Cleavage specificity of the poliovirus 3C protease is not restricted to Gln–Gly at the 3C/3D junction

Katherine M. Kean,* Natalya Teterina† and Marc Girard

Unité de Virologie Moléculaire (CNRS UA 545), Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris cedex 15, France

The 3C protease of poliovirus is distinguished from that of all other picornaviruses in that it only cleaves at Gln–Gly amino acid pairs within the viral polyprotein. To determine whether this strict cleavage specificity is an intrinsic property of the poliovirus 3C protease, amino acid substitutions were introduced at one of the Gln–Gly cleavage sites. Oligonucleotide-directed site-specific mutagenesis of an infectious poliovirus type 1 (Mahoney strain) cDNA was used to change the Gln–Gly site at the 3C/3D junction of the polyprotein into Gln–Val, Gln–Ala, Gln–Ser or Gin–Pro. The effects of these substitutions were studied in vivo after transfection of primate cells by the mutated cDNAs. The Gln–Gly to Gin–Pro substitution was lethal for virus growth, and the corresponding altered 3CD polypeptide expressed in insect cells using a recombinant baculovirus vector did not appear to undergo autocleavage. The Gln–Gly to Gln–Val change was also lethal, although production of virus was occasionally observed as a result of reverse mutations. Mutants with Gln–Ala and Gln–Ser sequences were viable, indicating that these dipeptides can be cleaved by the poliovirus protease in vivo. However, processing at the 3C/3D junction occurred relatively inefficiently in the case of the Gln–Ser virus. Furthermore, the Gln–Gly to Gln–Ala substitution seemed to result in an additional cleavage event within the N-terminal part of polypeptide 3D.

Introduction

Polioviruses are members of the Picornaviridae family. As such, their genome is a single RNA molecule of positive polarity that encodes a single large polyprotein (247K for poliovirus). All known picornavirus polypeptides are generated by nascent and post-translational cleavages of the polyprotein precursor, using virus-encoded proteolytic activities (for reviews, see Kraisslich & Wimmer, 1988; Wellink & van Kammen, 1988). In the case of poliovirus, three distinct types of cleavage are known.

A cotranslational primary cleavage event releases the precursor to the capsid polypeptides. This cleavage is carried out by the 2A protease at a Tyr–Gly site at the amino terminus of 2A itself (Toyoda et al., 1986). Only one of the 10 Tyr–Gly dipeptides present in the polyprotein is cleaved, to generate 3' and 3D', alternative products of the 3C protease and 3D replicase region of the polyprotein. A final capsid maturation occurs as the last step of virion morphogenesis after association of the viral RNA with the protein shell. This cleavage of VP0 into VP4 and VP2, which occurs at an Asn–Ser site, has been suggested to be autoproteolytic (Arnold et al., 1987).

The nine intermediate processing events are all carried out by the 3C protease, the major viral protease for all picornaviruses (Gorbalenya et al., 1979; Palmenberg et al., 1979; Hanecak et al., 1982). However, both mature 3C and at least one of its precursors, polypeptide 3CD, have proteolytic activity in vitro (Jore et al., 1988; Ypma-Wong et al., 1988). 3C-dependent processing appears to be extremely specific; the only cellular protein known to be cleaved is histone H3, by the 3C protease of foot-and-mouth disease virus (Falk et al., 1990; Tesar & Marquardt, 1990), and several potential cleavage sites in the picornavirus polypeptides are apparently never recognized (Kitamura et al., 1981; Pallansch et al., 1984).

A striking difference between the poliovirus 3C protease and the 3C proteases of other picornaviruses is the observed cleavage specificity. In nature, the poliovirus 3C protease cleaves only at Gln–Gly dipeptides within the viral polyprotein (Kitamura et al., 1981; Hanecak et al., 1982), whereas a broader range of amino acid pairs is recognized in all other picornaviruses. For the prototype strain of each virus, a specific subset of dipeptides from the following amino acids serve as cleavage sites: P1 Gln, Glu or Leu; P1' Gly, Ser, Ala,
Thr, Asn, Leu, Ile, Met or Val (Arnold et al., 1987; A. Palmenberg, personal communication).

To determine whether the stringency for cleavage at a Gln–Gly dipeptide is an intrinsic property which distinguishes the poliovirus 3C protease from that of other picornaviruses, we undertook specific substitution of one of the poliovirus 3C-specific cleavage sites. We found that the Gln–Gly site at the 3C/3D junction of the poliovirus type 1 polyprotein could be replaced by Gln–Ala or Gln–Ser without loss of virus infectivity. On the other hand, replacement of the same dipeptide by Gln–Val or Gln–Pro proved lethal for virus growth. Furthermore, 3CD which contained a Gln–Pro junction, rather than the natural Gln–Gly junction, showed loss of autoproteolytic activity when expressed in insect cells.

