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Relationship of p220 Cleavage during Picornavirus Infection to 2A Proteinase Sequencing

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Infection of HeLa cells by poliovirus results in an abrupt inhibition of host cell protein synthesis. It is thought that the mechanism of this inhibition involves proteolytic cleavage of the p220 component of the cap-binding protein complex, thereby causing functional inactivation of the cap-binding protein complex and preventing capped (cellular) mRNAs from binding ribosomes. Current data suggest that the viral proteinase 2A indirectly induces p220 cleavage via alteration or activation of a second proteinase of cellular origin. We present evidence that translation of poliovirus proteinase 2A sequences in vitro activates p220 cleavage. We have also aligned published picornavirus 2A amino acid sequences for maximum homology, and we show that the picornaviruses can be divided into two classes based on the presence or absence of a highly conserved 18-amino acid sequence in the carboxy-terminal portion of 2A. This conserved 2A sequence is homologous with the active site of the cysteine proteinase 3C common to all picornaviruses. We show that picornaviruses which contain the putative 2A active site sequence (e.g., enteroviruses and rhinoviruses) will induce cleavage of p220 in vivo. Conversely, we show that two cardioviruses (encephalomyocarditis virus and Theiler's encephalomyelitis virus) do not encode this putative proteinase sequence in the 2A region and do not induce cleavage of p220 in vivo. The foot-and-mouth disease virus (FMDV) 2A sequence represents an apparent deletion and consists of only 16 amino acids, most homologous with the carboxy terminus of the cardiovirus 2A sequence. It does not contain the putative cysteine proteinase active site. However, FMDV infection induces complete cleavage of BK cell p220, and translation of FMDV RNA in vitro induces an activity that cleaves HeLa cell p220. The data predict that an alternate FMDV viral protease is responsible for the induction of p220 cleavage.

The specific inhibition of host cell protein synthesis that occurs after poliovirus infection of HeLa cells is thought to result from the inactivation of the translational initiation factor called eIF-4F or the cap-binding protein complex (for reviews, see references 9, 33, and 35). This initiation factor identifies and binds the m^7G cap group in the 5′ ends of cellular mRNAs prior to their binding to the small ribosomal subunit. In poliovirus-infected cells, the largest subunit of eIF-4F, termed p220, is apparently cleaved to a set of antigenically related polypeptides of 100 to 130 kilodaltons kDa (12), and it is assumed that this structural alteration results in a loss of functional cap-binding protein activity.

The poliovirus genome codes for two identified proteinases, 3C and 2A, whose activities have been partially characterized (17, 38). Although no nonviral protein substrates were known for either of the viral proteinases, we tested the hypothesis that one of these enzymes was the mediator of p220 cleavage. These experiments involved the development of an in vitro assay for the conversion of p220 to the characteristic set of putative degradation products, as well as the partial purification of the p220-specific proteinase. It was of some surprise that neither proteinase 2A nor proteinase 3C copurified with the p220 cleavage activity; in fact, no viral protein could be implicated as being directly responsible for p220 cleavage (19, 21, 23). It was proposed that the cleavage of p220 was catalyzed by a latent, cellular proteinase, which was activated or induced following virus infection. Therefore, subsequent investigations of the role of poliovirus gene products in the inhibition of host cell protein synthesis were aimed at identifying viral proteins that could activate a cellular p220-specific proteinase.

In this report, we describe two approaches to identifying the viral mediator of the activation of p220 cleavage. First, a nested set of poliovirus RNA transcripts was generated from cloned poliovirus cDNA. These were translated in a mixed rabbit reticulocyte-HeLa cell extract in vitro, and the various translation products were tested for their ability to induce p220 cleavage activity. As was reported by Krausslich et al. (18) while this work was in progress, the synthesis of viral protein 2A correlated with p220 cleavage activation. These results are consistent with a previous report by Bernstein et al. (2), who presented genetic evidence suggesting that 2A was involved in the cleavage of p220. Their study described a mutant poliovirus, with a single amino acid insertion in 2A, which failed to induce p220 cleavage during infection and which manifested an altered phenotype with respect to inhibition of host cell protein synthesis.

