JOURNAL OF VIROLOGY, June 1992, p. 3330–3338
0022-538X/92/063330-09$02.00/0

Vol. 66, No. 6

Determinants of Substrate Recognition by Poliovirus 2A Proteinase

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Received 16 December 1991/Accepted 27 February 1992

Poliovirus proteinase 2A ($2A^{pro}$) is autocatalytically released from the viral polyprotein by cleavage in *cis* of a Tyr-Gly dipeptide at its own amino terminus, resulting in separation of the P1 structural and P2-P3 nonstructural protein precursors. A second Ty-Gly dipeptide within 3D polymerase is cleaved by $2A^{pro}$ in *trans*, but this is not essential for viral proliferation. The mechanism which limits cleavage to only 2 of the 10 Tyr-Gly dipeptides within the poliovirus polyprotein has not been characterized. We have therefore undertaken a systematic mutational analysis of the VP1-2A site to elucidate determinants of substrate recognition by $2A^{pro}$. The *P2* and *P1'* positions are important determinants for *cis* cleavage of this site, whereas a variety of substituents could be tolerated at the *P2'*, *P1*, and *P3* positions. The requirements for *trans* cleavage of this site were more stringent. We found that the $2A^{pro}$ of coxsackievirus type A21 and rhinoviruses 2 and 14 have stringent requirements similar to those of poliovirus $2A^{pro}$ for cleavage in *trans*.

The poliovirus genome encodes a single large polyprotein from which its capsid and nonstructural proteins are derived by proteolytic processing (26). All but one of the processing steps are catalyzed by the virally encoded proteinases $2A^{pro}$, $3C^{pro}$, and $3CD^{pro}$, which are themselves part of the polyprotein (11, 14). The initial rapid cleavage at the P1-$2A^{pro}$ junction separates the P1 capsid protein precursor from the nascent polyprotein. It results from cleavage at a Y-G dipeptide by $2A^{pro}$ at its own amino terminus (Fig. 1B) (44). This probably occurs in *cis* and is a prerequisite to subsequent proteolytic processing of P1 and consequently to assembly of the poliovirus capsid (16, 34). A second cleavage site recognized by $2A^{pro}$ lies within the viral 3D polymerase sequence (Fig. 1B); cleavage also occurs at a Y-G dipeptide, probably in *trans*, and leads to the appearance of the proteins 3C' and 3D', but it is not essential for viral proliferation (31).

A second function of 2A proteinase is in the rapid shutoff of host cell protein synthesis which occurs on infection with poliovirus and which is associated with proteolytic cleavage of the 220-kDa γ component of eukaryotic translation initiation factor 4F (eIF-4F) (3, 10, 28). $2A^{pro}$ is not directly responsible for cleavage of eIF-4Fγ but probably activates a latent cellular proteinase, which then cleaves eIF-4Fγ to produce three or four antigenically related polypeptides of 110 to 130 kDa (13, 28, 46, 48). Activation is likely to result from a proteolytic event, but neither the site of proteolytic cleavage nor even the putative cellular proteinase has been identified (46).

2A proteinases are characteristically encoded by all entero- and rhinoviruses immediately downstream of the P1 capsid precursor (41), and all are presumed to cleave the VP1-2A bond autocatalytically (40, 44) and to induce cleavage of p220 (9, 10, 32). The primary amino acid sequences of the various 2A proteinases are similar, and sequence alignment suggests that they may be structurally related to small trypsinlike serine proteinases, such as the α-lytic protease of *Lysobacter enzymogenes* (1). The presence of a cysteine residue in the catalytic triad of 2A proteinases in place of the serine residue that is characteristic of cellular trypsinlike proteinases is consistent with the results of inhibitor assays (27) and is supported by the results of site-directed mutagenesis (13, 40, 48).

Little is known about the specificity of 2A proteinases because, although they are all presumed to activate the same latent cellular p220-specific proteinase by a similar proteolytic mechanism (so that a similar cleavage specificity might be envisaged), there are in fact considerable differences between the amino acid residues flanking the various VP1-2A scissile bonds (Fig. 1C) (12, 39). Cleavage by $2A^{pro}$ of the poliovirus type 1 (Mahoney) [PV1(M)] polyprotein occurs at two Y-G dipeptides (between P1 and P2 and within $3D^{pol}$), yet there are eight additional Y-G dipeptides within it that are not cleaved. The mechanism which limits proteolytic processing to specific sites has not been fully characterized, but by analogy with other viral proteinases (6, 7, 23), it is likely that substrate recognition by poliovirus $2A^{pro}$ is determined both by the amino acids flanking the scissile bond and by the structural context of the cleavage site. Lee and Wimmer (31) noted that both bona fide $2A^{pro}$ cleavage sites are preceded by a Thr residue and have a Leu residue at the *P4* position, and in fact the residues at the *P4*, *P2*, *P1*, and *P1'* positions of the VP1-$2A^{pro}$ cleavage site have been found to be conserved in over 60 isolates of the three different serotypes of poliovirus (25, 35) (Fig. 1A). (In the nomenclature of Berger and Schechter [2], the newly generated carboxyterminus, after cleavage of the peptide bond, is designated *P1*, preceded by the *P2* residue, etc., and the newly generated amino terminus is designated *P1'*, followed by the *P2'* residue, etc.) A T-147→A substitution in 3D (which corresponds to a change at the *P2* position of the 3C'-3D' cleavage site) abolished proteolytic cleavage in vivo, whereas a Y-148→F substitution at the *P1* position had no effect (31). The specificity of the interaction of $2A^{pro}$ with its substrate may therefore vary at different subsites.