Methods

Construction of plasmids. Plasmid pKK4 (Fig. 1a) was constructed using the 3.6 kb BglII–EcoRI fragment of the infectious cDNA clone pKK17 (Kean et al., 1986) to create a subclone of the poliovirus type 1 (Mahoney strain) cDNA from nucleotide 5602 to the 3′ end of the genome.

Plasmid pKK7 was constructed such that the cDNA coding for the poliovirus 3CD polypeptide, followed by the 3′ non-coding region and poly(A) tail, was preceded by an in-phase ATG codon and flanked by BamHI sites (Fig. 1a). In pKK7 derivatives, the poliovirus-specific BglII–PvuII (position 5601 to position 7053) fragment came from pKK4 or from the different pKK4 mutants described below.

pAc–3CD plasmids (Fig. 1b) contain the poliovirus-specific BamHI fragment of pKK7 derivatives inserted into pVL941–poly, an Autographa californica nuclear polyhedrosis virus (AcNPV) shuttle vector derived from pVL941 (Luckow & Summers, 1989) by the insertion of a multi linker at the 3′ end of the BamHI site at position 170 of the polyhedrin gene (P. Gnone, personal communication). Detailed restriction mapping of these plasmids showed that no major rearrangements or deletions had occurred. In addition, the orientation of the insert was verified by sequencing over the junctions directly on the double-stranded plasmid DNA (Zagursky et al., 1985; T7 Sequencing Kit, Pharmacia LKB).

Escherichia coli strain 1106 (803 c.m.) was used for the propagation of plasmids. Recombinant DNA procedures used were essentially as described (Maniatis et al., 1982). Restriction and modification enzymes (Boehringer Mannheim, BRL or Biolabs) were used as directed by the manufacturers.

Oligonucleotide-directed site-specific mutagenesis. Equimolar ratios (0.03 pmol) of plasmid pKK4 linearized by PstI and dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim) and of the purified 6.5 kb fragment of pKK4 cut with EcoRV (positions 5805 and 6024 of the poliovirus cDNA; see F1; 1a) were added to an excess (20 pmol) of a mixture of phosphorylated synthetic oligonucleotides, 3′ TGAGTCTCAGTTNPACTTTAGGTCACC 5′ where N is A, C, G or T and P is A or G (Igolen, Pasteur Institute). After denaturation and renaturation by heating and cooling (Morinaga et al., 1984), the hybrid DNA molecules were repaired and ligated using Klenow enzyme and T4 DNA ligase [3 units (U) and 2.5 U respectively; Boehringer Mannheim] as described (Oostra et al., 1983). The clones obtained after two successive transformations of E. coli HB101 (Boyer & Roulland-Dussoix, 1969) were screened with a 5′ 32P-labelled oligonucleotide of the wild-type sequence of the region which had been mutagenized (5′ TCAGTCCACTTCTAG 5′). After in situ hybridization at 37 °C, mutants were selected by loss of the hybridization signal upon washing at 37 °C and then 39 °C. The sequence of mutant plasmid DNA was determined over the entire region repaired.

Virus strains and cells. Wild-type poliovirus type 1 (Mahoney strain) was obtained after transfection of confluent monolayers of cloned Vero cells by a reconstruction of the infectious plasmid pKK17 (Kean et al., 1986) in which the 3.6 kb BglII–EcoRI fragment came from a pKK4 plasmid that had undergone site-specific mutagenesis as described above without incorporating a synthetic oligonucleotide. Similarly, mutant viruses were obtained after transfection by derivatives of pKK17 in which the 3.6 kb BglII–EcoRI fragment was that of the different pKK4 mutants. For each full-length cDNA, the sequence over the site of mutagenesis was verified. In addition, detailed restriction mapping was carried out to check that no major rearrangements had occurred. Transfection was carried out at 33 °C, 37 °C or 39 °C in the presence of DEAE-dextran (Sompayrac & Danna, 1981) as previously described (Kean et al., 1988). Viruses were plaque-purified twice on the Hopkins strain of HeLa cells at 37 °C, then virus stocks were prepared. After transfection or infection, cells were grown under Dulbecco’s modified Eagle’s medium supplemented with 2% foetal calf serum (FCS) and, for plaque assays, with 50 mM-MgCl2 and 0.9% Noble agar.

