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Characterization of Poliovirus 2A Proteinase by Mutational Analysis: Residues Required for Autocatalytic Activity Are Essential for Induction of Cleavage of Eukaryotic Initiation Factor 4F Polypeptide p220

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The poliovirus proteinase 2A is autocatalytically released from the poliovirus polyprotein by cotranslational cleavage at its own amino terminus, resulting in separation of structural and nonstructural protein precursors. Cleavage is a prerequisite for further processing of the structural protein precursor and consequently for poliovirus encapsidation. A second function of $2A^{pro}$ is in the rapid shutoff of host cell protein synthesis that occurs upon infection with poliovirus. This is associated with proteolytic cleavage of the p220 component of eukaryotic initiation factor eIF-4F, which is induced but not directly catalyzed by $2A^{pro}$. We introduced single-amino-acid substitutions in the $2A^{pro}$-coding region of larger poliovirus precursors that were subsequently translated in vitro and thus demonstrated that His-20, Asp-38, and Cys-109 (which constitute the putative catalytic triad) are essential for, and that His-117 is an important determinant of, the autocatalytic activity of $2A^{pro}$. This is consistent with the proposal that $2A^{pro}$ is structurally related to a subclass of trypsinlike serine proteinases. Moreover, $2A^{pro}$ containing a Cys109Ser substitution retained a small but significant autocatalytic activity. Cleavage of p220 was not induced by those mutants that had reduced proteolytic activity, indicating that the cellular factor that cleaves p220 is probably activated by $2A^{pro}$-catalyzed proteolytic cleavage.

All poliovirus proteins are generated by proteolytic processing of a single polyprotein that is translated from the genomic RNA. All but one of the processing steps are catalyzed by two virally encoded proteinases, $2A^{pro}$ and $3C^{pro}$, which are themselves part of the polyprotein (12, 19). $2A^{pro}$ catalyzes cleavage of 2 of the 10 YG dipeptides within the polyprotein (34); all but one of the other cleavages are between QG dipeptides and are catalyzed by $3C^{pro}$ or its precursors. The initial rapid cleavage separates the capsid and nonstructural proteins and is a prerequisite to further processing of the P1 capsid protein precursor (25). It results from cleavage of $2A^{pro}$ at its own amino terminus, which occurs cotranslationally and probably in *cis* (we shall use *cis* cleavage to describe an intramolecular reaction, and *trans* cleavage to describe an intermolecular reaction). A second cleavage site recognized by $2A^{pro}$ lies within the viral 3D polymerase sequence; cleavage of a YG dipeptide leads to the appearance of the proteins 3C′ and 3D′ but is not essential for viral proliferation (21).

A likely second function of $2A^{pro}$ is in the rapid shutoff of host cell protein synthesis which occurs upon infection with poliovirus. This is associated with proteolytic cleavage of the 220-kDa component (p220) of eukaryotic translation initiation factor eIF-4F (8, 9). In poliovirus-infected cells, p220 is cleaved to yield three or four antigenically related polypeptides of 110 to 130 kDa. This cleavage is associated with changes in the composition and structure of the cap-binding complex and with a significantly lower cap-binding protein activity (10, 22). The cap-binding complex is probably required for attachment of capped mRNAs to the ribosomal 40S preinitiation complex, and thus most capped mRNAs are not translated in poliovirus-infected cell extracts. Poliovirus RNA does not contain the m^7Gppp cap of cellular mRNAs (26), and recent evidence indicates that initiation of translation of poliovirus RNA occurs by a novel cap-independent mechanism and thus does not require intact eIF-4F (reviewed in references 14, 15, 31, and 32). The ability of poliovirus to inhibit host cell protein synthesis and to initiate translation by a cap-independent mechanism provides poliovirus RNA facilitated access to the host cell translational machinery by avoiding competition from host mRNAs.

Proteolysis of p220 is induced by $2A^{pro}$ in vitro (2, 18, 23) and in vivo (33) following activation of a cellular factor. Poliovirus mutants that have been constructed to map in $2A^{pro}$ have an altered phenotype with regard to p220 cleavage and shutoff of host cell protein synthesis (2, 27). However, proteolysis of p220 could not be directly attributed to the proteolytic activity of $2A^{pro}$ (18), and in fact the p220 protease activity separates during purification from $2A^{pro}$ (24). It has therefore been proposed that $2A^{pro}$ activates a cellular proteinase, possibly as a result of a proteolytic event, which then cleaves p220. This putative cellular proteinase has not been identified, but eukaryotic translation initiation factor eIF-3 has been shown to be required for the p220 cleavage activity and may even contain the p220 cleavage active site (36).

