Poliovirus Proteinase 2A Induces Cleavage of Eucaryotic Initiation Factor 4F Polypeptide p220

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Poliovirus infection of HeLa cells induces rapid shutoff of host protein synthesis, whereas translation of poliovirus RNA is not inhibited. It is presumed that shutoff is the result of proteolytic cleavage of component p220 of eucaryotic initiation factor 4F. To study whether poliovirus proteinase 2A is involved in this cleavage, we translated synthetic RNAs that contained the coding region for poliovirus-specific polypeptides P1 and 2A in vitro and assayed for cleavage of p220. We report here that cleavage of p220 occurred in all cases when active proteinase 2A was translated and that disruption of the coding sequence of 2A by linker insertion or deletion prevented processing of p220 in vitro. Activity of 2A was determined by its ability to cleave at the P1-P2 site of a segment of the poliovirus polyprotein. We also constructed a plasmid in which the 3'-most 500 nucleotides of the nontranslated region of encephalomyocarditis virus were linked to the coding sequence for poliovirus polypeptide 2A. Translation of the RNA transcript of this clone was very efficient and yielded a fusion protein that included 2A; this polypeptide also induced cleavage of p220. In vitro translation in the presence of antibodies against 2A specifically inhibited processing of p220, whereas incubation of in vitro translation products with antibodies against 2A after translation was completed did not prevent proteolysis of p220.

Poliovirus infection of HeLa cells induces shutoff of host cell protein synthesis (for reviews, see references 6 and 37). Studies of poliovirus-infected cells showed that translation of cellular mRNAs declines before substantial synthesis of viral proteins has occurred. It was shown that synthesis of host cell proteins is blocked at the initiation step (17, 21) and that one of the protein synthesis initiation factors, eucaryotic initiation factor 4F (eIF-4F, previously called cap-binding protein complex CBPII), is both structurally and functionally altered (10, 11). This complex is considered to be required for attachment of capped mRNAs to the ribosomal 40S preinitiation complex (for a review, see reference 34). Poliovirus-infected cell extracts do not support the translation of capped mRNAs (14, 32), but this activity can be restored by addition of purified eIF-4F from uninfected cells (5, 12, 40). Poliovirus RNA, like other picornavirus RNAs, does not contain the m⁷Gppp cap of cellular mRNAs (15, 27), and it is generally believed that poliovirus RNA can initiate translation by a mechanism that does not require intact eIF-4F (reviewed in reference 37).

eIF-4F is a multisubunit complex containing at least three polypeptides of molecular masses 24, 50, and 200 to 220 kilodaltons (kDa) (5, 12, 40). The 24-kDa polypeptide can be specifically cross-linked to the oxidized, labeled cap structure (38, 39). The 50-kDa polypeptide, which is similar if not identical to eIF-4A (5, 12), was shown to have an ATP-dependent mRNA-unwinding activity (31). The function of the largest component of eIF-4F (termed p220) has not been determined.

Structural studies of eIF-4F by immunoblot analysis showed that in poliovirus-infected cells p220 is absent and several smaller polypeptides (100 to 130 kDa) antigenically

related to p220 are found (11). Presumably these smaller polypeptides are generated by proteolytic cleavage of p220, and this cleavage is possibly responsible for inactivation of capped mRNA recognition in infected cells.

Poliovirus RNA encodes a single open reading frame of 2,208 codons (18), and the primary translation product (247 kDa) is subsequently processed to yield mature capsid and noncapsid proteins (for reviews of processing, see references 26 and 43). These proteolytic cleavages are mediated by two virus-encoded proteinases, polypeptides 3C and 2A. Polypeptide 3C cleaves the polyprotein at glutamine-glycine sites (13), whereas 2A cleaves at two tyrosine-glycine sites (42), one of which severs the viral capsid precursor from the noncapsid proteins.