Confluent monolayers of Spodoptera frugiperda IPLB-SF21-AE clonal isolate 9 cells (SF9 cells) were cotransfected with wild-type AcNPV DNA (0.4 µg/106 cells) and with one of the pAc–3CD shuttle vectors (10 µg DNA/106 cells) using the calcium phosphate precipitation technique (Graham & van der Eb, 1973; Summers & Smith, 1987). SF9 cells were grown at 27 °C in Grace’s medium (Gibco) supplemented with 10% heat-inactivated FCS and, in the case of plaque assays, with 1% indubiose (IBF). Three days after transfection, the medium was replaced by fresh medium. Extracellular virus was harvested 5 to 6 days after transfection, when occlusion bodies were harvested in 90 to 100% of the cells. Recombinant baculoviruses were subjected to four rounds of plaque purification on SF9 cells, then virus stocks were prepared and stored at 4 °C. The integrity of the poliovirus 3C-specific sequences in the genome of these viruses was verified.

Viral DNA was extracted from infected cells (Summers & Smith, 1987) and digested with BamHI to release the passenger DNA (see Fig. 1b). The digestion products were separated on an agarose gel and hybridized to a 32P-labelled cDNA probe specific for poliovirus nucleotides 5602 to 7053.

Sequencing of polioviral RNA. HeLa cell monolayers were infected at an m.o.i. of 20 p.f.u./cell. After incubation for 5 to 6 h at 37 °C, cytoplasmic extracts were prepared by cell lysis with NP40 (Macc et al., 1987). Cytoplasmic RNA (10 µg) was annealed to a 5′ 32P-labelled deoxyoligonucleotide primer complementary to bases 6041 to 6055 of the viral RNA (20 pmol). Sequence reactions were carried out using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) as described (Geliebter et al., 1986).

Labelling of infected cells with [35S]methionine and immunoprecipitations. Confluent HeLa cell monolayers were infected with poliovirus at an m.o.i. of 50 p.f.u./cell, in the presence of actinomycin D (2.5 µg/ml; Calbiochem). The cells were incubated in methionine-free medium for 30 min before labelling with 30 µCi/ml of [35S]methionine (>1000 Ci/mmol; Amersham). In pulse-chase experiments, cells were labelled for 15 min, then washed and fed with medium containing 2 mg/ml unlabelled methionine.

Confluent SF9 cell monolayers were infected with baculovirus at an m.o.i. of 10 p.f.u./cell. The cells were incubated in methionine-free medium for 60 min prior to labelling with 45 µCi/ml of [35S]methionine.
Cytoplasmic extracts were prepared by lysis with NP40 (Bellocq et al., 1984). Samples of cytoplasmic extracts were immunoprecipitated with antibodies either raised against polypeptide 3C (Hanecak et al., 1982) or against a TrpE-polypeptide 3C-3D fusion protein (Richards et al., 1987). Activated Staphylococcus aureus cells were used to recover immunoprecipitated proteins (Semler et al., 1982). Proteins were analysed by electrophoresis on a 15% SDS-polyacrylamide gel (Laemmli, 1970) and in the case of immunoprecipitations the gels were fluorographed using Amplify (Amersham).

Results

Construction of mutant poliovirus cDNAs

To determine whether the strict specificity for Gln-Gly dipeptides at poliovirus 3C cleavage sites is required for virus viability, we modified the sequence which codes for the Gln-Gly cleavage site at the 3C/3D junction by oligonucleotide-directed site-specific mutagenesis of an infectious plasmid carrying the full-length poliovirus cDNA. The Gly residue was substituted in preference to the Gin residue, since the compilation of all known naturally occurring picornaviral 3C cleavage sites (Arnold et al., 1987; A. Palmenberg, personal communication) and the results of mutagenesis of encephalomyocarditis (EMC) virus 3C cleavage sites (Parks & Palmenberg, 1987; Parks et al., 1989) suggest that the specificity at the PI' position is rather more flexible than that at the P1 position.

To facilitate the mutagenesis, it was performed on plasmid pKK4 which contains only the last 1840 nucleotides of the poliovirus cDNA (see Methods). The Gly residue, which is part of the cleavage site at the 3C/3D junction of the poliovirus polyprotein and which is the first residue of the viral replicase, could be changed into any one of Ala, Val, Leu, Ser, Ile, Thr, Phe or Pro residues by using a mixture of synthetic oligonucleotides in which multiple bases had been incorporated at two adjacent positions (see Methods). However, during the synthesis of such a mixture of oligonucleotides it is unlikely that the multiple bases would each be incorporated in equimolar ratios at a single position. Furthermore, the mutants obtained were screened by loss of hybridization to the wild-type oligonucleotide. It is thus not surprising that only four mutant cDNAs (pKK41 to pKK44) were obtained in practice, coding for Val, Ala, Ser and Pro (Table 1).