We have undertaken a systematic and extensive mutational analysis of the VP1-2A junction of PV1(M) to characterize the determinants of cleavage site recognition and processing by $2A^{pro}$ in *cis* of substrates expressed by translation in vitro, some results of which we have reported

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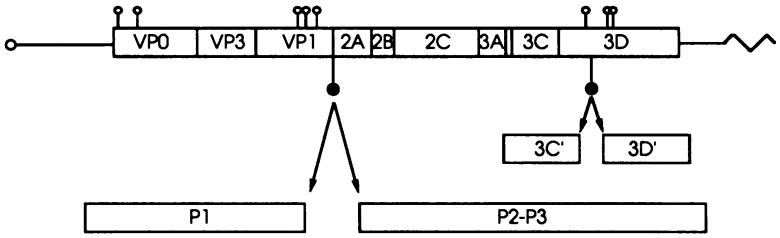
A

Poliovirus isolates	Amino acid sequence																			
	P10				*P5*					*P1*	*P1'*			*P5'*						*P10'*
PV1/Mahoney	P	L	S	T	K	D	L	T	T	Y	G	F	G	H	Q	N	K	A	V	Y
PV1/1266/USA 79	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-	-	-
PV1/16006/IND 82	-	-	-	-	-	-	-	-	-	-	-	Y	-	-	-	-	R	-	-	-
PV1/7231/ELS 87	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-
PV1/6535/NEP 86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-
PV2/SABIN 2/USA 54	-	-	P	E	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PV2/LANSING/V667	-	-	P	G	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PV2/11-710/KEN 71	-	-	P	E	-	G	-	-	-	-	-	L	-	-	-	-	-	-	-	-
PV2/W-2	-	-	P	E	-	G	-	I	-	-	-	-	-	-	-	-	-	-	-	-
PV3/SABIN/USA 37	-	-	-	E	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PV3/SAUKETT/USA 52	-	-	-	A	-	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PV3/USOL-D-bac	-	-	-	V	-	G	-	-	-	-	-	Y	-	-	-	-	-	-	-	-
PV3/6879/COL 86	-	-	-	-	-	G	-	-	-	-	-	Y	-	-	-	-	-	-	-	-
PV3/7840/PER 86	-	-	-	-	-	G	-	-	-	-	-	-	-	A	-	-	-	-	-	-

B



C

Picornavirus	Amino acid sequence																			
	P10				*P5*					*P1*	*P1'*			*P5'*						*P10'*
PV1/Mahoney	P	L	S	T	K	D	L	T	T	Y	G	F	G	H	Q	N	K	A	V	Y
HRV2	I	V	T	R	P	I	I	-	-	A	-	P	S	D	M	Y	V	H	G	N
HRV14	K	K	R	K	G	-	I	K	S	-	-	L	-	P	R	Y	G	G	I	-
CAV21	L	T	K	V	D	S	I	-	-	F	-	-	-	-	-	-	-	-	-	-

FIG. 1. Processing of picornavirus polyproteins by 2A proteinase. (A) Amino acid sequence at the poliovirus VP1 capsid protein/2A proteinase boundary. The VP1-2A cleavage site of PV1(M) is shown (26) in the single-letter amino acid code. Sequences of other isolates are described in references 25, 30, and 35. Dashes indicate amino acid residues identical to those shown for PV1(M). The position of residues is described by the nomenclature of Berger and Schechter (2). (B) Schematic representation of the poliovirus polyprotein to show sites of proteolytic processing by 2A proteinase. Cotranslational intramolecular cleavage by $2A^{pro}$ occurs at a Y-G dipeptide at its amino terminus. Intermolecular cleavage by $2A^{pro}$ occurs at a Y-G dipeptide within 3CD to yield 3C' and 3D'. Tyr-Gly dipeptides that are cleaved by $2A^{pro}$ are indicated by solid circles below the genome, whereas those that are not cleaved are indicated by smaller open circles above the genome. (C) Amino acid sequence at the VP1 capsid protein/2A proteinase boundary of the four picornaviruses used in this study. The VP1-2A cleavage site of PV1(M) is shown (26) in the single-letter amino acid code. The sequences of HRV2, HRV14, and CAV21 are described in references 38, 4, and 19, respectively. Dashes indicate amino acids identical to those shown for PV1(M). The position of residues is described by the nomenclature of Berger and Schechter (2).

previously (15). We have used these and similar substrates to distinguish between the requirements for cleavage in *cis* and in *trans* by homologous $2A^{pro}$ as well as to distinguish between the requirements for cleavage in *trans* by $2A^{pro}$ encoded by poliovirus, coxsackievirus A21 (CAV21), and human rhinoviruses 2 (HRV2) and 14 (HRV14).

MATERIALS AND METHODS

Genetic engineering of DNA. Restriction enzymes and DNA-modifying enzymes were purchased from New England BioLabs, Bethesda Research Laboratories, Inc., Stratagene, and Boehringer Mannheim. Sequencing primers and mutagenic oligonucleotides were synthesized on an Applied Biosystems apparatus. DNA manipulations were done by standard procedures (36).

Plasmid construction and site-directed mutagenesis. To analyze the effects of substitution of residues surrounding the VP1-2A scissile bond on its autocatalytic cleavage by $2A^{pro}$, a vector, pBS^{-}(VP1-2A), was constructed from which mRNA that encodes $2A^{pro}$ in the form of a VP1-2A zymogen can be transcribed in vitro. A termination codon

(UGA) was introduced immediately after the sequence encoding $2A^{pro}$ in pBS$^-$(VP1-2ABΔ2C) (13) by site-directed mutagenesis with the deoxyoligonucleotide 5′-GGTGAT TCATTGTTC-3′ and the method described below. Thus, pBS$^-$(VP1-2A) contains PV1(M) cDNA encoding two amino acids of VP3, all of VP1, all of 2A (nucleotides [nt] 2474 to 3827), a termination codon in place of the first amino acid of 2B, the remainder of 2B, and part of 2C (nt 3831 to 4600), placed under the control of part of the encephalomyocarditis virus (EMCV) 5′ nontranslated region (nt 260 to 841). These poliovirus and EMCV cDNA segments were cloned between the *Eco*RI and *Bam*HI sites in the polylinker region of pBluescribe M13(−) (Stratagene), which contains an M13 origin as well as a ColE1 origin and a *bla* gene for amplification in *Escherichia coli*.