Since poliovirus codes for two proteinases, it was investigated whether one of these proteinases mediates the cleavage of p220. Studies with polypeptide 3C (20, 22) and more recently with 2A (23) showed that neither enzyme copurified with p220 cleavage activity, and antibodies to neither proteinase inhibited cleavage of p220 in vitro. However, a poliovirus mutant has been described (2) that contains a single amino acid insertion in 2A yet fails to inhibit host cell protein synthesis selectively and to induce cleavage of p220. Taken together, these observations suggest that poliovirus proteinase 2A is indirectly involved in the cleavage of p220. The objective of this study was to provide direct evidence that poliovirus-encoded proteinase 2A induces cleavage of the cellular polypeptide p220. No attempt has been made to correlate the cleavage of p220 with the mechanism(s) of shutoff of cellular protein synthesis.

MATERIALS AND METHODS

Construction of plasmids. Restriction enzymes and enzymes used in cloning and modifying DNAs were purchased from New England BioLabs, Inc., and Bethesda Research Laboratories, Inc. DNA manipulations were done by stan-

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FIG. 1. Construction of plasmid pS32A. (A) The locations of several restriction endonuclease sites are shown for pE5LVPO, pVS(3)2819, and pS32A. The orientation of the β -lactamase gene of pBR322, the poliovirus type 3 (Sabin) genomic sequence (solid bar), and the EMCV genomic sequence (open bar) is indicated by arrows. The phage T7 promoter sequence is depicted as a solid arrow. The construction of pS32A is described in Materials and Methods. The RNA sequence at the EMCV-poliovirus junction was verified by sequence analysis by the dideoxy chain termination method (8, 33). (B) Structure of the fusion protein derived from pS32A. The hatched bar indicates five amino acids of the EMCV leader peptide, the solid bar indicates two amino acids contributed by the vector. Poliovirus type 3 (Sabin) amino acid sequences are depicted as an open bar; the 2A region is characterized by the Y-G site at its amino terminus (N) and the Q-G site at its carboxy terminus (C). 2B $^{\Delta}$ indicates 24 amino acids of the 2B sequence. bp, Base pair.

dard procedures (24). All transformations were performed with *Escherichia coli* C600 (1).

Plasmids pMN22 and pMN27 to pMN30 were derived from the vector pAR2106 (A. Rosenberg, J. J. Dunn, and F. W. Studier, unpublished data), which contains the promoter of gene 10 of phage T7 inserted counterclockwise into the *Bam*HI site of pBR322. Construction of plasmids pMN22 and pMN27 to pMN29 is outlined elsewhere (25a). Briefly, plasmid pMN22 contains a truncated 5' nontranslated region (NTR) and the P1-coding region (nucleotides 671 to 3381) of poliovirus type 1 (Mahoney), followed by a stop codon (see Fig. 2C). Plasmids pMN27 to pMN29 also contain the truncated 5' NTR and P1-coding region of poliovirus type 1

(Mahoney), followed in frame by a segment of the P2-coding region (nucleotides 3374 to 4243) of poliovirus type 3 (Sabin). Plasmid pMN27 was derived from pVS(3)2801 (42) and contains the coding sequence of 2A and 2B and 44 amino acids of that of 2C (see Fig. 2C). Plasmids pMN28 and pMN29 were derived from pVS(3)2813 and pVS(3)2820 (42) and have an insertion of four codons close to the 5' end and a deletion of nine codons close to the 3' end of the 2A region, respectively (see Fig. 2C).

Plasmid pMN30 was constructed by ligating the P1 region of pMN27 and the P2 region of pVS(3)2819 (nucleotides 3374 to 3918; containing the coding sequence for 2A and 32 amino acids of 2B of poliovirus type 3 [Sabin]; 42) at their homologous *Nde*I site (see Fig. 2C).