Consequently, the deduced amino acid sequences of various 2A polypeptides from members of the four genera of picornaviruses have been aligned and analyzed for the presence or absence of a putative thiol proteinase consensus sequence which we propose is required for activation of p220 cleavage activity. We have previously identified this sequence in poliovirus 2A as being homologous to the cysteine proteinase active site common to all picornavirus 3C proteinases (1, 3, 23). Infected-cell extracts were tested directly for p220 cleavage by the various viruses. It was found that enteroviruses and rhinoviruses have conserved this 18-amino-acid sequence in the carboxy-terminal portion of 2A

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and that its presence correlates with the ability to activate p220 cleavage in their respective host cells. Two cardiovirus 2A sequences lack the conserved sequence and fail to induce p220 cleavage. Interestingly, foot-and-mouth disease virus (FMDV), which encodes only a 16-amino-acid stub of 2A that does not include the putative thiol protease active site, nevertheless does induce cleavage of p220, both in vivo and in vitro. A different proteinase sequence is encoded in the leader region of the aphthovirus genome, and it is likely that the leader protease is responsible for activation of the p220 cleavage activity.

MATERIALS AND METHODS

Cells and viruses. HeLa S3 cells were grown in suspension in Joklik modified minimum essential medium (Irvine Scientific Sales Co., Inc., Santa Ana, Calif.) containing penicillin and streptomycin and supplemented with 7% calf serum. Human amnion WISH cells or BK cells were grown in monolayer in Eagle minimum essential medium supplemented with 7% fetal bovine serum and 4 μg of gentamicin per ml or 5% calf serum, penicillin, and streptomycin, respectively. Growth in HeLa cells and purification of the Mahoney strain of poliovirus have been previously described (15). FMDV (type A_{12}) was grown in BK cells as described previously (13). Theiler's murine encephalomyelitis virus-infected cells were kindly supplied by R. Roos, University of Chicago. Coxsackievirus B3-infected HeLa cells were generously provided by M. Schultz, Department of Microbiology, Hahnemann University School of Medicine. Human enterovirus 70 was generously supplied by G. J. Stanton and was grown in human amnion WISH cells as previously described (36). Ribosomal salt washes containing concentrated p220 antigens were prepared from uninfected or poliovirus-infected HeLa cells as described (15).

Sequence analyses. The following published amino acid sequences deduced from cloned virus cDNAs were used for sequence comparisons: poliovirus type 1, Mahoney strain consensus (7); rhinovirus type 14 (4); coxsackievirus type B3 (20); hepatitis A virus (HAV) (5); encephalomyocarditis virus (28); Theiler's murine encephalomyelitis virus BeAn (30); and FMDV type A_{12} (32). The segments of those sequences corresponding to the 2A coding region were aligned for maximum homology by using the ALIGN program available through BIONET.

Antibody and immunoblot analysis. Immunoblot analysis of p220 antigens was performed as previously described by using either mouse monoclonal antibody directed against p220 (10) or a rabbit polyclonal antibody directed against the p220 degradation products from poliovirus-infected HeLa cells (22).

Restriction endonucleases and enzymes. Restriction endonucleases and enzymes used in cloning were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.; and New England BioLabs, Inc., Beverly, Mass. Manipulations of DNA were performed by using standard procedures (24), and transformations were performed with *Escherichia coli* C600. SP6 RNA polymerase and RNasin were purchased from Promega Biotec, Madison, Wis. Bacteriophage T7 RNA polymerase was purified by B. Szewczyk, University of Utah.