Single-stranded DNA substituted with uracil was prepared by passage through *E. coli* BW313 (*dut ung*) by the method of Kunkel (29). Mismatched, partly degenerate synthetic deoxyoligonucleotides were annealed to the DNA, and second-strand synthesis and transformation into *E. coli* C600 were done as described previously (29). Mutant sequences were identified by sequence analysis by the dideoxynucleotide chain termination method (37).

To analyze the ability of $2A^{pro}$ to cleave wild-type (wt) and mutated variants of the VP1-2A cleavage site in *trans*, a series of vectors were constructed by ligating the smaller of the two *Pst*I fragments of pBS$^-$(VP1-2ABΔ2C)(2A Cys-109→Ala) (13) to the larger of the two fragments generated from various mutated derivatives of pBS$^-$(VP1-2A) after digestion with the same enzyme. The partial poliovirus polyproteins encoded by mRNA transcripts derived from this series of plasmids therefore contain substitutions at one residue of the VP1-2A cleavage site and an alanine residue in place of cysteine 109 of $2A^{pro}$. This residue is a component of the putative catalytic triad of $2A^{pro}$, and its substitution by alanine inactivates $2A^{pro}$ (13).

Substitutions at the VP1-2A cleavage site were introduced into a full-length poliovirus transcription vector, pT7XL (a derivative of pT7PV1-5 [45]), by a two-stage cloning procedure, which was necessary to replace the termination codon that follows the 2A coding sequence in pBS$^-$(VP1-2A) by the first amino acid of 2B. Initially, the smaller of the two *Pst*I fragments of the desired derivative of pBS$^-$(VP1-2A) was ligated to the larger of the two *Pst*I fragments of pBS$^-$(VP1-2ABΔ2C) (13). The smaller of the two *Bst*EII fragments of the resulting plasmid was then ligated to the larger of the two *Bst*EII fragments of pT7XL.

The presence of nucleotide substitutions in all plasmids that included cDNA fragments derived from mutated versions of pBS$^-$(VP1-2A) was confirmed by sequence analysis.

Transcription, translation, and cleavage assays. All but one of the derivatives of plasmids pBS$^-$(VP1-2A) and pBS$^-$(VP1-2ABΔ2C)(2A Cys-109→Ala) were linearized by digestion with *Bam*HI and transcribed in vitro with T7 RNA polymerase. The exception was pBS$^-$(VP1-2A)(2A Phe-2→Ser), which contains a *Bam*HI site within $2A^{pro}$ and was therefore linearized with *Pvu*II. Synthetic mRNA transcripts were translated in a rabbit reticulocyte lysate (RRL; Promega) in the presence of [^{35}S]methionine for 60 min at 30°C. The resulting products and those proteins immunoprecipitated by sera raised against VP1 and $2A^{pro}$ were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34). Preparation of antisera was described previously (34, 44).

To assay cleavage of the VP1-2A bond by $2A^{pro}$ in *trans*, mutated VP1-2ABΔ2C precursors were translated for 60 min at 30°C in the presence of [^{35}S]methionine. RNase A, cycloheximide, and methionine were added to 3-μl aliquots of each translation reaction mix to final concentrations of 10 g/ml, 1 mg/ml, and 0.5 mM, respectively. Translation mixtures were mixed with 3-μl aliquots of S10 fractions of uninfected, enterovirus-infected, or rhinovirus-infected HeLa cell lysates, as indicated in the text. Mixtures were incubated for 90 min at 30°C. The amounts of the VP1-2A precursor and of the cleavage products generated by *cis* or *trans* cleavage were quantitated by scanning X-ray films with an LKB laser densitometer.

Preparation of HeLa cell postmitochondrial (S10) fractions. Postmitochondrial supernatants (S10) from mock-infected HeLa cells or HeLa cells infected with PV1(M), CAV21, HRV2, or HRV14 were prepared essentially as described before (5). The sources of virus isolates have been described previously (8).

Approximately 5×10^8 HeLa S3 spinner cells were harvested after incubation for 4 h at 37°C after infection with 250 PFU of PV1(M) or HRV2 per cell or after incubation for 5.5 h at 34°C after infection with 250 PFU of HRV14 or CAV21 per cell. Cells were swollen for 15 min hypotonic buffer (10 mM NaCl, 5 mM dithiothreitol, 1.5 mM $MgCl_2$, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.5]) and disrupted by Dounce homogenization. Cell debris and mitochondria were removed by centrifugation at 10,000 × *g* for 15 min. The supernatant extracts were adjusted to 10% (vol/vol) glycerol and stored at −80°C.

Characterization of poliovirus mutants. HeLa R19 cells were maintained as monolayers in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (GIBCO). Wt PV1(M) was derived from a single plaque, resulting from transfection with RNA transcripts from the poliovirus cDNA clone pT7XL. Derivatives of pT7XL that encoded single amino acid substitutions at the VP1-2A cleavage site were linearized with *Eco*RI and transcribed with T7 polymerase in vitro. Synthetic mRNA transcripts were transfected into HeLa cells by the DEAE-dextran method (45), and HeLa cells were then incubated at 37°C. Medium and debris from transfected cells were collected when cytopathic effects (CPE) became apparent or after 3 days, as appropriate, and virus was released by three cycles of freeze-thawing. Cell debris was removed by low-speed centrifugation, and the supernatant was stored as the viral stock. Virus contained in the stock was plaque-purified three times and amplified on monolayers of HeLa R19 cells. Plaque assays were carried out in 35-mm petri dishes under semisolid medium (0.9% agarose). Cells were incubated at 37°C for 40 to 50 h before being stained with crystal violet (18). RNA was extracted from purified virus particles, and its nucleotide sequence in the vicinity of the VP1-2A cleavage site was determined by the dideoxynucleotide method with avian myeloblastosis virus reverse transcriptase (Promega Biotec) and the oligonucleotide primer 5′-CCTG AGTGGCCAAGTGGTAGTTGC-3′ [which is complementary in sequence to nt 3435 to 3458 of PV1(M)]. Virus yields were determined by plaque assay, and protein synthesis was examined by pulse-labeling with a mixture of [^{35}S]cysteine and [^{35}S]methionine (Translabel; ICN Biomedicals, Inc.) as described by Lee and Wimmer (31).