Plasmid pS32A was derived from pVS(3)2819 and pE5LVPO (29), which contains a segment of the 5' NTR and the coding sequence for the leader peptide and capsid protein VP0 of encephalomyocarditis virus (EMCV) (nucleotides 260 to 2012) inserted into the vector pSPT18 (Pharmacia). Plasmid pSPT18 is a derivative of pUC18 that contains a T7 promoter and a polylinker cloning site. Plasmid pE5LVPO DNA was kindly provided by Ann Palmenberg. The construction of pS32A is outlined in Fig. 1A. The *Hinc*II-*Nco*I fragment (polylinker from pSPT18 to nucleotide 848 of EMCV) of pE5LVPO and the *Nde*I-*Eco*RV fragment (nucleotides 3381 to 3897 of the poliovirus sequence) of pVS(3)2819 were reacted with DNA polymerase I Klenow fragment in the presence of all four deoxynucleoside triphosphates and ligated. The predicted protein product, after transcription and translation of the plasmid, is a fusion protein with 5 amino acids of the L protein of EMCV at its amino terminus, followed by 1 amino acid of P1, the entire 2A sequence, and 24 amino acids of 2B of poliovirus type 3 (Sabin; Fig. 1B). Since the plasmid was linearized with *Pst*I, which cleaves in the polylinker (from pSPT18), two carboxy-terminal amino acids are contributed by the vector.

Preparation of HeLa cell postmitochondrial (S10) fractions.

A postmitochondrial cytoplasmic extract (S10) of mock-infected and poliovirus-infected HeLa cells was prepared as described previously (4). Approximately 5×10^8 cells were infected with poliovirus at a multiplicity of infection of 400 PFU per cell. Cells were harvested 3.5 h after infection, swollen for 15 min in hypotonic buffer (10 mM NaCl, 5 mM dithiothreitol, 1.5 mM MgCl₂, 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 $10,000 \times g$ for 15 min. The extracts were adjusted to 10% (vol/vol) glycerol and stored at -60°C .

Purification of IgG. Preparation of antisera was described previously (7, 42). The immunoglobulin G (IgG) fraction was purified as previously described (13). Concentration of protein was assayed by colorimetry of complexes between protein and brilliant blue G-250 (Bio-Rad Protein Assay). Protein was concentrated to approximately 15 mg/ml with a vacuum centrifuge.

In vitro transcription and translation. Plasmids were purified by standard procedures (24). Plasmids pMN22 and pMN27 to pMN30 were linearized with *Dra*I, which cleaves the vector at nucleotide 3943 of pBR322. Plasmid pS32A was linearized with *Pst*I. Transcription reactions were performed as described previously (44). T₇ RNA polymerase was the kind gift of John J. Dunn. Samples of 10 μg each of RNA were made, dried in a vacuum centrifuge, and stored dry. RNA was redissolved in H₂O to a concentration of 1 $\mu\text{g}/\mu\text{l}$

FIG. 2. Products of in vitro translation of synthetic RNAs and effect on p220 processing. (A) Samples (1.5 μ l) of translation mixtures were diluted to 50 μ l in loading buffer (19) and analyzed on an SDS-12.5% polyacrylamide gel. Note that a protein with a

mobility very similar to that of VP0 was present in the no-RNA lane. This protein has been described previously (16). Lanes M represent a lysate of [³⁵S]methionine-labeled poliovirus-infected HeLa cells.

(B) For the left panel, 12- μ l samples of each translation mixture were incubated with 6 μ l of S10 extract from mock-infected HeLa cells for 2 h at 30°C. A 9- μ l portion of each sample was diluted to 40 μ l in loading buffer, analyzed on an SDS-8% polyacrylamide gel, and transferred to nitrocellulose sheets. The blots were probed with a monoclonal antibody directed against p220. U-S10 represents a 3 μ l of S10 extract from mock-infected HeLa cells. I-S10 represents 3 μ l of S10 extract from poliovirus-infected HeLa cells. C.P. refers to cleavage products of p220. For the right panel, 6 μ l of a translation mixture programmed with pMN30 RNA and 0.5 μ l of a translation mixture programmed with pS32A RNA were incubated with 3 μ l of S10 extract as described above and analyzed by immunoblotting. (C) Diagram of synthetic RNAs. The top bar represents a segment of a hypothetical hybrid poliovirus genome in which the P2 region is derived from the type 3 (Sabin) virus and the rest is from type 1 (Mahoney). Solid bars represent the 5' NTR, open bars indicate the poliovirus type 1 coding sequence, and hatched bars represent the poliovirus type 3 coding sequence. The calculated molecular mass(es) of the expected protein product(s) from each RNA is indicated on the right. For clarity, this diagram is not to scale (modification of the diagram published by Nicklin et al. [25a]).

immediately preceding in vitro translation, heated for 5 min at 65°C, and cooled rapidly on ice.