Construction of plasmids. Plasmids pPV-16 and pT7-1 were kindly supplied by B. L. Semler. Plasmid pPV-16 contains the entire poliovirus genome and is described elsewhere (34). The construction of pT7-1 has also been described (40); pT7-1 contains the entire poliovirus genome inserted into the pGEM-1 transcription vector (Promega Biotec). pSPOV was constructed in our laboratory by D. Brown. Briefly, the whole poliovirus cDNA was excised from pPV-16 by digestion with *Eco*RI, gel purified, and ligated into the multiple cloning site of the commercial transcription vector pSP65 (Promega Biotec) to form plasmid pSPOV. pT7-1 and pSPOV contain the insert oriented so that transcription reactions catalyzed with T7 or SP6 polymerase, respectively, yield positive-strand poliovirus RNA of full length. Thus, plasmids pT7-1 and pSPOV contained identical copies of the poliovirus genome, with the single exception that pT7-1 was further engineered to shorten the guanosine linker on the 5′ end of the genome to 3 residues (pSPOV has 18 residues) (40). pTPOV-3916 was derived from pT7-1 by digestion with *Hin*cII and religation of the largest fragment into the multiple cloning site. This procedure deleted all poliovirus sequences 3′ of the *Hin*cII site at position 3916 (in the 2B coding region). Similarly, pSPOV-2957, kindly supplied by K. Bienkowska-Szewczyk, was derived from pSPOV by digestion with *Sna*BI and *Sma*I and religation of the largest fragment. This procedure deleted all poliovirus sequences 3′ of the *Sna*BI site at position 2957 (in the 1D coding region).

In vitro transcription. Prior to transcription, the DNA template plasmids were linearized with the appropriate restriction endonuclease, extracted with phenol-$CHCl_3$, and precipitated in ethanol. Linearized templates were dissolved in water (0.5 μg/ml) and stored at −70°C. Transcriptions took place in a 50-μl reaction volume with either SP6 or T7 RNA polymerase as specified by the manufacturer (Promega Biotec). Product RNAs were twice extracted with phenol-$CHCl_3$ and precipitated in ethanol before storage at −70°C.

In vitro translation. Poliovirus RNA or FMDV RNA was extracted directly from purified virions, dissolved in water (0.5 μg/ml) or precipitated in 70% ethanol, respectively, and stored at −70°C. Translations of poliovirus RNAs were carried out by using a commercial rabbit reticulocyte translation kit (Promega Biotec) or as previously described (22) in rabbit reticulocyte lysates obtained from Green Hectares, Oregon, Wis. Most translation reactions (in 25-μl volumes) were supplemented with 5 μl of HeLa cell cytoplasmic extracts which had been treated with micrococcal nuclease (29). After 3 h of incubation at 30°C, translation reactions were terminated by freezing at −70°C or addition of sodium dodecyl sulfate to 1%. In some instances, additional HeLa cell cytoplasmic extract was added after 3 h of incubation, and the mixture was incubated at 30°C for an additional 2 h. Translation of FMDV RNA was performed as previously described (39).

RESULTS

Translation of transcripts of cloned poliovirus cDNA. A nested set of poliovirus RNAs were transcribed from linearized plasmids containing viral cDNA inserted adjacent to an SP6 or T7 promoter (Fig. 1). The RNA transcripts were analyzed on agarose gels to confirm their relative sizes (data not shown). Each RNA was then translated in a mixed rabbit reticulocyte-HeLa cell extract, which has been shown previously to effect accurate initiation and relatively efficient translation and processing of the product polypeptides (8, 22, 31, 40). Figure 2 shows the products of the translation reactions programmed with these poliovirus RNAs. Poliovirus proteins synthesized in infected HeLa cells and labeled with [^{35}S]methionine are displayed for comparison in lane i.