TABLE 1. Efficiency of processing of mutated VP1-2A cleavage sites in *cis* by poliovirus $2A^{pro}$[a]

Site	Residues	Cleavage efficiency (%)	Viable virus produced[b]
WT	TY↓GF	94	+
P2'	TY↓GS	93	
P1'	TY↓A	85	
	TY↓S	73	
	TY↓C	32	
	TY↓L	31	
	TY↓T	23	
P1	TL↓G	95	
	TF↓G	94	
	TI↓G	94	
	TR↓G	92	
	TQ↓G	91	
	TP↓G	23	−
P2	AY↓G	94	
	SY↓G	93	+
	NY↓G	91	
	QY↓G	90	
	PY↓G	83	
	IY↓G	50	Revertant
	LY↓G	50	Revertant
	EY↓G	22	
	RY↓G	14	
P3	STY↓G	91	
P5	ALTTY↓G	92	

[a] Amino acid residues that differ from those constituting the wt VP1-2A boundary in PV1(M) are underlined; the scissile bond is indicated by an arrow. Efficiency of cleavage is defined as the amount of authentic VP1 and 2A produced after incubation of translation mixtures for 1 h at 30°C as a a percentage of all translation products (whether cleaved normally, aberrantly, or not at all). Values given are the means of three to five independent assays.

[b] Viable virus was obtained after transfection of HeLa cells with three of four mutated derivatives of pT7XL and is described as + if the mutated amino acid residue was maintained and revertant if the residue reverted to wt.

RESULTS

Generation of mutants. To assay the effects of substitution of residues surrounding the VP1-2A scissile bond on its autocatalytic cleavage by $2A^{pro}$, we constructed a vector, pBS^-(VP1-2A), which encodes the enzyme as part of a precursor that consists of the VP1 structural protein and $2A^{pro}$. This precursor was placed under the translational control of nt 260 to 841 of the 5′ untranslated region of EMCV RNA in such a way that initiation of translation occurred at AUG-11, the authentic initiation site (22). This portion of the EMCV 5′ untranslated region has previously been shown to direct highly efficient translation of poliovirus proteins in RRL (11, 13, 28) and renders the in vitro-synthesized mRNA cap independent for translation (20, and references therein).

Single amino acid substitutions at the *P5*, *P3*, *P2*, *P1*, *P1'*, and *P2'* positions of the VP1-2A cleavage site of PV1(M) were made by site-directed mutagenesis (Table 1), and mutations were identified by nucleotide sequence analysis.

Autocatalytic processing of the VP1-$2A^{pro}$ precursor and a mutated derivative. The wild-type 50-kDa precursor encoded by pBS^-(VP1-2A) was cleaved to yield equimolar amounts of 33.5-kDa VP1 and 17-kDa $2A^{pro}$ cleavage products during translation in RRL (Fig. 2, lane 2). The identities of these two products were confirmed by immunoprecipitation with antiserum directed against VP1 or 2A (Fig. 2, lanes 3 and 4). A small amount of uncleaved VP1-2A precursor was always apparent, but this never exceeded 5% of the total. A similar residual proportion was also observed when the VP1 coding sequence was replaced by that of the entire P1 capsid protein precursor and when the 2A coding sequence was replaced by that of most of the P2 nonstructural protein precursor (data not shown). We therefore concluded that translation of a VP1-2A precursor provided a reliable assay for analysis of autocatalytic cleavage of the VP1-2A bond by $2A^{pro}$.

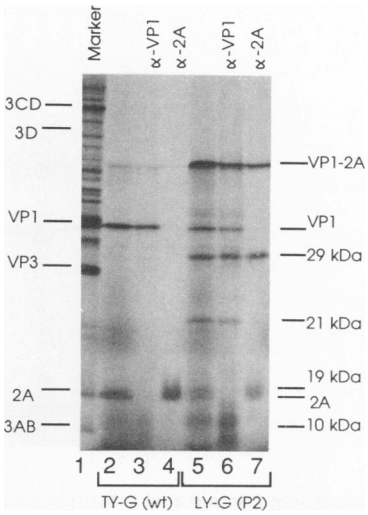


FIG. 2. Products of normal and aberrant proteolytic cleavage of the VP1-2A precursor polyprotein. RNAs transcribed from pBS^-(VP1-2A) (lanes 2 to 4) and the mutant derivative pBS^-(VP1-2A)[VP1(T-301→L)] (lanes 5 to 7) were translated for 1 h at 30°C in RRL. Aliquots (3 μl) were removed from both translation mixtures prior to immunoprecipitation (lanes 2 and 5). Similar aliquots were immunoprecipitated with antiserum raised against VP1 (lanes 3 and 6) and against 2A (lanes 4 and 7). The translation products were resolved by electrophoresis in a 12.5% polyacrylamide gel. The gel was dried and exposed to X-ray film at −80°C for 24 h. A number of poliovirus-encoded proteins are indicated at the left-hand side; the VP1-2A precursor and the various cleavage products are indicated on the right-hand side. Aberrant cleavage products are identified by molecular mass. The marker is a [^{35}S]methionine-labeled cell lysate prepared from HeLa cells infected with PV1(M).