Synthetic RNAs (final concentration, 80 μ g/ml) were translated in rabbit reticulocyte lysate (Promega Biotech) essentially as described by the supplier. The reaction mixtures (25- μ l total volume) were incubated for 90 min at 30°C. Incorporation of ³⁵S was determined by the standard method (16). Typically, the added RNA gave an 8- to 10-fold stimulation over the message-free control. In vitro translation products were analyzed on sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels (12 by 13 by 0.15 cm) with the buffer system of Laemmli (19) modified as described by Nicklin et al. (25a). Dried gels were exposed to Kodak XAR-5 film for 18 h.

p220 cleavage assay and immunoblot analysis. To assay for p220 cleavage activity, 6 μ l of postmitochondrial cytoplasmic extract from mock-infected HeLa cells (U-S10) was mixed with 12 μ l of freshly prepared in vitro translation products and incubated for 2 h at 30°C. A 9- μ l portion of each sample was withdrawn, diluted to 40 μ l with loading buffer (19), and analyzed on an 8% SDS-polyacrylamide gel (13 by 9 by 0.15 cm).

Preparation of the monoclonal antibody against p220 is described elsewhere (8a). Immunoblot analysis of p220 was a modification of a previously published procedure (22, 41). Protein was transferred electrophoretically for 4 h at 30 V to nitrocellulose sheets (Schleicher & Schuell, Inc.), and the blots were prehybridized in 3% bovine serum albumin in TNN (10 mM Tris hydrochloride [pH 7.5], 0.5% Nonidet P-40, 150 mM NaCl) for 30 min at 20°C. The blots were treated with anti-p220 monoclonal antiserum (diluted 1:5,000 in TNN containing 0.5% bovine serum albumin) overnight at 20°C and then with alkaline phosphatase-conjugated goat anti-mouse IgG (Tago Immunochemicals; 1:1,000 dilution in TNN with 0.5% bovine serum albumin) for 2 h at 20°C. The blots were washed extensively in TNN and incubated with 10 ml of freshly prepared substrate for alkaline phosphatase, as previously described (36): 0.1 mg of *p*-nitro blue tetrazolium chloride (U.S. Biochem. Corp.) per ml-50 μ g of 5-bromo-4-chloro-3-indolyl phosphate (U.S. Biochem.) per ml-5 mM MgCl₂-50 mM glycine-NaOH, pH 9.6. The reac-

tion mixture was immediately preceding in vitro translation, heated for 5 min at 65°C, and cooled rapidly on ice.

tion was terminated after color developed (approximately 3 to 5 min) by washing the blots in water.

RESULTS

Translation of synthetic RNAs and assay for p220 cleavage.

To investigate the hypothetical role of poliovirus proteinase 2A in the proteolytic cleavage of the eIF-4F component p220, we translated different synthetic RNAs containing the coding region for 2A *in vitro* in rabbit reticulocyte lysates and incubated the products of translation with a postmitochondrial cytoplasmic extract (S10) of mock-infected HeLa cells. A diagram of the RNA transcripts of the plasmids used in this study is shown in Fig. 2C. In another study (25a), we demonstrated that truncation of the 5' NTR of synthetic poliovirus RNAs resulted in more efficient translation *in vitro*; therefore, all RNAs used in this experiment contained a truncated 5' NTR starting at nucleotide 671.

Figure 2A shows the products of *in vitro* translation. Translation of pMN22 RNA yielded P1, and translation of pMN27 RNA produced P1 and a smaller protein which is likely to be 2ABC^A, the other cleavage product of the precursor polypeptide (25a). Similarly, translation of pMN30 RNA yielded 2A activity that cleaved the tyrosine-glycine bond between P1 and P2, and a protein of approximately 20 kDa corresponding to 2AB^A was produced (Fig. 2A). Linker insertion in the coding sequence of 2A (pMN28; Fig. 2A) or deletion of nine codons, including the putative active-site histidine of 2A (pMN29; Fig. 2A), rendered the 2A proteinase partially or completely inactive, respectively, and a precursor protein containing P1-2ABC^A was observed (25a).