The DNA of each clone was sequenced between nucleotides 5800 and 6100 to verify that no other mutation had been introduced into the region of DNA repaired during the mutagenesis procedure. However, a much larger fragment than this was used for the reconstruction of a full-length cDNA (see Methods). The presence of second-site spontaneous mutations could not be entirely ruled out, as we did not sequence the entire poliovirus cDNA. Therefore, for each mutant other than Ser, several independent clones were used to reconstruct full-length cDNAs in the infectious plasmid pKK17 (pKK17-41 to pKK17-44). Only one Ser mutant was obtained and it should be noted that it did not have the sequence predicted from the oligonucleotide synthesized (TCT), but was encoded by an AGT sequence (see Table 1). This mutant presumably arose from a fortuitous mutation introduced during the repair of pKK4 after mutagenesis.

Analysis of the infectivity of mutant cDNA molecules

To study the effects of the substitutions on virus viability, Vero cells were transfected with plasmids pKK17-41 to 44. pKK17-44, which carried the Gln–Gly to Gln–Pro substitution, did not give rise to virus (Table 1) although four independent reconstructions of this plasmid were transfected onto Vero cells more than 100 times in total. This was regardless of the transfection temperature (33 °C, 37 °C or 39 °C; data not shown). We concluded that this substitution was lethal for virus growth.

Virus was obtained systematically after transfection with both pKK17-42 (Gln–Ala) and pKK17-43 (Gln–Ser) and these viruses were named vG2-3CD17-42 and vG1-3CD17-43, according to the nomenclature

Table 1. Mutants obtained after mutagenesis at the 3C/3D cleavage site

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>cDNA sequence</th>
<th>Virus production</th>
<th>Viral RNA sequence</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK17</td>
<td>CAA GGT</td>
<td>+</td>
<td>CAA GGU</td>
<td>Wild-type control</td>
</tr>
<tr>
<td></td>
<td>Gln/Gly</td>
<td></td>
<td></td>
<td>Same sequence found six times for three independent clones</td>
</tr>
<tr>
<td>pKK17-41</td>
<td>CAA GTT</td>
<td>Occasionally</td>
<td>CAA GCU</td>
<td>Four independent clones tested</td>
</tr>
<tr>
<td></td>
<td>Gln/Val</td>
<td></td>
<td>Gln/Ala</td>
<td></td>
</tr>
<tr>
<td>pKK17-42</td>
<td>CAA GCT</td>
<td>+</td>
<td>CAA GCU</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln/Ala</td>
<td></td>
<td>Gln/Ala</td>
<td></td>
</tr>
<tr>
<td>pKK17-43</td>
<td>CAA AGT</td>
<td>+</td>
<td>CAA AGU</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln/Ser</td>
<td></td>
<td>Gln/Ser</td>
<td></td>
</tr>
<tr>
<td>pKK17-44</td>
<td>CAA CCT</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gln/Pro</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
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Fig. 1. Structural organization of plasmids which contain 3CD sequences. (a) The poliovirus cDNA of pKK4 (solid box) and the SV40 transcription termination signals (open box) came from pKK17 (Kean et al., 1986). The 3C-specific cDNA of pKK7 (horizontally hatched box) upstream of the NarI site (position 5823 of the poliovirus cDNA) came from pEXCalibur (Ivanoff et al. 1986) and the 3C and 3D-specific cDNA (vertically hatched box) downstream from this site came from pSW3004 (a gift of S. van der Werf). In both pKK4 and pKK7, the poliovirus-specific cDNA was inserted into pBR327 (solid lines). The fragment of pKK7 replaced by that of plasmid pKK4 and its mutated derivatives is shown by dotted lines. (b) The changes in the AcNPV polyhedrin gene (diagonally hatched box) in the cases of the pVL941-poly baculovirus shuttle vector and the pAc-3CD plasmids are shown. The poliovirus cDNA coding for 3C and 3D are shown as horizontally and vertically hatched boxes, respectively. The polyhedrin and poliovirus non-coding regions are shown as solid lines. The nucleotide sequence at the junction between the polyhedrin gene and the 5' end of the region coding for poliovirus 3CD is indicated, as is the natural double stop codon at the end of the region coding for 3CD. The amino acid sequence at the 3C/3D junction of the various pAc-3CD plasmids is shown and transcription initiation is indicated by *.

proposed by Bernstein et al. (1986). They will be referred to as the Gln-Ala and Gln-Ser mutants. The sequence of the viral RNA was determined in the region coding for the 3C/3D junction to check that these two mutants had each retained the original codon from pKK17-42 and pKK17-43 (Table 1).

After transfection with pKK17-41 (Gln-Val), virus was occasionally recovered. This was a sporadic event, occurring in about 75% of the plates, regardless of whether transfections were carried out at 33 °C, 37 °C or 39 °C (data not shown). However, when the RNA of these viruses was sequenced over the 3C/3D junction, it was found that they coded for a Gln-Ala junction in all cases (Table 1). It thus appeared that the Gln-Gly to Gln-Val substitution was lethal for virus growth.