A number of mutated precursors were processed in an aberrant manner, yielding reduced levels of VP1 and 2A and various amounts of four novel polypeptides of 10, 19, 21, and 29 kDa (Fig. 2, lane 5). Aberrant cleavage correlated with accumulation of the mutated VP1-2A precursors and was presumably a consequence of inhibition of cleavage at the bona fide VP1-2A site. The 10-kDa and 21-kDa polypeptides were antigenically related to VP1, the 19-kDa polypeptide was antigenically related to 2A, and the 29-kDa polypeptide was antigenically related to both VP1 and 2A (Fig. 2, lanes 6 and 7). Similar aberrant cleavage of these mutants (for example, resulting in the appearance of the 29-kDa polypeptide) was observed when the VP1 coding sequence was replaced by that of the entire P1 capsid protein precursor (data not shown). We conclude that VP1 contains two cryptic sites at which cleavage can occur if scission at the bona fide cleavage site is inhibited. Cleavage between the Tyr-187–Gly-188 and Tyr-280–Gly-281 pairs in VP1 (which

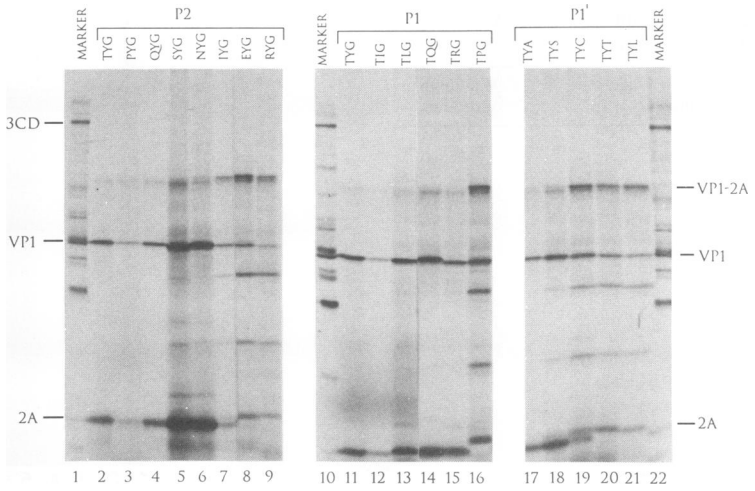


FIG. 3. Effects of single amino acid substitutions at the *P2*, *P1*, and *P1'* positions of the VP1-2A cleavage site on autocatalytic processing of a VP1-2A polyprotein precursor. Aliquots (3 μl) of RRL translation mixture containing products translated from transcripts of wt pBS$^-$(VP1-2A) and representative mutated derivatives thereof were resolved by electrophoresis in 12.5% polyacrylamide gels. Gels were dried and exposed to X-ray film at −80°C for 24 h. Lanes are labeled to indicate the amino acid substitutions made at the VP1-2A boundary. The primary translation product (VP1-2A) and the VP1 and 2A cleavage products are indicated on the right-hand side. The marker is a [^{35}S]methionine-labeled cell lysate prepared from HeLa cells infected with PV1(M).

are preceded by Thr and Tyr residues, respectively) would be compatible with the observed molecular masses of the aberrant cleavage products. However, we have not yet confirmed the exact sites of cleavage by direct determination of the amino-terminal amino acid sequences of these novel species.

Effect of cleavage site variation on processing of mutated VP1-2A in *cis*. The Gly residue at the *P1'* position of the VP1-2A cleavage site is conserved in all isolates of the three serotypes of poliovirus (Fig. 1A) and indeed in all other members of the enterovirus and rhinovirus genera examined to date (12) (Fig. 1C). To examine the influence of the *P1'* position on the efficiency of autocatalytic cleavage by 2A^{pro}, this Gly residue was systematically substituted by the amino acid residues shown in Table 1. Substitution of Gly by Ala (which is only slightly larger by virtue of its methyl group side chain) resulted in a small reduction in cleavage efficiency. Substitution by increasingly larger residues (Ser, Cys, Leu, and Thr) resulted in a proportionately greater decrease in cleavage efficiency (Table 1; Fig. 3, lanes 18 to 21). Thus, the presence of a Thr residue at the *P1'* position reduced the efficiency of cleavage to a quarter of that at the wt cleavage site. Replacement of the Phe residue at the *P2'* position by a Ser residue did not affect the extent (Table 1) or the fidelity (data not shown) of proteolytic processing.

The *P1* position tolerated the presence of a variety of residues without significantly affecting the extent of proteolytic *cis* cleavage. Thus, substitution of the Tyr residue (which occurs in the *P1* position in all poliovirus isolates) by Leu or Ile (which have aliphatic side chains; Fig. 3, lanes 12 and 13), by Phe (which has an aromatic side chain; Table 1 and data not shown), by Arg (which has a basic side chain; Fig. 3, lane 15), or by Glu (which has a neutral polar side chain; Fig. 3, lane 14) did not reduce the extent of cleavage by more than 5%. However, the presence of proline in the *P1* position reduced cleavage fourfold (Table 1; Fig. 3, lane 16).

The *P2* position was less tolerant of substitution than the *P1* position. Whereas introduction of Leu or Ile residues at the *P1* position had no effect (see above), introduction of these residues at the *P2* position halved the extent of cleavage (Table 1). The presence of residues with charged side chains (e.g., Glu or Arg) reduced the efficiency of cleavage four- to sixfold. Substitution of the wt Thr residue by a number of amino acid residues with small side chains (Ala, Ser, Asn, Gln, or Pro) caused only minor reductions in *cis* cleavage efficiency.