It is likely that $2A^{pro}$ has an active-site thiol group, since

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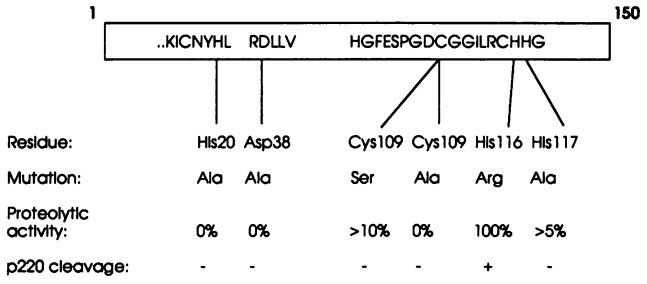

FIG. 1. Schematic diagram of 2A proteinase of poliovirus type 1 (Mahoney) showing amino acid substitutions and the positions at which they were made. The numbering of residues is based on the published sequence (16). The proteolytic activity of mutant 2A proteinases is given as a percentage of the activity of wild-type $2A^{pro}$. The ability to induce cleavage of p220 is indicated by +, and the failure to do so is indicated by −.

its proteolytic activity is inhibited by the alkylating agents iodoacetamide and *N*-ethylmaleimide (17). These observations support an earlier preliminary classification of $2A^{pro}$ as a cysteine proteinase, which was suggested by the presence of a conserved sequence, PGDCGGXLXCXHG, in $2A^{pro}$ of polioviruses and rhinovirus types 2 and 14 (3, 34). The underlined residues, which could act as part of the catalytic center of a cysteine proteinase, are conserved in all enteroviruses and rhinoviruses. Intramolecular activity of poliovirus $2A^{pro}$ was abolished by deletion of nine amino acids from its carboxy-terminal portion, which include the conserved His residue of the sequence noted above (34). Substitution of the underlined Cys residue by Ser and of His by Gly in $2A^{pro}$ of rhinovirus type 2 similarly abolished its intramolecular activity (30). More recent analysis of aligned sequences suggested that $2A^{pro}$ is structurally related to the small subclass of trypsinlike serine proteinases (e.g., *Lysobacter enzymogenes* alpha-lytic protease) and, more specifically, that His-20, Asp-38, and Cys-109 form the catalytic triad (1). The proposed substitution of a cysteine residue for a serine residue in the viral catalytic triad is a notable difference between the cellular trypsinlike and viral trypsinlike proteinases. The assignment of catalytic function to His-20 contrasts with the earlier suggestion that His-117 could act as a catalytic residue (34).

We introduced single-amino-acid substitutions at these four conserved positions and at one nonconserved position in poliovirus $2A^{pro}$ and noted their effects in vitro on the *cis* activity of a $2A^{pro}$ zymogen and on proteolytic cleavage of the p220 component of the cap-binding complex (Fig. 1). We also investigated whether cDNA that incorporated the coding sequence of those $2A^{pro}$ mutants that retained proteolytic activity could be transcribed to yield infectious RNA.

MATERIALS AND METHODS

Genetic engineering of DNA. Restriction enzymes and DNA-modifying enzymes were purchased from either New England BioLabs or Bethesda Research Laboratories, Inc. Sequencing primers and mutagenic oligonucleotides were synthesized on an Applied Biosystems apparatus. DNA was manipulated by standard procedures (28).

Bacterial strains and plasmid construction. For analysis of autocatalytic activity, a vector [pBS^-(VP1-2ABΔC)] was constructed that contained poliovirus type 1 (Mahoney) cDNA encoding two amino acids of VP3, all of VP1, 2A, and 2B, and one-half of 2C (nucleotides 2474 to 4600) placed under the control of part of the encephalomyocarditis virus 5′-nontranslated sequence (nucleotides 260 to 841, derived from pS32A [18]). These sequences were cloned between the *Eco*RI and *Bam*HI sites in the polylinker region of pBluescribe M13(−) (Stratagene). Synthetic deoxyoligonucleotides were annealed to single-stranded DNA substituted with uracil that had been prepared by passage through *Escherichia coli* BW313 (*dut ung*). Second-strand synthesis and transformation into *E. coli* C600 were done as previously described (20). Mutant sequences were identified by sequence analysis, using the dideoxynucleotide chain termination method (29).