Expression of substituted 3CD polypeptides in insect cells

To determine whether the lethal substitutions at the 3C/3D junction reflected a cleavage defect at the altered site, we constructed recombinant baculoviruses in which the poliovirus 3CD polypeptide was placed under the transcriptional regulation of the strong polyhedrin gene promoter. Thus, 3CD could be expressed in a transient fashion in infected S. frugiperda (Sf9) cells. It has been reported repeatedly that foreign proteins are expressed to extremely high levels in recombinant baculoviruses and that their biological properties are conserved (for a review, see Luckow & Summers, 1988).

Fig. 2. Analysis of poliovirus 3C- and 3D-related proteins expressed in insect cells. Sf9 cells were infected with wild-type AcNPV (lanes 4, 12 and 13), with the wild-type (Gln-Gly) 3CD recombinant baculovirus (lanes 3, 10 and 11), or with two independent Gln-Pro substituted 3CD recombinant baculoviruses (lanes 1, 2, 6, 7, 8 and 9) and labelled with [35S]methionine for 3 h at 25 h post-infection. Aliquots of cytoplasmic extracts corresponding to 3 x 10⁶ cells were immunoprecipitated with antibodies directed against poliovirus 3D (lanes 6, 8, 10 and 12) or 3C (lanes 7, 9, 11 and 13) before electrophoresis on a 15% SDS-polyacrylamide gel. Alternatively, extracts corresponding to 2 x 10⁴ cells were analysed directly (lanes 1 to 4). Mr markers and poliovirus proteins from infected HeLa cell extracts run in parallel are shown on the left and right, respectively.
The pVL941–poly shuttle vector was used, since this vector has been reported to promote particularly high level expression of non-fused foreign genes (Luckow & Summers, 1989). The substitution into the polyhedrin gene began +35 nucleotides downstream from the polyhedrin initiation codon which was changed to an ATT (Fig. 1b). pEXCalibur was used as the source of the 5' extremity of the insert as this plasmid supplies an in-phase ATG directly preceding the 3CD coding region (Ivanoff et al., 1986). The authentic double stop codon at the end of the 3CD coding region was conserved, as was the poliovirus 3' non-coding region and the genome-encoded poly(A) sequence (Fig. 1b, see also Methods). In addition to the wild-type pAc–3CD plasmid, two independent pAc–3CD plasmids were constructed for each of the Gln–Val and Gln–Pro mutants using independent isolates of pKK41 and pKK44 and were cotransfected into Sf9 cells with wild-type AcNPV genomic DNA.

Stocks of recombinant baculovirus that encoded the Gln–Val 3CD could not be prepared, most probably because this polypeptide was toxic for Sf9 cells (data not shown). Therefore, expression of only the Gln–Pro 3CD was compared to expression of the wild-type Gln–Gly 3CD. The maximum level of expression of poliovirus 3CD in Sf9 cells was observed between 25 and 30 h post-infection, after which progressive cell death occurred (data not shown). As shown in Fig. 2, in the case of the wild-type Gln–Gly Ac–3CD virus, a protein was observed which comigrated with authentic 3CD from poliovirus-infected HeLa cells on an SDS–polyacrylamide gel and which was specifically immunoprecipitated with antibodies directed against either 3C or 3D. In addition, small amounts of a 3D-specific polypeptide which comigrated with authentic 3D and of a 3C-specific polypeptide which comigrated with authentic 3C could be detected (Fig. 2). The relative amounts of the cleavage products were not noticeably increased when proteins were labelled for longer times or when pulse–chase experiments were carried out (data not shown). Limited autoproteolytic cleavage of 3CD was evident therefore in Sf9 cells. It is possible that the cleavage of 3CD in this system is limited because of the low temperature at which Sf9 cells are cultivated (27 to 28 °C) relative to a usual poliovirus infection (37 °C).

A protein immunoprecipitated by antibodies directed against either 3C or 3D was also observed in the case of both Gln–Pro Ac–3CD viruses, but this migrated significantly more rapidly than wild-type 3CD (Fig. 2). We attributed this abnormal migration on SDS–polycrylamide gels to the lower temperature at which Sf9 cells are cultivated.