Processing of mutated VP1-2A cleavage sites in *trans*. A second cleavage site within the poliovirus polyprotein that is recognized by 2A^{pro} lies within the viral 3D polymerase sequence. Substitution of the wt Thr residue by an Ala residue at the *P2* position was sufficient to block *trans* cleavage at this Tyr-Gly dipeptide (31). In light of the results presented above (in particular, the lack of effect of an identical Thr-Ala substitution at the *P2* position of the VP1-2A cleavage site on the extent of cleavage at that site in vitro), it would appear that the requirements for cleavage by 2A^{pro} in *trans* are more stringent than for cleavage in *cis*. To investigate intermolecular cleavage of the VP1-2A scissile bond further, we have used an approach initially developed by Nicklin et al. (34): incubation of a polyprotein precursor that is incapable of cleavage in *cis* with a source of exogenous proteinase. We therefore introduced an additional inactivating Cys-109→Ala mutation (15) into the 2A^{pro} sequence of VP1-2ABΔ2C polyprotein precursors that already contained a mutation at the VP1/2A boundary. Such polyproteins were then incubated with postmitochondrial S10 extracts of uninfected HeLa cells or with S10 extracts of HeLa cells that had been infected with PV1(M), CAV21, HRV2, or HRV14. The presence of active 2A^{pro} in each of these virus-infected extracts was verified by their ability to induce cleavage of eIF-4Fγ (data not shown).

We and others have previously noted that in addition to the expected VP1-2ABΔ2C precursor, several unexpected polypeptide species appeared on translation of mRNA transcripts containing mutations in the coding sequence of 2A^{pro} (13, 48). As in these previous experiments, we found that the 2ABΔ2C cleavage product was slightly less prominent than expected (Fig. 4), which may be due to proteolytic degrada-

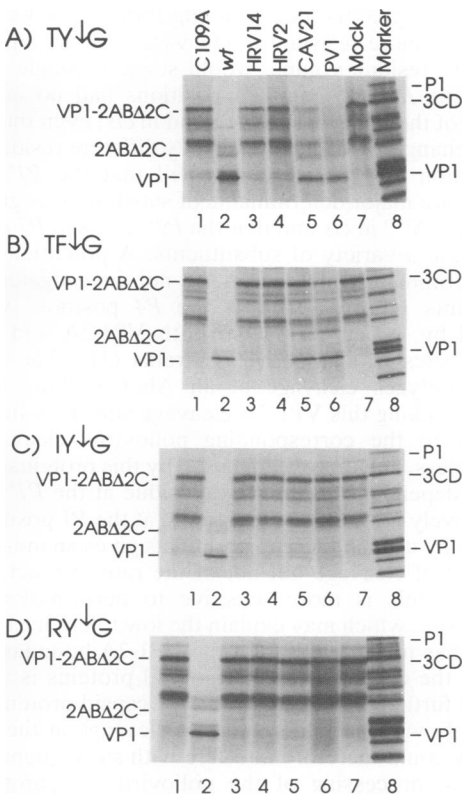


FIG. 4. Effect of incubation of wt and mutated VP1-2ABΔ2C precursors with uninfected and virus-infected HeLa S10 fractions. Aliquots (3 μl) of translation mixtures containing products translated from wt pBS⁻(VP1-2ABΔ2C) are shown in lane 2 of panels A through D. Lanes 1 and 3 to 7 of panel A show products translated from mRNA transcripts of a derivative of pBS⁻(VP1-2ABΔ2C) that contains a proteinase active-site Cys-109→Ala mutation and is inactive in *cis* cleavage. The products in the corresponding lanes of panels B, C, and D are derived from variants of this Cys-109→Ala plasmid with additional changes: Tyr to Phe at the *P1* position (B), Thr to Ile at the *P2* position (C), and Thr to Arg substitutions at the *P2* position (D). The RRL and translation products in lanes 1 and 2 of all panels received no further additions; the RRL and translation products in lanes 3, 4, 5, 6, and 7 had been mixed with equivalent volumes of HeLa S10 fractions from HRV14-infected cells, HRV2-infected cells, CAV21-infected cells, PV1(M)-infected cells, and mock-infected cells, respectively, and had then been incubated for 1 h at 30°C. Lane 8 of each panel contains marker proteins prepared from a [^{35}S]methionine-labeled lysate of HeLa cells that had been infected with PV1(M). Proteins were resolved in 10 to 20% polyacrylamide–SDS gradient gels, which were dried and exposed to X-ray film at −80°C for 24 h. The primary translation product (VP1-2ABΔ2C) and the VP1 and 2ABΔ2C cleavage products are indicated on the left-hand side.

tion. We have therefore defined the efficiency of proteolytic cleavage in *trans* as the proportion of the VP1-2ABΔ2C precursor that was cleaved to yield VP1.

Over 90% of the inactivated precursor was cleaved after incubation with exogenous poliovirus 2A^{pro} for 90 min at 30°C (Fig. 4A, lane 6; Table 2). The proportion of precursor remaining uncleaved is thus the same as that which remained after *cis* cleavage (see above). Substitution of the Gly residue at the *P1'* position by Ala halved the extent of cleavage of the VP1-2ABΔ2C precursor. The extent of cleavage of a precursor containing changes in the *P1* position

TABLE 2. Effects of mutations at the VP1-2A boundary on efficiency of cleavage in *trans* of a partial poliovirus polyprotein[a]

VP1-2A cleavage site	Cleavage efficiency (%)			
	PV1(M)	CAV21	HRV2	HRV14
TY↓G	92	81	4	13
TY↓A	44	38	6	12
TF↓G	74	67	3	15
TQ↓G	33	29	3	9
TP↓G	0	0	0	0
AY↓G	4	2	0	0
IY↓G	4	6	0	0
RY↓G	2	4	1	0
EY↓G	1	0	0	0

[a] Postmitochondrial S10 fractions of HeLa cells infected with PV1(M), CAV21, HRV2, and HRV14 were used as sources of exogenous 2A^{pro}. Cleavage sites are described as in Table 1. The efficiency of cleavage is defined as the amount of VP1 processed from the VP1-2ABΔC precursor as a percentage of the total of this precursor and the VP1 cleavage product.

depended on the residues in this position. Whereas a Phe residue exerted a moderate effect (Fig. 4B, lane 6; Table 2), a Gln in this position strongly inhibited and a Pro in this position completely prevented processing in *trans* (Table 2). All substitutions (Ala, Ile, Arg, or Glu) of the Thr residue at the *P2* position rendered the VP1-2ABΔ2C precursor virtually resistant to cleavage in *trans* (Table 2; Fig. 4C and D, lane 6).