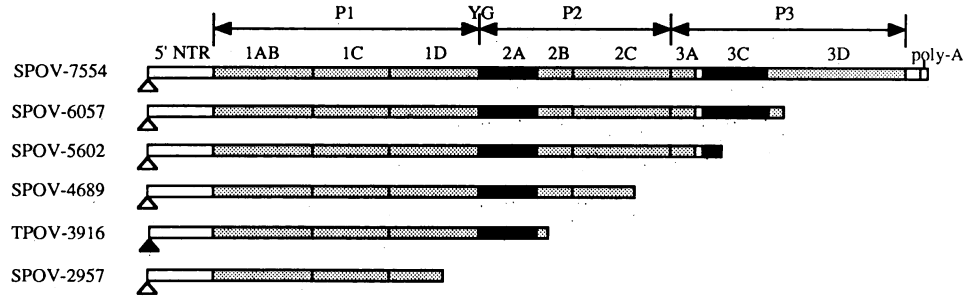

FIG. 1. Poliovirus RNAs transcribed in vitro. Diagram of the expected set of nested RNA transcripts generated from linearization of the plasmids pSPOV, pTPOV-3916, and pSPOV-2957 with selected restriction endonucleases and subsequent transcription with T7 or SP6 polymerases. In the notation used in this article, plasmids are distinguished from their transcribed product RNAs by the prefix p. All transcribed poliovirus RNAs included the 5' nontranslated region of the genome. Poliovirus genes are labeled, and the positions of the two poliovirus proteinases are highlighted in black. The numbers in parentheses refer to the number of the 3'-terminal poliovirus nucleotide included in the transcript RNA. Also shown is the position of codons for the tyrosine-glycine site where proteinase 2A cotranslationally cleaves the precursor polypeptide.

RNA extracted from purified virions resulted in the synthesis of a protein profile (lane a) that corresponds quite closely to that made in infected cells, and translation of the full-length RNA transcript from pSPOV yielded protein products (lane c) almost identical to those directed by viral RNA. Translation of all RNAs except SPOV-2957 produced the P1 precursor polypeptide 1ABCD (lanes c to g) as a result of processing by active proteinase 2A (38). SPOV-2957 RNA generated an 84-kilodalton polypeptide, which is the predicted molecular mass of 1ABCD* (where the asterisk denotes an incomplete product [Fig. 1]).

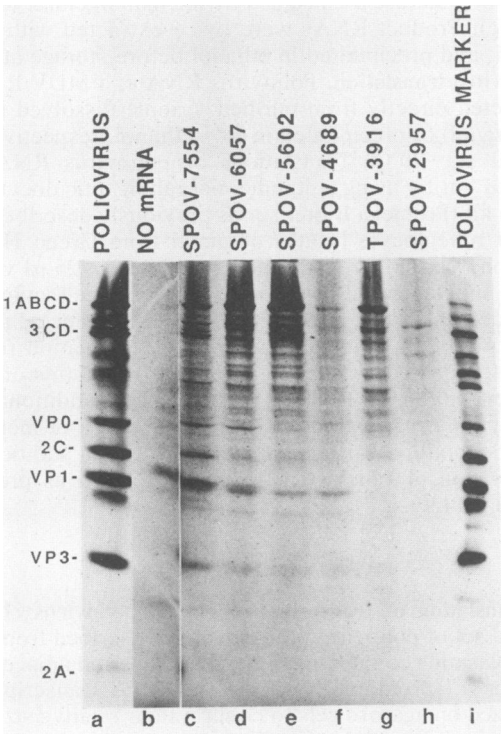

FIG. 2. In vitro translation of transcribed poliovirus RNAs. The products of in vitro translation reactions programmed with poliovirus RNAs described in Fig. 1 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Lane a shows products directed by viral RNA extracted from purified virions. Lane i shows cytoplasmic extract from poliovirus-infected HeLa cells pulse-labeled with [^{35}S]methionine 3.5 to 4.5 h postinfection.

Translation of full-length RNAs (virion or SPOV) yielded proteins VP0, VP1, VP3, and 2C, which indicated synthesis of active 3C proteinase, since each of these polypeptides is processed from precursors by 3C-mediated reactions. SPOV-6057 RNA includes all poliovirus sequences except those coding for the carboxy terminus of 3D polymerase. Examination of the translation products (lane d) showed that cleavage of the carboxy terminus of VP0 occurred efficiently, but other 3C-catalyzed cleavage reactions occurred less efficiently or not at all. This alteration in cleavage pattern was probably due to the inability to produce an intact 3CD precursor protein, which is required for efficient processing of the P1 capsid protein precursor to capsid proteins (40). Translation of other poliovirus RNAs lacking 3C coding sequences (lanes e to h) did not produce detectable cleaved capsid proteins or 2C, as expected, since no 3C proteinase was synthesized.