The products of *in vitro* translation of these synthetic RNAs were incubated with S10 extracts from mock-infected HeLa cells to assay for p220 cleavage activity. These extracts were used to provide a source of p220 since our monoclonal antibody directed against human p220 gave only a weak signal in response to the rabbit p220 present in reticulocyte lysates. p220 was completely stable to incubation for 2 h at 30°C with a translation mixture programmed with either no RNA (Fig. 2B) or pMN22 RNA (Fig. 2B). Incubation with a translation mixture containing an unmutated 2A sequence, however, induced complete cleavage of p220 to yield the antigenic products described previously (100 to 130 kDa; Fig. 2B, pMN27 and pMN30). Linker insertion or deletion in the coding sequence of 2A abolished this effect completely (Fig. 2B, pMN28 and pMN29), whereas deletion of most of the coding sequence of polypeptide 2B in this construct had no effect on the processing of p220 (Fig. 2B, pMN30).

Effect of deletion of capsid proteins on cleavage of p220. The results described so far show that *in vitro* translation of P1-P2 hybrid proteins of poliovirus produces an activity that induces cleavage of p220. Intact proteinase 2A, but not polypeptide 2B, is necessary for this cleavage. Although P1 without 2A clearly does not induce proteolysis of p220, we cannot rule out a possible involvement of capsid proteins in this cleavage since the P1 region was present in all of the constructions used. We therefore constructed a plasmid that contained the complete coding sequence of 2A but no P1 segments. Plasmid pS32A was derived from pE5LVP0 (29), which spans the EMCV sequence from nucleotides 260 to 2012. EMCV RNA translates with high efficiency in reticulocyte extracts (30, 35), and it was shown recently that the 3'-most 500 nucleotides of the NTR of EMCV suffice to allow efficient translation of synthetic RNAs *in vitro* (29; G. M. Duke and A. C. Palmenberg, manuscript in prepara-

tion). We therefore joined the 5' NTR of EMCV and the coding sequence of poliovirus polypeptide 2A to produce plasmid pS32A (Fig. 1A; see Materials and Methods). The predicted fusion protein derived from the transcript of pS32A is described in Fig. 1B.

Translation of pS32A RNA (yielding a polypeptide containing 7 methionine residues) and pMN30 RNA (yielding a polypeptide containing 30 methionine residues) gave approximately equal incorporation of [³⁵S]methionine into translation products (data not shown), a result demonstrating that pS32A RNA was translated highly efficiently. Migration of the translation product of pS32A RNA on an SDS-polyacrylamide gel was very similar to that of the 2AB^A product of pMN30 RNA (20 kDa; data not shown). To assay for 2A activity in the translation product, we incubated the mixture programmed with pMN29 RNA (containing a stable P1-2ABC^A precursor; Fig. 2A) together with the mixture programmed with pS32A RNA. We found that the fusion protein translated from pS32A RNA induced cleavage of the P1-P2 bond *in trans* (data not shown).

Incubation of the translation mixture programmed with pS32A RNA with S10 extracts from mock-infected HeLa cells induced complete cleavage of p220, a pattern indistinguishable from the effect induced by pMN30 RNA translation (Fig. 2B, right panel). These data demonstrate that the presence of capsid proteins is not required for processing of p220. Whereas 6 µl of a translation mixture programmed with pMN30 RNA was necessary to induce complete cleavage of the p220 present in S10 extract, as little as 0.5 µl of pS32A RNA translation products produced the same effect (Fig. 2B, right panel).