The cDNA segments containing Cys109Ser and His116 Arg mutations were cloned into a full-length poliovirus transcription vector by inserting the smaller *Bst*EII-*Bst*EII fragment of pBS^-(VP1-2ABΔC) (corresponding to nucleotides 3235 to 3925 of the poliovirus cDNA) into the larger *Bst*EII-*Bst*EII fragment of plasmid pT7XL derived from plasmid pT7 PV1-5 (35). Mutations were subsequently verified by sequence analysis.

Transcription, translation, and cleavage assays. Plasmid pBS^-(VP1-2ABΔC) (wild type and mutated derivatives thereof) was linearized by digestion with *Bam*HI and was transcribed in vitro with T7 RNA polymerase. Synthetic mRNA transcripts were translated in 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, $2A^{pro}$, and 2C were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25). Preparation of antisera was described previously (7, 25, 34).

To assay cleavage of the VP1-2A bond by $2A^{pro}$ in *trans*, we translated mutated VP1-2ABΔC precursors 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 mixture to final concentrations of 10 μg/ml, 1 mg/ml, and 0.5 mM, respectively, which were then mixed either with 3-μl aliquots of RRL translation mixture devoid of exogenous RNA or with 3-μl aliquots of RRL in which RNA transcripts derived from linearized pS(3) 2A III (18) had been translated to generate unlabeled $2A^{pro}$. Mixtures were incubated for 90 min at 30°C.

Purified pT7XL and its mutagenized derivatives were linearized by digestion with *Eco*RI and transcribed in vitro with T7 RNA polymerase.

Transfections. Synthetic RNA derived by transcription of linearized pT7XL was transfected into HeLa R19 cells by the DEAE-dextran method described previously (35). Cells were maintained until complete cytopathic effects (CPE) were observed; HeLa cell monolayers that did not show CPE 3 days after transfection were harvested, and lysates were used for infection of fresh monolayers and in plaque assays.

p220 cleavage assays. The ability of mutated $2A^{pro}$ to induce cleavage of p220 was determined as described by Kräusslich et al. (18). Intact p220 and fragments thereof were detected by the Western blotting (immunoblotting) technique, using a monoclonal antibody directed against p220 (kind gift from D. Etchison).

RESULTS

Generation of mutants. To assay the autocatalytic activity of $2A^{pro}$, we constructed the vector pBS^-(VP1-2ABΔC), which encodes the enzyme as part of a larger precursor