When compared with virion RNA, the transcripts demonstrated relatively poor messenger activity (e.g., compare lanes a and c, which contained similar amounts of RNA in the translation reaction). For this reason, several small poliovirus proteins, including 2A, could not be detected. SPOV-4689 and SPOV-2957 (lanes f and h) were especially poorly translated; it is not clear whether this was due to partially degraded RNA preparations or to an inherent problem in translating these RNAs. The presence of numerous other polypeptides in minor but significant amounts is unexplained in these and other (18) similar studies.

The results shown in Fig. 2 demonstrate that poliovirus RNAs that contain coding sequences for 2A proteinase yield 2A proteinase activity in vitro; RNAs that encode 3C sequences manifest 3C proteinase activity in vitro. We therefore examined those translation reactions for their ability to activate p220 cleavage activity.

p220 cleavage by poliovirus translation products. The reticulocyte lysate translation reactions described above were supplemented with extracts from uninfected HeLa cells to improve the fidelity of initiation of translation. It was therefore possible, by performing a direct immunoblot of the translation reaction, to determine whether the translation products were capable of activation of p220-specific proteinase and p220 cleavage. Figure 3 shows the immunoblot of the

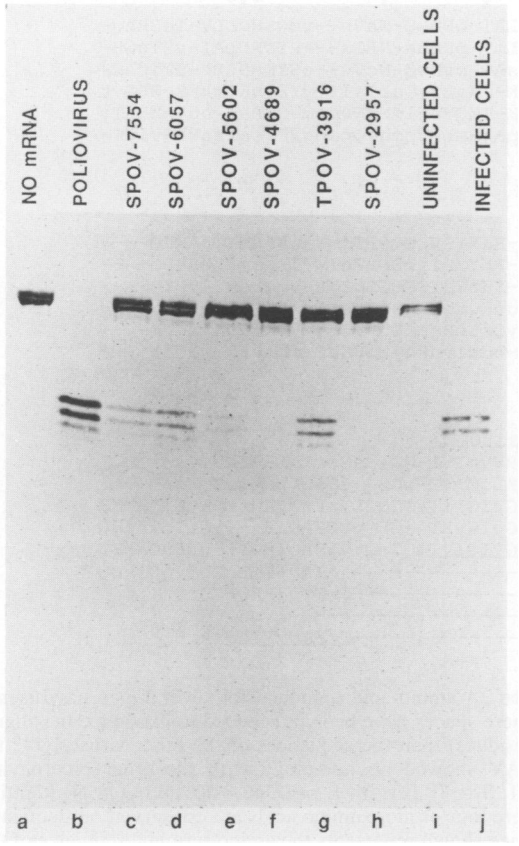

FIG. 3. Immunoblot analysis for p220 cleavage activity induced by translation products of poliovirus RNAs. Translation reactions shown in Fig. 2 were analyzed by immunoblot analysis with anti-p220 antibody. Lanes i and j contain cytoplasmic extract from uninfected or poliovirus-infected HeLa cells, respectively.

same translation reactions whose products are displayed in Fig. 2. The anti-p220 monoclonal antibody used in this immunoblot has very poor reactivity with rabbit p220, so that only HeLa cell p220 and its induced cleavage products are detected (22). p220 cleavage was induced in all translation reactions except the one programmed with SPOV-2957 RNA (lane h), which has no 2A coding sequence, and produced no 2A proteinase activity. The products of translation of virion RNA efficiently induced p220 cleavage (lane b), whereas the translation products of the full-length poliovirus RNA transcript were less efficient, consistent with the reduced translation activity of the transcript, shown above. Two RNAs which did not contain complete 3C coding sequences, SPOV-5602 and SPOV-4689 (lanes e and f), produced detectable but greatly reduced p220 cleavage activity. Since the C terminus of 2A is normally produced by 3C cleavage, these RNAs would generate 2A moieties unnaturally linked to larger polypeptides at their carboxy termini (2ABC3ABC* and 2ABC*) and thus may have reduced *trans* cleavage activity, even though these proteins do catalyze intramolecular autocatalytic cleavage to release 1ABCD (Fig. 2). p220 cleavage activity reached higher levels in the lysate programmed with TPOV-3916 (Fig. 3, lane g), which would yield a significantly smaller 2AB*. These results suggested that a functional 2A was both necessary and sufficient for the activation of p220 cleavage activity. They are in agreement with those reported in a recent study by Krausslich et al. (18), who additionally showed that disruption of the 2A coding sequence by either insertion or deletion prevented cleavage of p220 in vitro. These investigators also showed that anti-2A serum inhibited p220 cleavage if added during the in vitro translation reaction, but did not inhibit p220 cleavage if added after activation of the p220-specific proteinase had occurred.