Table 2. Phenotypes of viable 3C/3D cleavage site mutants

<table>
<thead>
<tr>
<th>Phenotypic* properties</th>
<th>Temperature (°C)</th>
<th>Gln/Gly (wild-type)</th>
<th>Gln/Ala</th>
<th>Gln/Ser</th>
</tr>
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<tbody>
<tr>
<td>Temperature sensitivity†</td>
<td>33</td>
<td>8-2</td>
<td>8-7</td>
<td>8-5</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>8-3</td>
<td>8-8</td>
<td>8-3</td>
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<td></td>
<td>39</td>
<td>8-2</td>
<td>8-8</td>
<td>8-5</td>
</tr>
<tr>
<td>Plaque diameter‡</td>
<td>37</td>
<td>1-9</td>
<td>2-1</td>
<td>2-0</td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>2-6</td>
<td>2-7</td>
<td>2-5</td>
</tr>
<tr>
<td>Virus production§</td>
<td>37</td>
<td>8-6</td>
<td>8-3</td>
<td>8-5</td>
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<tr>
<td></td>
<td>39</td>
<td>8-2</td>
<td>8-3</td>
<td>8-7</td>
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<tr>
<td>Level of protein synthesis</td>
<td>37</td>
<td>100</td>
<td>113</td>
<td>97</td>
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<td></td>
<td>39</td>
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<td>110</td>
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<td>Level of viral RNA synthesis¶</td>
<td>37</td>
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<td></td>
<td>39</td>
<td>100</td>
<td>108</td>
<td>52</td>
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<tr>
<td>Heat-stability of virions**</td>
<td>37</td>
<td>1-0</td>
<td>0-6</td>
<td>1-0</td>
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<tr>
<td>Host cell restriction††</td>
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<td></td>
<td>39</td>
<td>0-3</td>
<td>0-3</td>
<td>0-23††</td>
</tr>
</tbody>
</table>

* Phenotypic properties were determined on HeLa cells.
† Titres are expressed as log p.f.u./ml.
‡ The diameter in mm was measured 40 h post-infection.
§ Total virus (logio p.f.u./ml) was recovered after 8 h infection at an m.o.i. of 20 p.f.u./cell.
¶ Levels were measured between 3 and 4.5 h post-infection by incorporation of [35S]methionine into TCA-precipitable material. The results are expressed as a percentage of those obtained after infection with wild-type virus at 37 °C (6.7 x 10^6 c.p.m./7 x 10^4 cells).
† Incorporation of [3H]uridine into TCA-precipitable material in the presence of actinomycin D was measured at 6 h post-infection at 37 °C and 5 h post-infection at 39 °C. Values are expressed as a percentage of the incorporation after infection with wild-type virus (4.2 x 10^4 c.p.m. and 2.8 x 10^4 c.p.m./10^6 cells at 37 °C and 39 °C, respectively).
** The loss of infectivity after heating to 45 °C for 5 min is expressed as logio p.f.u./ml.
†† The relative titre of Vero cells/HeLa cells was determined.
††† Small plaques on Vero cells.
acrylamide gels to the Pro residue substitution, since abnormal migration of poliovirus 3CD after single amino acid substitutions is well documented (Bellocq et al., 1987; Dewalt & Semler, 1987; Kean et al., 1988). However, as we have not sequenced the entire 3D coding region of the pAc–3CD plasmids, the possibility that premature stop codons were introduced into both Gln–Pro mutant pAc–3CD plasmids within 3D cannot be excluded. A faint band corresponding to a product that migrated faster than authentic 3D could be observed when proteins from Sf9 cells infected with the Gln–Pro Ac–3CD viruses were immunoprecipitated by antibodies directed against 3D (Fig. 2). However, it seems unlikely that this reflected cleavage of the substituted 3CD. No corresponding 3C-related cleavage product could be detected even when the gel was highly overexposed (not shown). In addition, increased exposure of the gel revealed the presence of this band in all immunoprecipitations following both wild-type and mutant Ac–3CD infections. In other experiments, a polypeptide migrating at this position was also observed when proteins from baculovirus-infected or mock-infected cells were immunoprecipitated by antibodies directed against 3D (data not shown). It would seem therefore that the Gln–Pro 3CD does not process itself.

Phenotypic properties of the Gln–Ala and Gln–Ser viruses

A wide range of phenotypic properties was studied for each of the two viable 3C/3D junction mutants (Table 2), because the engineered substitutions could be expected to have pleiotropic effects. In general, the two mutant viruses behaved like wild-type virus, both for properties indicative of correct capsid precursor processing and assembly such as the heat stability of capsids, and for properties related to replication such as single-cycle growth kinetics and plaque diameter (Table 2). The only defects observed were for the Gln–Ser mutant, which synthesized relatively low levels of virus-specific RNA and proteins at 39 °C. No such defects were observed at lower temperatures (Table 2). In addition, this mutant had a small plaque phenotype on Vero cells, but again, only at high temperatures (data not shown).

Analysis of protein synthesis in HeLa cells infected with the viable mutants was carried out to investigate whether the substitution of the 3C/3D cleavage site had any effect on the use of this site or on the activity of the protease in vivo. The amino acid sequence of 3CD of these mutants is altered and it has been reported that this polypeptide is the form of the 3C protease required for cleavage of the precursor to the capsid polypeptides (Jore et al., 1988; Ypma-Wong et al., 1988).