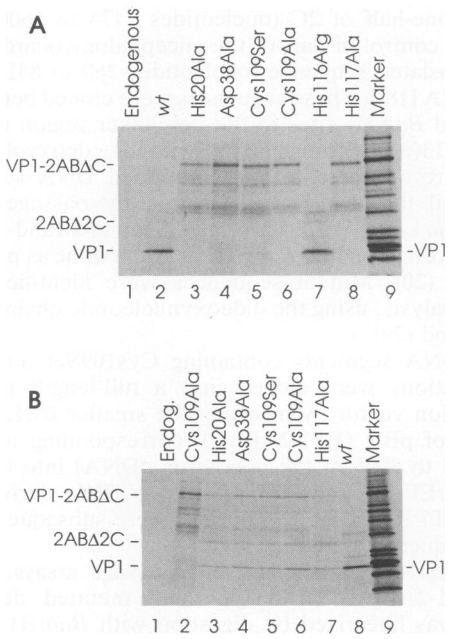

FIG. 2. Effects of single-amino-acid substitutions in $2A^{pro}$ on processing of a VP1-2ABΔC polyprotein precursor. (A) Autocatalytic processing of VP1-2ABΔC precursors. Aliquots (3 μl) of RRL translation mixtures containing products translated from transcripts of wild-type (*wt*) pBS⁻(VP1-2ABΔC) and mutated derivatives thereof were resolved by electrophoresis in a 10 to 20% SDS-polyacrylamide gradient gel. (B) Proteolytic processing of VP1-2ABΔC precursors by $2A^{pro}$ in *trans*. Aliquots (3 μl) of RRL translation mixtures containing products translated from the RNA transcripts of wild-type pBS⁻(VP1-2ABΔC) and mutated derivatives thereof were incubated for 90 min at 30°C with aliquots (3 μl) of RRL translation mixtures devoid of exogenous RNA (lanes 2 and 8) or with aliquots of RRL translation mixture containing unlabeled $2A^{pro}$ (lanes 3 to 7). Gels were dried and exposed to X-ray film at −80°C for 24 h. Lanes are labeled to indicate the substitutions made in the amino acid sequence of $2A^{pro}$. The primary translation product (VP1-2ABΔC) and the VP1 and 2ABΔ2C cleavage products are indicated on the left-hand side. The marker is a [^{35}S]methionine-labeled cell lysate prepared from HeLa cells infected with poliovirus type 1 (Mahoney).

comprising segments of both the P1 structural protein and P2 nonstructural protein regions. The open reading frame of the partial poliovirus polyprotein was placed under the translational control of approximately 600 nt of the 5′-nontranslated sequence of encephalomyocarditis virus RNA. This region, which contains the internal ribosomal entry site, has previously been shown to direct highly efficient translation of poliovirus proteins in RRL (18) and renders the in vitro-synthesized mRNAs cap independent for translation (15, and references therein).

Single-amino-acid substitutions of residues His-20, Asp-38, Cys-109, His-116, and His-117 in $2A^{pro}$ of poliovirus type 1 (Mahoney) were made by site-directed mutagenesis, and mutations were identified by nucleotide sequence analysis.

Proteolytic activity of mutants. The wild-type precursor encoded by pBS⁻(VP1-2ABΔC) was cleaved to completion during translation in RRL, yielding the expected VP1 and 2ABΔ2C cleavage products (Fig. 2A, lane 2). The prominence of the 2ABΔ2C cleavage product was slightly less than expected, which may be due to proteolytic degradation. Several unexpected bands were apparent that had electrophoretic mobilities intermediate between those of the VP1-2ABΔC precursor and the 2ABΔ2C cleavage product. They were particularly prominent in aliquots of RRL in which precursors containing inactive proteinases had been translated, but the reason for this is not known. Similar aberrant proteins have been described as resulting from translation of synthetic mRNA transcripts derived from related poliovirus-containing plasmid constructs (18, 25). They might be degradation products of VP1-2ABΔC or may result from aberrant initiation, which is a characteristic of poliovirus translation in RRL (4, 5).

The Cys109Ser mutant precursor retained up to 10% of its autocatalytic activity (Fig. 2A, lane 5), but cleavage of the Cys109Ala mutant precursor was completely abolished (Fig. 2A, lane 6), resulting in accumulation of the uncleaved VP1-2ABΔC polyprotein. The efficiency of cleavage of the His116 Arg mutant precursor was unchanged from that of the wild-type precursor, whereas processing of the His117Ala mutant precursor was reduced by over 95% (Fig. 2A, lanes 7 and 8, respectively). His20Ala and Asp38Ala mutant precursors had both lost virtually all detectable autoproteolytic activity, and the uncleaved VP1-2ABΔC polyprotein precursor therefore accumulated (Fig. 2A, lanes 3 and 4, respectively).

To demonstrate that these mutations did not affect the conformation of the VP1-2A cleavage site, we assayed cleavage in *trans* by exogenous $2A^{pro}$. Partial cleavage of the mutated VP1-2ABΔC precursors by $2A^{pro}$ to VP1 and 2ABΔ2C occurred to a similar extent in all instances (Fig. 2B, lanes 3 to 7). The mobility of the VP1 cleavage product was very slightly less than that of authentic VP1 synthesized by poliovirus type 1 (Mahoney) in vivo (Fig. 2B, lanes 8 and 9). This is consistent with the expected presence of two additional amino acids at the amino terminus of the VP1 product derived by cleavage in vitro of the precursor encoded by transcripts derived from pBS⁻(VP1-2ABΔC). We therefore concluded that cleavage of the VP1-2A junction by $2A^{pro}$ occurred accurately in *trans*. The identities of the VP1 and 2ABΔC cleavage products were confirmed by immunoprecipitation with antisera directed against VP1, 2A, or 2C, as appropriate (data not shown).