Comparison of picornavirus 2A coding regions. Poliovirus 2A proteinase contains, near its carboxyl terminus, an amino acid sequence of 18 residues which is very similar to the active site of 3C cysteine proteinase of poliovirus and other picornaviruses (23). It has been shown that poliovirus 2A catalyzes proteolytic cleavages of the poliovirus polyprotein, and although no direct biochemical evidence exists, the sequence homology with proteinase 3C makes it likely that poliovirus 2A is also a cysteine proteinase. Figure 4 shows a computer-matched sequence alignment of the 2A amino acid sequence of several picornaviruses. The putative cysteine proteinase active site sequence was found in the carboxyl half of the 2A coding region of poliovirus, rhinovirus, and coxsackievirus (Fig. 4, box). The sequence homology within this site among the three viruses is high, containing 15 of 18 identical residues. Conversely, neither 2A genes nor 1D and 2B genes of HAV, encephalomyocarditis virus, Theiler's murine encephalomyelitis virus, and FMDV contain a similar cysteine proteinase active-site sequence (data not shown). Thus, picornaviruses can be divided into two apparent classes based on the presence of the putative cysteine proteinase active site sequence in the 2A gene. This site is found in poliovirus, rhinovirus, and coxsackievirus, but it has been deleted from the 2A region of the aphthoviruses and cardioviruses.

Overall, the picornavirus 2A genes are not highly conserved among the four genera. However, considerable homology exists in a small region near their carboxy termini in the putative cysteine proteinase region and in two flanking regions (Fig. 4, regions 1 and 2). Although they lack a cysteine proteinase sequence, cardiovirus and FMDV 2A genes show conservation of a different amino acid sequence not found in the enteroviruses or rhinovirus (Fig. 4, region 1). The FMDV 2A gene product consists exclusively of this latter region. In addition, all picornaviruses except cardioviruses and HAV contain some homology in a third region (Fig. 4, region 2). Interestingly, in FMDV this sequence is not included in 2A but occurs at the beginning of the 2B gene product.

p220 cleavage during picornavirus infection. Cytoplasmic extracts from several picornavirus-infected cells were prepared during the mid to late phases of the virus growth cycles. Proteins in these extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and then probed with anti-p220 serum to determine whether each virus induced p220 cleavage. Figure 5 shows the resulting immunoblots from several infected-cell extracts, as well as control, uninfected extracts. All enteroviruses tested (poliovirus, coxsackievirus B3, and enterovirus 70) induced rapid and total cleavage of p220 (Fig. 5, lanes b, f, and h). Previously, rhinovirus 14 infection also has been shown to induce p220 cleavage in HeLa cells (11). In contrast, Theiler's murine encephalomyelitis virus, which is now considered a cardiovirus (26, 27, 30), did not cause detectable cleavage of p220 (lane d). This result was in accordance with a previous study, which determined that another cardiovirus, encephalomyocarditis virus, also does not cause cleavage of p220 (25). The immunoblots shown in Fig. 5, lanes a to d, were probed with a polyclonal serum that

FIG. 4. Sequence comparisons of picornavirus 2A gene regions. The deduced 2A amino acid sequences of several picornaviruses were aligned by computer as described in Materials and Methods. Dashes indicate where spaces have been introduced to maximize the alignment homology. The published 2A sequence for HAV is considerably longer (189 residues) than the sequences of the other viruses (142 to 150 residues); therefore, several gaps have been introduced in areas where HAV showed no homology with the other picornaviruses. Conservative amino acid substitutions allowed in this analysis were V = L = I, S = T, F = Y, E = D, R = K, and Q = N. Identical or conservative amino acids are shown in capitals. Asterisks indicate positions where four or more amino acids are conserved or identical. The putative active-site serine or cysteine is shown (◇). The italicized FMDV sequence shown represents the amino-terminal sequence of the 2B polypeptide. Also shown are related amino acid sequences from trypsin (trp) and the capsid proteinase of Sindbis virus (sbv) (16). Other abbreviations: pol, poliovirus; hrv, human rhinovirus; cox, coxsackievirus; emcv, encephalomyocarditis virus; tmev, Theiler's murine encephalomyelitis virus.