The electrophoretic profile of total proteins synthesized in cells infected with the Gln–Ala mutant (Fig. 3) showed that this virus produced all wild-type polypeptides efficiently, including two polypeptides that comigrated with wild-type 3C and 3D and were specifically immunoprecipitated by antibodies directed against 3C and 3D (see Fig. 4). Strikingly, the presence of two additional virus-specific polypeptides was observed systematically. One of these migrated slightly more
Fig. 4. 3C-related and 3D-related proteins in cells infected with the Gln-Ala mutant. HeLa cells infected at 37 °C by wild-type poliovirus (lane 1) or by the Gln-Ala mutant (lanes 2 and 3) were labelled with [35S]methionine between 3 and 4.75 h post-infection. Aliquots of cytoplasmic extracts corresponding to $10^5$ cells were immunoprecipitated with antibodies directed against poliovirus 3C (a, 3C) or 3D (b and c, 3D) before electrophoresis on 15% SDS–polyacrylamide gels. Alternatively, extracts corresponding to $10^4$ cells were analysed directly (total). (c) is an overexposure of the bottom part of the gel shown in (b). See text for explanation of arrows. $M$, markers (M) are shown in (a).
slowly than polypeptide 3C while the other migrated slightly more rapidly than polypeptide 'P3-7d' (arrows, Fig. 3). 'P3-7d' corresponds to the N-terminal part of polypeptide 3D, being generated by cleavage at the 3C/3D junction and at the 2A-specific site between 3C' and 3D' (Pallansch et al., 1984). Immunoprecipitation of the Gln-Ala mutant polypeptides (Fig. 4) showed that the polypeptide which migrates more slowly than polypeptide 3C was specifically immunoprecipitated by antibodies directed against polypeptide 3C (Fig. 4a) but not by antibodies directed against polypeptide 3D (Fig. 4b). The converse appeared to be true of the polypeptide which migrates more rapidly than 'P3-7d', although gels had to be overexposed even for total cytoplasmic extracts, to demonstrate the presence of the 'P3-7d' doublet (Fig. 4, compare panels b and c). Neither of these two polypeptides was immunoprecipitated by antibodies directed against polypeptide 3B (data not shown).

The analysis of total proteins synthesized in cells infected by the Gln-Ser mutant showed that polypeptides 3C and 3D were present in reduced amounts (data not shown). Therefore, pulse-chase analysis was used to examine more precisely processing of the mutant polypeptides (Fig. 5). All processing events were carried out efficiently, with the exception of cleavage at the 3C/3D junction. Inefficient cleavage at this substituted site was indicated by the low levels of the three polypeptides that are generated by use of this site i.e. polypeptides that comigrate with wild-type polypeptides 3C, 3D and, on overexposure of gels (not shown), polypeptide 'P3-7d'. The identity of these three polypeptides was confirmed by immunoprecipitation with 3C- and 3D-specific antibodies (data not shown). Surprisingly, the cleavage defect was observed at both 37 °C and at 39 °C although it was less pronounced at 37 °C (Fig. 5), whereas the phenotypic defects of the Gln-Ser mutant were observed only at the higher temperature. Interestingly, no secondary processing defects were observed. This contrasts with defects of P2 processing that we have previously reported as being exhibited by a different mutant defective for cleavage at the 3C/3D junction (Kean et al., 1988).

**Discussion**

The discovery that the cDNA of poliovirus is infectious (Racaniello & Baltimore, 1981) has allowed precise
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3C-specific site has ever been described for any picornavirus (A. Palmenberg, personal communication). We are currently investigating whether the substituted 3CD precursor has retained the ability to cleave at other sites of the polyprotein in trans. It would also be interesting to verify that this site cannot be cleaved by exogenously supplied wild-type protease.

The genomic cDNA which carries the Gln–Val substitution was also non-infectious. However, revertant virus was obtained sporadically after transfection of primate cells with this cDNA. It should be noted that the three independent clones of such revertants sequenced corresponded to primary site revertants to Gln–Ala. True revertants to Gln–Gly were not observed, although the change of the original Val codon to either Ala or Gly would require only a single base change (see Table 1). To our knowledge, such reversion has never been reported after transfection with cDNA molecules, although it has been described after transfection with non-infectious poliovirus RNA transcripts which were replication-competent (Marc et al., 1989). It was suggested that reversion could have occurred during RNA replication in the transfected cells (Marc et al., 1989). We have also observed extremely rapid reversion after transfection with a mutant cDNA that encodes a minute plaque poliovirus (K. M. Kean, N. Teterina & M. Girard, unpublished results). These observations suggest that the Gln–Val mutant would be capable of some limited replication during which spontaneous reversion could occur. This implies some slight cleavage at the Gln–Val site to liberate the viral replicase. To address this question we inserted the substituted 3CD into a baculovirus vector, but the corresponding recombinant virus could not be isolated, despite extensive screening. Experiments are in progress to analyse the autoproteolytic cleavage of the Gln–Val 3CD in other expression systems.