Effect of anti-2A IgG on p220 processing. If poliovirus proteinase 2A does induce cleavage of p220, an antibody directed against 2A might inhibit this cleavage. We therefore translated pMN30 RNA in the presence of increasing concentrations of anti-2A IgG. Anti-2A IgG at 0.25 mg/ml in the translation mixture inhibited cleavage of the P1-P2 bond severely (Fig. 3A, lane 3), and in the presence of 1.5 mg of anti-2A IgG per ml almost no cleavage of the P1-2AB^A precursor protein was observed (Fig. 3A, lane 5). Increasing the concentration of anti-2A IgG to 5 mg/ml abolished cleavage completely, as demonstrated by the disappearance of the 2AB^A product (Fig. 3A, lane 7). Normal rabbit IgG at concentrations of up to 5 mg/ml did not inhibit cleavage of the P1-P2 bond (Fig. 3A, lanes 8 to 10). This effect of the anti-2A antibody was not seen in a previous study (42), in which anti-2A IgG was added to a poliovirus-specific translation complex prepared from poliovirus-infected HeLa cells. Hence, we wanted to distinguish whether this inhibition of cleavage at the P1-P2 bond by anti-2A antibodies is due to inhibition of the proteolytic activity of 2A or alteration of the availability of the cleavage site. We therefore performed *in vitro* translation of pMN30 RNA in the presence of anti-2A IgG, anti-VP1 IgG, and anti-VP3 IgG. Whereas anti-2A antibodies strongly inhibited cleavage of the P1-P2 bond (Fig. 4, lanes 2 to 4), no such effect was seen with either anti-VP1 IgG (Fig. 4, lanes 5 to 7) or anti-VP3 IgG (Fig. 4, lanes 8 to 10). These data suggest that the inhibitory effect of anti-2A antibodies on the cleavage of the P1-2AB^A precursor protein is due to inhibition of the proteolytic activity of 2A.

To study whether this inhibition of 2A activity has an effect on the cleavage of p220, we translated pMN30 RNA *in vitro* in the presence of increasing concentrations of anti-2A IgG and incubated the translation mixture with S10 extract from mock-infected HeLa cells. A concentration of 0.25 mg

FIG. 3. Effect of anti-2A IgG on the processing of p220 in vitro. (A) Samples (1.5 μ l) of translation mixtures were diluted to 50 μ l in loading buffer and analyzed on an SDS-12.5% polyacrylamide gel. Lane 1 shows a translation mixture without RNA, and lanes 2 to 10 were programmed with pMN30 RNA. Antibody concentrations in the translation mixtures were as follows (final concentrations) for

FIG. 4. Effects of anti-2A, -VP1, and -VP3 IgGs on the processing of a synthetic poliovirus precursor in vitro. Samples (1.5 μ l) of translation mixtures programmed with pMN30 RNA were diluted to 50 μ l and analyzed on an SDS-12.5% polyacrylamide gel. Antibody concentrations in the translation mixtures were as follows (final concentrations) for anti-2A IgG (lanes): 2, 0.25 mg/ml; 3, 1.5 mg/ml; 4, 3 mg/ml. For anti-VP1 IgG they were as follows (lanes): 5, 0.25 mg/ml; 6, 1.5 mg/ml; 7, 3 mg/ml. For anti-VP3 IgG they were as follows (lanes): 8, 0.25 mg/ml; 9, 1.5 mg/ml; 10, 3 mg/ml. Lane 1 contained no antibody, and lanes M represent an extract of [35 S]methionine-labeled poliovirus-infected HeLa cells.

of anti-2A IgG per ml in the translation mixture inhibited the cleavage of p220 severely (Fig. 3B, lane 2), and in the presence of 3 mg of anti-2A antibodies per ml no cleavage of p220 occurred (Fig. 3B, lane 5). Normal rabbit serum at concentrations of up to 5 mg/ml did not inhibit cleavage of p220 (Fig. 3B, lanes 7 to 9).