had been prepared against poliovirus-infected HeLa cell p220 cleavage products (22), whereas a monoclonal antibody prepared against HeLa cell p220 (10) was used in the immunoblots shown in lanes e to h. Use of a polyclonal serum was necessary because the monoclonal antibody failed to react with p220 in the BHK host used for Theiler's murine encephalomyelitis virus infection. The reactivity of even the polyclonal antiserum with hampster p220 was quite weak, and the high concentration of serum used in this study resulted in the appearance of several nonspecific background bands (lanes c and d). This antiserum also consistently reacts with an unidentified protein of approximately 150 kilodaltons. A comparison of the 2A sequences shown in Fig. 4 with the analysis of p220 cleavage activities of the several picornaviruses that have been tested indicate that the presence of the cysteine proteinase active-site sequences in 2A positively correlates with the ability to induce p220 cleavage for three of the four picornavirus genera. The results of analyses of the aphthovirus FMDV are described below.

p220 cleavage by induced FMDV in vivo and in vitro. The alignment of the 2A coding sequences presented in Fig. 4 shows that FMDV lacks a complete 2A protein but codes for a 16-amino-acid polypeptide fragment that most closely resembles the extreme carboxy terminus of the cardioviral 2A protein. Since the putative cysteine proteinase active-site sequence is not present, our initial prediction was that FMDV would not induce p220 cleavage in infected cells. Immunoblot analysis of a cytoplasmic extract prepared from FMDV-infected BK cells showed, however, that p220 was completely cleaved (Fig. 6A, lanes c and d). Again, high concentrations of serum were required for reactivity with the bovine (BK) cell p220, thus producing a significant background of nonspecific signal, but clearly showing the complete absence of p220. New, antigenically related polypeptides were detected with mobilities similar to, but not identical with, the cleavage products produced in poliovirus-infected HeLa cells (Fig. 6A, lane b), although differences in antigen-antibody reactivity may complicate a direct comparison of the p220 products formed after infection of the two cell types with the different viruses.

The above results indicated that FMDV infection caused cleavage of the host cell p220. In that case, we reasoned that FMDV-infected cell extracts should contain an active p220-specific proteinase. Indeed, when FMDV-infected cell extracts were incubated with uninfected HeLa cell cytoplasm, the infected-cell extract was able to induce cleavage of the HeLa cell p220 in vitro (Fig. 6B, lane e). No cleavage occurred when the HeLa cell cytoplasmic extract was incubated with either buffer (lane c) or uninfected BK cell extract (lane d). The products of HeLa cell p220 cleavage by FMDV-induced, p220-specific proteinase did not react immunologically in the same way as those produced by poliovirus-induced HeLa cell-specific proteinase (lane f), although in other experiments, antigenically reactive products were detected in the same size range as those produced in poliovirus-infected cells. It should be noted that these ex-

