane 7). Gly163 was also replaced with Ala but in this case, the precursor protein was fully cleaved to the mature form (Fig. 2, lane 6). These results are consistent with the sequence alignment data8,9 that Gly164 is equivalent to Gly216 of chymotrypsin (Fig. 1). The residue of 3Cpro equivalent to Gly226 of chymotrypsin is either Gly16910 or Ala17211 (Fig. 1). Mutations of these residues to larger amino acids generated 3 mutants, Gly169 to Ala (G169A), Gly169 to Leu (G169L), and Ala172 to Leu (A172L). The autocleavage activity of 3Cpro was greatly impaired in all these mutants (Fig. 2, lanes 8–10). These results indicate that both Gly169 and Ala172 are important for 3Cpro activity and it has not been discovered whether Gly169 or Ala172 is equivalent to Gly226 of chymotrypsin.

The results obtained here support the prediction that Thr142, His161, and Gly164 of 3Cpro constitute the substrate-binding pocket. Interestingly, all these 3 residues and Gly163 are conserved in Staphylococcus aureus protease7 (SAP) (Fig. 1), which is a serine protease with a specificity for Glu in the S1 pocket.17 On the other hand, 3Cpro has a specificity for Gln.1–3 It is therefore considered that there must be other residue(s) contributing to the substrate specificity. While preparing this manuscript, the X-ray crystal structure of 3Cpro of hepatitis A virus (HAV) was presented.18 In that report, the authors indicated that in HAV 3Cpro, the residue Glu132 plays a role in the discrimination between Glu and Gln at the P1 position of the substrate. However, this residue is not conserved among 3Cpro of other picornaviruses. Further mutagenic analyses would present some information about the Gln preference of 3Cpro of other picornaviruses containing CBV3.

References