Effects of mutations on virus viability. The Cys109Ser and His116Arg amino acid substitutions in $2A^{pro}$ (for both of which some proteolytic activity was detectable in vitro) were tested for their effect on virus viability. Transfection of wild type and pT7XL (2A, His116Arg) RNA resulted in complete CPE within 24 h, whereas in the instance of pT7XL (2A, Cys109Ser) RNA, no CPE were observed following seven independent transfections. Cell lysates obtained 3 days after transfection of HeLa cells with pT7XL (2A, Cys109Ser) RNA were used for infection of fresh HeLa cell monolayers, but no CPE were observed. Plaque formation was not detected with medium or cell lysates recovered at any stage within 72 h of transfection of HeLa cells with pT7XL (2A, Cys109Ser) RNA. The phenotypes of virus derived from pT7XL and of the W1-2A-H116R derivative were indistinguishable with respect to time of onset of CPE, plaque size, and titer of virus stocks grown in HeLa cells at 37°C.

Effects of mutations on p220 cleavage. Complete cleavage of p220 occurred upon incubation of HeLa cell extract with wild-type $2A^{pro}$, and virtually complete cleavage was induced by incubation with His116Arg $2A^{pro}$ (Fig. 3), whereas incubation of this extract with His20Ala, Asp38Ala, Cys109 Ser, Cys109Ala, or His117Ala mutant $2A^{pro}$ had no effect. Complete cleavage of p220 was induced by incubation with a

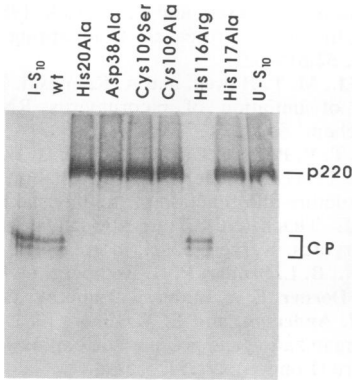

FIG. 3. Effect of mutations in $2A^{pro}$ on its ability to induce cleavage of p220. Aliquots of RRL in which transcripts of pBS^- (VP1-2ABΔC) encoding wild-type (wt) or mutated $2A^{pro}$ (as indicated) had been translated were incubated with an S10 extract of mock-infected HeLa S3 cells, separated by SDS-PAGE, and transferred to a nitrocellulose sheet as described by Kräusslich et al. (18). The blot was probed with a monoclonal antibody to p220. U-S_{10} represents an S10 extract from mock-infected HeLa cells, and I-S_{10} represents an S10 extract from poliovirus-infected HeLa S3 cells. CP, cleavage products of p220.

lysate of poliovirus-infected HeLa cells, whereas incubation with a lysate of uninfected cells had no effect.

DISCUSSION

We identified residues that are required for the proteolytic activity of poliovirus $2A^{pro}$ and that may constitute its putative catalytic triad by introducing single-amino-acid substitutions in $2A^{pro}$ and subsequently determining the *cis* cleavage activity of mutant proteinase zymogens (summarized in Fig. 1). His-20, Asp-38, and Cys-109 were found to be essential for the proteolytic activity of $2A^{pro}$, in that substitutions could be made at these positions that abolished the ability of $2A^{pro}$ to be excised from a polyprotein, presumably by cleavage in *cis*. It has been suggested that these residues constitute the catalytic triad of $2A^{pro}$, and our results are consistent with this proposal. The proposal that His-20 is the basic residue of the putative catalytic triad and that His-117 forms part of the substrate-binding pocket (1) contrasts with earlier suggestions that His-117 is a constituent of the putative catalytic triad (2, 34) but is consistent with the His117Ala mutant having substantially greater activity than the His-20 mutant. The His-116 residue is not conserved, occurring in poliovirus types 1 (Sabin) and (Mahoney) but not in other serotypes. The results described above indicate that it is clearly not involved in catalysis, nor does it play any other role essential to the function of $2A^{pro}$ in vivo.