In a previous study it was shown that incubation of S10 extracts from poliovirus-infected HeLa cells with anti-2A does not prevent cleavage of p220 in vitro and that the enzyme activity that cleaves p220 does not copurify with 2A (23). It was therefore suggested that proteinase 2A does not

anti-2A IgG (lanes): 2, 0 mg/ml; 3, 0.25 mg/ml; 4, 0.5 mg/ml; 5, 1.5 mg/ml; 6, 3 mg/ml; 7, 5 mg/ml. For normal rabbit IgG they were as follows (lanes): 8, 0.25 mg/ml; 9, 1.5 mg/ml; 10, 5 mg/ml. Lanes M represent a lysate of [35 S]methionine-labeled poliovirus-infected HeLa cells. (B) A 12- μ l sample of each translation mixture was incubated with 6 μ l of S10 extract from mock-infected HeLa cells and subjected to immunoblot analysis as for Fig. 2B. Antibody concentrations in the translation mixtures were as follows for anti-2A IgG (lanes): 1, 0 mg/ml; 2, 0.25 mg/ml; 3, 0.5 mg/ml; 4, 1.5 mg/ml; 5, 3 mg/ml; 6, 5 mg/ml. For normal rabbit IgG they were as follows (lanes): 7, 0.25 mg/ml; 8, 1.5 mg/ml; 9, 5 mg/ml. U-S10 represents S10 extract from mock-infected HeLa cells, I-S10 represents S10 extract from poliovirus-infected HeLa cells. C.P. refers to cleavage products of p220. (C) In vitro translation of pMN30 RNA was performed for 90 min. Samples (12 μ l) of the translation mixture were incubated for 30 min at 30°C with (final concentrations) the following (lanes): 1, 0.75 mg of anti-2A IgG per ml; 2, 2.5 mg of anti-2A IgG per ml; 3, 0.75 mg of normal rabbit IgG per ml; 4, 2.5 mg of normal rabbit IgG per ml. Incubation with S10 extract from mock-infected HeLa cells and immunoblot analysis were performed as described in the legend to Fig. 2B.

cleave p220 directly. If this were true, incubation of the translation mixture with anti-2A antibodies, after translation, would not result in the same inhibitory effect on the proteolysis of p220 as that observed when anti-2A IgG was present in the translation reaction. This is because activation of a second proteolytic factor should already have occurred by the time the antibody was added. Accordingly, we performed *in vitro* translation of pMN30 RNA, followed by preincubation of the translation mixture with anti-2A antibodies or normal rabbit IgG for 30 min, followed by incubation of the mixture with S10 extract from mock-infected HeLa cells. Neither anti-2A antibodies nor normal rabbit serum had any detectable effect on the cleavage of p220 (Fig. 3C, lanes 1 to 4).

DISCUSSION

In this work we investigated whether the second proteinase encoded by poliovirus, proteinase 2A (42), plays a role in the proteolytic degradation of the cellular protein p220. A previous study had demonstrated that, in cells infected with an engineered mutant poliovirus that contained a single amino acid insertion in the amino-terminal part of polypeptide 2A, neither the characteristic poliovirus-specific shutoff of host cell protein synthesis nor processing of p220 occurred (2). By contrast, another study showed that the proteolytic activity that cleaves p220 did not copurify with 2A and that antibodies to 2A did not inhibit the processing of p220 *in vitro* (23). It was therefore considered likely that proteinase 2A does not cleave p220 directly. The genetic and immunochemical studies, however, do not rule out the possibility that polypeptide 2A activates a second proteolytic factor, of cellular or viral origin, which then cleaves p220. Presumably, activation of this factor by polypeptide 2A would occur early after infection of the cell with poliovirus; hence, antibodies directed against 2A would not inhibit cleavage of p220 in HeLa cell extracts prepared 3.5 to 4.5 h after infection.

Previous *in vitro* studies of the cleavage of p220 were hampered by the fact that, in crude extracts from poliovirus-infected cells, all protein products of the viral genome were present and therefore no specific effect of a single viral protein could be studied. We recently developed an efficient *in vitro* translation system which allowed us to express segments of the viral genome by translation of synthetic RNAs *in vitro* (25a). In the present study, we translated synthetic RNAs encoding the capsomer precursor (P1) and segments of the P2 region *in vitro* and incubated the translation products with cytoplasmic extracts from mock-infected HeLa cells. The p220 provided by these extracts was completely cleaved in all cases when active 2A proteinase was synthesized. The activity of 2A was determined by its ability to cleave at the tyrosine-glycine bond between P1 and P2 (25a). Fusion proteins that had a four-amino-acid insertion in the amino-terminal part of 2A or a nine-amino-acid deletion in the carboxy-terminal part of 2A completely failed to induce cleavage of p220 *in vitro*. The deletion of nine amino acids affects the histidine residue in the putative active site of 2A (42). This mutation rendered the proteinase completely inactive in regard to processing at the P1-P2 site. The four-amino-acid insertion inactivated 2A only partially. Cleavage of p220, however, was completely abolished by both the deletion mutation and the insertion mutation. These data are in accord with the result described previously that a poliovirus mutant with an amino acid insertion in the amino-terminal part of 2A was infectious and therefore did induce