Both the Gln–Ala and Gln–Ser substitutions were viable, although in both cases the protein processing profiles observed were abnormal. In the case of the Gln–Ala substitution, two additional polypeptides were observed. Their apparent Mr, and their specific reactions with antibodies directed against polypeptides 3C and 3D (see Fig. 4) suggested that these polypeptides could be the result of a new alternative cleavage within the N-terminal part of polypeptide 3D instead of at the C terminus of polypeptide 3C. It remains to be determined whether these polypeptides arise because of a change in the specificity of an active substituted 3C–protease precursor, or whether they reflect the unmasking of a cryptic cleavage site in the substituted polyprotein. However, there are no alternative Gln–Gly or Tyr–Gly sites within this part of the polyprotein. The most likely candidate for a 3C-specific cleavage site within this region would be a Glu–Gly dipeptide 35 amino acids after the start of polypeptide 3D. Such a sequence, in the correct context, can be recognized as a 3C cleavage site in the case of numerous other picornaviruses (Arnold et al., 1987). The context of the specific Glu–Gly dipeptide in question does not resemble closely any single 3C-specific poliovirus cleavage site, but the residues in positions P3 (V), P2 (F), P1 (E), P1' (G) and P2' (V) could be considered to form a composite cleavage site. However, it should be noted that the residue found in position P4 (Y) does not conform to the accepted consensus (see Wellink & van Kammen, 1988).

The protein profiles observed for the Gln–Ser mutant indicated that cleavage at the substituted site occurred inefficiently. Interestingly, this defect was observed both
at 37 °C and at 39 °C, whereas slight defects in the levels of protein and RNA synthesis could be detected at 39 °C but not at 37 °C. A similar temperature-dependent defect in RNA synthesis has been described by Dewalt & Semler (1989) in the case of a 3C mutant which exhibits a temperature-independent 3C/3D cleavage defect. These authors suggest that this pleiotropic phenotype may reflect a role of 3CD in viral replication and that the effects on viral replication of misfolding of this precursor may be exacerbated at increased temperature. It could also be proposed that in the case of the Gln–Ser mutant enough of the 3D replicase is produced to ensure a normal level of viral replication at 37 °C, whereas this would not be the case at 39 °C. However, it cannot be excluded that the replication defects of this mutant are the result of a second, unidentified mutation. It should be noted that we have only studied a single clone of the Gln–Ser mutant and the sequence of its entire genome has not been established.

Although we have no formal proof that the Gln–Ala and Gln–Ser mutants are effectively cleaved at the substituted 3C/3D junctions, this seems to be the most likely explanation of the observed protein profiles. In particular, production of an authentic 3C polypeptide is strongly indicated by the existence of polypeptides which comigrate with the wild-type 3C polypeptide and which are specifically immunoprecipitated with antibodies directed against polypeptide 3C.

Thus, our data strongly suggest that the poliovirus 3C protease is capable of processing dipeptides that are not found naturally at the 3C-specific cleavage sites when these residues are positioned in normal cleavage site contexts. This is similar to results reported for processing by the EMC virus protease in vitro (Parks & Palmenberg, 1987; Parks et al., 1989). The dipeptides processed (Gln–Ser, Gln–Ala and perhaps Gln–Val) are all recognized cleavage sites for other picornaviruses (Arnold et al., 1987; A. Palmenberg, personal communication). Nevertheless, it must be emphasized that our results reflect the analysis of cleavage at the poliovirus 3C/3D junction only, a cleavage event that has been proposed to be monomolecular (Hanecak et al., 1984). It would be interesting to extend these experiments to include sites that are cleaved only in trans. In this context, it should be noted that the deletion of amino acids one to four of the capsid polypeptide VP1, which changes the VP3/VP1 3C-dependent cleavage site to a Gln–Met dipeptide, has no apparent effect on proteolytic processing (Kirkegaard & Nelson, 1990).

We thank Pascale Gonet for the gift of pVL941–poly and for helpful advice during the baculovirus expression experiments and Philippe Desprès for designing the structural map of the baculovirus transfer vectors. We are grateful to Ann Palmenberg for kindly communicating her unpublished protein alignments. We appreciate the gifts of pEXCalibur from Bruce Korant, of anti-3D antibodies from Cara Burns and Ellie Ehrenfeld and of anti-3C antibodies from Eckard Wimmer. We are particularly grateful to Sylvie van der Werf for critical reading of the manuscript and for the gift of pSW3004. This work was supported in part by grant 87.C.0421 from the MRT. N.T. was the recipient of a FEBS fellowship.

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