A CAV21-infected HeLa S10 extract catalyzed cleavage at Tyr-Gly, Phe-Gly, Gln-Gly, and Tyr-Ala scissile bonds, albeit less efficiently in each instance than the PV1(M)-infected HeLa S10 extract. Cleavage of precursors containing Ala, Ile, Arg, or Glu substitutions at the *P2* position or a Pro substitution at the *P1* position was negligible or nonexistent (Table 2; Figure 4, lanes 5). It should be noted that the 2A^{pro} of CAV21 separates the P1 and P2 regions of the CAV21 polyprotein by cleavage of a Phe-Gly scissile bond (19). HRV2- and HRV14-infected HeLa S10 extracts cleaved the wt, VP1(Y-302→F), VP1(Y-302→Q), and 2A(T-1→A) mutant poliovirus polyproteins very inefficiently (Fig. 4A and B, lanes 3 and 4; Table 2). Precursor polypeptides containing Ala, Ile, Arg, or Glu substitutions at the *P2* position or a Pro substitution at the *P1* position were resistant to cleavage in *trans* by these extracts (Table 2; Fig. 4C and D, lanes 3 and 4).

Generation and characterization of mutant viruses. To assess the functional importance of residues at some of the positions that constitute the VP1-2A cleavage site, we engineered nucleotide substitutions into this region of an infectious poliovirus cDNA clone and characterized the viruses obtained after transfection of full-length RNA transcripts into HeLa cells. Substitutions were made to replace the Thr residue at the *P2* position by Ser, Ile, or Leu, to replace the Tyr residue at the *P1* position by a Pro residue, and to replace the Gly residue at the *P1'* position by a Cys residue.

Transfection of wt and pT7XL [VP1(T-301→S)] RNA resulted in complete CPE within 24 h. Lysates prepared from HeLa cells 3 days after transfection with the five other mutant transcripts were used to infect fresh HeLa cell monolayers. Lysates were prepared from these cells 3 days after infection and used to infect fresh HeLa cell monolayers, which were then covered with semisolid medium (0.9% agarose), resulting in plaque formation in four out of five instances. Neither plaque formation nor CPE was observed in six independent experiments with pT7XL [VP1(Y-

302→P)] RNA used for transfection, followed by attempts to amplify virus from cell lysates prepared as described above after transfection with this RNA. We therefore concluded that this substitution was lethal to virus growth. The viruses picked from plaques formed after infection of HeLa cell monolayers with amplified stocks of the four other primary transfectant lysates were all primary-site revertants. Two revertants derived from pT7XL [VP1(T-301→I)] (in which the wt ACA encoding Thr had been mutated to ATC, encoding Ile) were sequenced; one had reverted to ACC and the other to ACA, both of which changes restored the wt amino acid sequence. Reversion of other mutations was to the wt nucleotide sequence.

Virus derived from pT7XL [VP1(T-301→S)] was amplified in a 1-liter suspension culture of HeLa S3 cells after three cycles of plaque purification. It retained the nucleotide substitution (UCA in place of the wt ACA) that had been introduced into the poliovirus cDNA clone, and this virus was therefore named W1-VP1-T301S according to the nomenclature proposed by Bernstein et al. (3). The phenotypes of virus derived from pT7XL and of the W1-VP1-T301S derivative were indistinguishable in time of onset of CPE, protein synthesis, and proteolytic processing in HeLa cells and in plaque size and titer of virus stocks grown in HeLa cells at 32, 37, or 39°C (data not shown).

DISCUSSION

The poliovirus P1 capsid protein precursor is separated from the nascent polyprotein by the poliovirus 2A proteinase, following scission of a Tyr-Gly bond in *cis*. Although the amino acid residues flanking the VP1-2A scissile bond are highly conserved at several positions in different poliovirus isolates (Fig. 1A), comparison of these positions in other enteroviruses and rhinoviruses reveals considerable heterogeneity (Fig. 1C) (12, 39). This suggests significant covariation of enzyme and substrate in these rapidly evolving RNA viruses (42). Little is known of the substrate specificity of poliovirus 2A proteinase, and we have therefore undertaken a systematic mutational analysis of the VP1-2A cleavage site to characterize the determinants of cleavage site recognition.

Recognition of the VP1-2A cleavage site by $2A^{pro}$ in *cis* is surprisingly permissive, and no single amino acid substitution resulted in a total inhibition of cleavage at this site. However, efficient cleavage was tolerant of only minor changes to residues at some positions, an observation indicating that the VP1-2A cleavage site is defined by the presence of specific amino acid residues at specific positions. The size of the residue at the *P1'* position is of paramount importance; a Gly residue occurs at this position in the wt sequence, and even small increases in residue volume (as defined by Zamyatnin [49]) reduced the extent of cleavage. The pronounced decrease in cleavage efficiency resulting from additional small increases in residue volume over that of serine may indicate a limit to the size of the *S1'* pocket of the substrate-binding site. By contrast, the *P1* position readily accepted substitution by a variety of amino acid residues with divergent physical properties without significant reductions in the extent of precursor cleavage. Indeed, of six mutant precursors tested, only one (containing a Pro substitution) was significantly resistant to *cis* cleavage. The extent of precursor cleavage was approximately inversely proportional to the volume of the residue at the *P2* position, and it is therefore also an important determinant of cleavage site recognition in *cis*. Other constraints in addition to size are clearly imposed on this position by residues lining the *S2* pocket, since precursors containing Pro, Ile, or Leu substituents were more resistant to cleavage than a simple correlation with residue volume would suggest. Single substitutions at the *P2'*, *P3*, and *P5* positions had no impact on cleavage of the VP1-2A scissile bond in *cis*, even though two of these changes introduced radically different residues. This investigation has therefore revealed that the *P1'* and *P2* positions are major determinants of substrate recognition by poliovirus $2A^{pro}$ in *cis* and that the *P2'*, *P1*, and *P5* positions can tolerate a variety of substituents. A potentially important determinant which we have not investigated in the experiments reported here is the *P4* position, which is occupied by a Leu residue in both VP1-2A and 3C'-3D' cleavage sites in all poliovirus strains (31). The $2A^{pro}$ of HRV2 catalyzes cleavage at an Ala-Gly dipeptide, and residues flanking this VP1-2A cleavage site show little overt similarity to the corresponding poliovirus cleavage site. Nevertheless, substrate recognition by this proteinase is also strongly dependent on the (Gly) residue at the *P1'* position and relatively tolerant of substitution at the *P1* position (39).