cleavage of the P1-P2 bond but apparently did not induce cleavage of p220 (2). It seems likely that, besides the putative active-site region of 2A, there are other structural requirements in the amino-terminal part of 2A necessary for the induction of cleavage of p220.

We determined that the P1 precursor is not required for processing of p220 *in vitro* by constructing a plasmid that joins together the 5' NTR of EMCV and the coding sequence for poliovirus polypeptide 2A. This construction allowed us to use the unusually high translational efficiency of EMCV RNA for translation of a poliovirus protein. Since the fusion protein translated from this RNA contained active 2A proteinase (as determined by its ability to cleave the P1-P2 bond *in trans*) but no other viral proteins, and since synthesis of this fusion protein induced complete cleavage of p220, we conclude that active 2A proteinase is sufficient to induce proteolysis of p220 *in vitro* without involvement of other viral proteins.

Studies with antibodies directed against 2A were in accordance with these data. The presence of anti-2A antibodies in the translation mixture completely abolished p220 cleavage. However, the antibodies had no effect on p220 cleavage when they were incubated with the translation reaction mixture after translation had been completed. These data are in agreement with the observation that cleavage of p220 *in vitro* by cytoplasmic extracts from poliovirus-infected cells is not inhibited by antibodies directed against 2A (23). Our data show that proteinase 2A is the only viral protein required for processing of p220 and that this cleavage occurs via induction of a second proteolytic factor unrelated to 2A. This second factor is most likely of cellular origin. The presence of active 2A is apparently not required once this activation has occurred. Thus, our data demonstrate a specific interaction between a defined poliovirus protein and a cellular factor. In this study, the proteolytic factor was induced in rabbit reticulocytes. Recent experiments with highly purified proteinase 2A (H. König and B. Rosenwirth, submitted for publication) showed that a similar proteolytic factor, cleaving p220, was induced in HeLa cells when 2A was added (Kräusslich and Wimmer, unpublished data).

It is important to note that the data on the cleavage of p220 *in vitro* at present apply only to poliovirus. However, cleavage of p220 to similar degradation products has also been observed in cells infected with human rhinovirus 14 (9). Human rhinovirus 14 and poliovirus have closely related 2A polypeptides (23). EMCV, however, does not contain a homologous 2A polypeptide, and EMCV infection does not induce cleavage of p220 (25). Considering that a mutant poliovirus with an amino acid insertion in 2A showed an alteration in the shutoff of host cell protein synthesis and a lack of cleavage of p220 (2), it seems intriguing that 2A may mediate shutoff via induction of proteolysis of p220. At present, however, we cannot be certain that cleavage of p220 is the mechanism of shutoff *in vivo* because the actual function of the p220 component of eIF-4F is unknown and it has not been shown that cleavage of p220 causes inactivation of the complex rather than representing a secondary effect of the enzyme that inactivates eIF-4F. Recent evidence suggested that cleavage of p220 is necessary but may not be sufficient to induce complete shutoff in poliovirus-infected cells (3). EMCV infection induces double-stranded RNA-independent phosphorylation of eIF-2 (28), which leads to an overall decrease of protein synthesis. EMCV RNA, being a good messenger (30, 35), may profit from this decrease. We do not know whether similar mechanisms apply for poliovi-

rus or whether the shutoff of host cell protein synthesis is initiated differently by disparate picornaviruses. Further studies are needed to determine whether our extracts, in which p220 was cleaved, retain their ability to translate capped mRNA and to identify the proteolytic factor induced by poliovirus proteinase 2A.

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