The residual activity of the Cys109Ser mutant suggests that the hydroxy group of the serine residue can function in the same fashion as the thiol group of the cysteine residue in the nucleophilic attack on the amide bond that occurs on proteolysis. This mechanism is consistent with the complete loss of activity of the Cys109Ala mutant. Cys, Ser, and Ala residues are of similar size, so that substitution of one by another is unlikely to cause significant misfolding of $2A^{pro}$. In this regard, it is noteworthy that Ser and Cys residues can function in catalyzing proteolytic cleavage by wild-type and mutant trypsin moieties, respectively, albeit at greatly differing rates (13). Similarly, the activity of tobacco etch virus 49-kDa protease was reduced but not eliminated by replacement of the putative active-site Cys residue by Ser (6). However, we have found that substitution of the putative active-site Cys residue by Ser in poliovirus $3C^{pro}$ (which is probably also related to trypsinlike serine proteinases) abolished the activity of this enzyme in a variety of assays (11).

Impairment of autoproteolysis of the $2A^{pro}$ zymogen by mutation of specific residues demonstrates only that they are essential for activity but does not show that they are involved in catalysis itself. Mutations could equally impair activity as a consequence of structural alterations, and strictly, we are unable to distinguish between these possibilities. However, we were able to conclude that these mutations do not affect the structure of the cleavage site recognized by $2A^{pro}$ per se. We demonstrated this by using an approach initially developed by Nicklin et al. (25) to investigate intermolecular cleavage of the VP1-2A bond: all mutated VP1-2ABΔC precursors were cleaved accurately (to yield VP1 and 2ABΔ2C cleavage products) and with equal efficiency by exogenous $2A^{pro}$.

The apparent discrepancy between the residual activity of the Cys109Ser and His117Ala poliovirus (type 1) $2A^{pro}$ mutants that were translated in RRL and the complete lack of activity of equivalent Cys106Ser and His114Gly rhinovirus (type 2) $2A^{pro}$ mutants expressed in *E. coli* (30) has a precedent in the observation made by Nicklin et al. (25): a mutated poliovirus $2A^{pro}$ expressed in *E. coli* that completely lacked proteolytic activity paradoxically retained partial activity when translated in RRL.

The system used to assay cleavage in vitro gives an indication of the extent of cleavage but not of the rate of reaction, which probably undergoes a larger decrease. Rapid separation of the capsid and nonstructural proteins has been shown to be a prerequisite for further processing of the P1 capsid protein precursor (25). In vivo, a reduced catalytic rate could therefore lead to abortive processing of the structural precursor if the $2A^{pro}$ moiety was not removed rapidly enough. This may explain why the residual autocatalytic activity of the Cys109Ser mutant in $2A^{pro}$ in vitro was not sufficient to support infectivity of poliovirus in vivo.

A second function of $2A^{pro}$ in poliovirus infection of HeLa cells is in inducing cleavage of the 220-kDa polypeptide (p220) component of eukaryotic translation initiation factor eIF-4F (8, 9), presumably by activating a cellular factor (2, 18, 24). We showed that amino acid substitutions that affected the proteolytic activity of $2A^{pro}$ also affected its ability to induce cleavage of p220. Activation of the cellular factor that degrades p220 is therefore probably a direct result of proteolytic cleavage of this factor by $2A^{pro}$. Substitutions near the carboxy terminus of $2A^{pro}$ (i.e., Cys109Ser and His117Ala) completely abolished cleavage of p220, although autoproteolytic activity was only partially inhibited. These data are consistent with results described previously that insertions near the carboxy terminus (2, 27, 33) and near the amino terminus (18) that rendered the proteinase either partially inactive or even sufficiently active to cleave the VP1-2A bond during infection by mutant poliovirus in vivo were nevertheless unable to induce cleavage of p220. The lack of effect of the His116Arg substitution was expected, since, as noted above, the His-116 residue is not conserved among poliovirus serotypes and could be substituted in an infectious clone without effect on the resulting phenotype.

There are subtle differences between the VP1-2A cleavage sites recognized by $2A^{pro}$ encoded by various members of the enterovirus and rhinovirus groups, yet these enzymes

are all able to induce cleavage of p220. It is therefore possible that the cleavage site within the cellular factor whose scission activates p220 cleavage is not optimally recognized by poliovirus $2A^{pro}$. Since there are also likely to be mechanistic differences in the interaction between $2A^{pro}$ and its cognate substrates in *cis* and *trans* reactions, it is quite possible that mutations in $2A^{pro}$ that reduce *cis* cleavage of a homologous substrate could abolish *trans* cleavage of heterologous substrates, such as the putative activator of the p220 proteinase.

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