The assay used in these experiments gives an indication of the extent of cleavage but not of the rate of reaction. It is likely that this is more sensitive to perturbation of the cleavage site, which may explain the low tolerance that virus viability has to substitution at the VP1-2A boundary. Separation of the capsid and nonstructural proteins is a prerequisite for further processing of the P1 capsid protein precursor (34). In vivo, a reduced rate of cleavage at the VP1-2A boundary could therefore interfere with subsequent steps in proteolytic processing of the poliovirus polyprotein. We have noted previously that this may explain why the residual autocatalytic activity of an active-site mutant of $2A^{pro}$ in vitro was not sufficient to support infectivity of poliovirus in vivo (13). An additional contributory factor to the lethal nature of mutations such as VP1(Y-1302→P) is suggested by the observation that two cryptic sites within VP1 are efficiently cleaved in vitro if scission at the bona fide cleavage site is inhibited. This may be a consequence of an increase in the half-life of precursors and of the proximity of the enzyme to authentic and cryptic cleavage sites. Similar aberrant *cis* cleavage has recently been reported to be catalyzed by $3C^{pro}$ close to its carboxy terminus following substitution of residues at the 3C-3D cleavage site (24). Candidate locations for the two cryptic $2A^{pro}$ cleavage sites in VP1 have been identified, between Tyr-187 and Gly-188 and between Tyr-280 and Gly-281. Ypma-Wong et al. (47) have demonstrated that QG dipeptides within the β-strands of the poliovirus capsid proteins are not cleaved by $3C^{pro}$, but since the two putative $2A^{pro}$ cleavage sites are located in the loop between the F and G β-strands and in the flexible carboxy terminus of VP1 (17), they are potentially accessible to $2A^{pro}$.

A T-147→A substitution within 3D at the *P2* position of the 3C'-3D' cleavage site abolished proteolytic cleavage by $2A^{pro}$ in vivo (31). This reaction presumably occurs in *trans*, and its sensitivity to alteration in the cleavage site is thus much greater than that of cleavage at the VP1-2A site in *cis*. By incubating several different mutated VP1-2A precursors with exogenous $2A^{pro}$, we have found that this difference is not restricted to the 3C'-3D' cleavage site and have therefore confirmed that the requirements for cleavage by $2A^{pro}$ in *trans* are much more restrictive than for cleavage in *cis*. This is a very interesting observation. Less-stringent substrate-subsite interactions may be either a general requirement for *cis* excision of a viral proteinase from a polyprotein prior to subsequent *trans* cleavage steps or, more simply, a consequence of enzyme and substrate being the same molecule.

The mechanism by which *cis* cleavage occurs is not known, but it is probably governed by the local folding of the single polypeptide chain that comprises both the enzyme and the substrate. As long as the overall folding of this precursor is not grossly disturbed, a peptide bond positioned in the vicinity of the catalytic triad may be cleaved, regardless of the nature of the amino acid residues forming the scissile bond. After proteolytic release from this precursor, $2A^{pro}$ may adopt a "mature" structure that differs from its folding pattern while part of a precursor. Such differences could in turn cause changes in the interactions of the enzyme with its substrate. On the basis of the homology between picornavirus 2A proteinases and bacterial serine proteinases (such as α-lytic proteinase), Bazan and Fletterick (1) have suggested that the 10 carboxy-terminal residues of VP1 form a β-strand that is equivalent to the βA_1 strand of cellular serine proteinases. These residues are of course separated from 2A as a consequence of *cis* cleavage.

The proteolytic activities encoded by CAV21, HRV2, and HRV14 that cleave the poliovirus VP1-2A site in *trans* have substrate requirements that resemble those of poliovirus $2A^{pro}$, specifically in being tolerant of various residues at the *P1* position and in requiring a small residue at the *P2* position. The similarity between the activity encoded by CAV21 and 2A of poliovirus is striking. The wt poliovirus VP1-2A site expressed in vivo as part of a larger polyprotein by using a recombinant vaccinia virus is cleaved efficiently on coinfection with HRV14 (21). Thus, although the lower activities of the two rhinovirus-infected cell lysates used in these experiments may reflect differences from poliovirus $2A^{pro}$ in substrate recognition, we cannot exclude the possibility that they are simply a consequence of a lower enzyme concentration caused by the two rhinoviruses having grown to lower titers than CAV21 and PV1(M).

The results reported here extend our previous studies of proteolytic processing by poliovirus proteinase (15). They suggest that the *P2* and *P1'* positions are critical determinants for the recognition of a cleavage site by $2A^{pro}$ and that the stringency of the requirement for these determinants is greater for cleavage in *trans* than in *cis*. Additionally, we have found that the $2A^{pro}$ of CAV21, HRV2, and HRV14 have stringent requirements similar to those of poliovirus $2A^{pro}$ for cleavage of polyprotein substrates in *trans*.

ACKNOWLEDGMENTS

We thank J. Dunn for the T7 polymerase, C. Helmke for photography, and P. Kissel for synthesis of oligonucleotides.

This work was supported in part by Public Health Service grants AI-15122 and CA-28146 to E.W. from the National Institutes of Health and by a grant from Boehringer Ingelheim Pharmaceuticals.

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