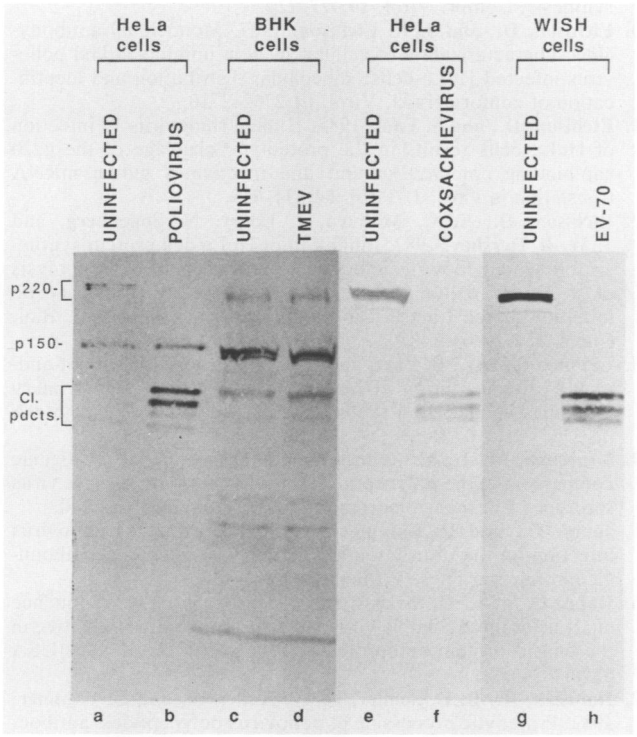

FIG. 5. p220 cleavage during picornavirus infections. Cell lysates prepared from uninfected or virus-infected cells were analyzed by immunoblot methods with anti-p220 antibody. Samples in lanes a to d were reacted with rabbit antiserum made against HeLa cell p220 cleavage products (22), and those in lanes e to h were incubated with anti-p220 monoclonal antibody (10). Lysates of picornavirus-infected cells were collected in mid to late phases of the virus growth cycles.

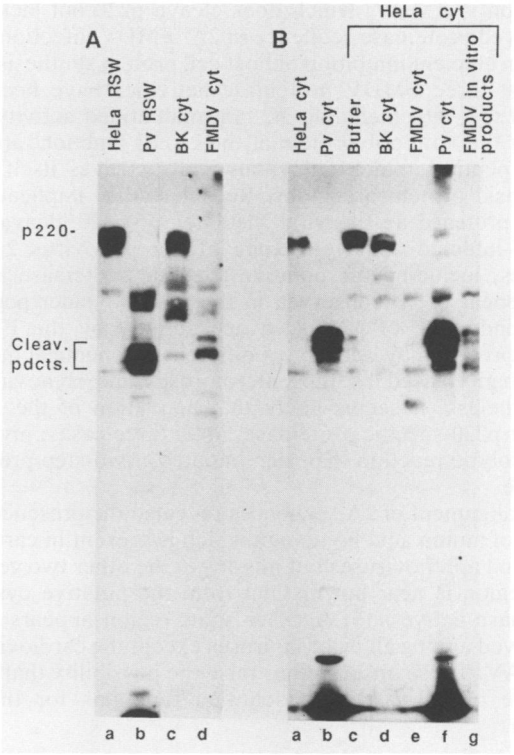

FIG. 6. FMDV-induced cleavage of p220. (A) Cleavage of p220 in vivo. Lanes a and b show immunoblot analysis (with polyclonal serum) of ribosomal salt washes (RSW) from uninfected HeLa or poliovirus-infected HeLa cells, respectively. Lanes c and d show a similar immunoblot analysis of cytoplasmic extracts (cyt) from uninfected or FMDV-infected BK cells. (B) Cleavage of p220 by cell extracts in vitro. Uninfected HeLa cell extract (10 μl) was incubated for 3 h at 37°C with buffer (lane c) or extracts (20 μl) from uninfected or FMDV-infected BK cells (lanes d and e) or poliovirus-infected HeLa cells (lane f). Lane g shows a similar assay with 10 μl of HeLa cell extract incubated with 20 μl of reticulocyte lysate at the completion of an in vitro translation reaction programmed with FMDV RNA. All samples were analyzed by immunoblot procedures, using polyclonal antibody.

periments did not distinguish between p220 cleavage catalyzed by proteinase derived from BK cells or HeLa cells or both.

Finally, FMDV RNA was translated in a reticulocyte lysate, and the translation products were tested for their ability to induce p220 cleavage, as was done for polioviral RNA translation products (Fig. 3, lane b). The FMDV RNA translation products caused efficient cleavage of HeLa cell p220 (Fig. 6B, lane g), as well as BK cell p220 (data not shown). Thus, despite the absence of the proposed proteinase sequence that is conserved in the 2A genes of the other picornaviruses that induce p220 cleavage in their infected host cells, FMDV does induce a p220-specific proteinase activity.

DISCUSSION

Recently published work by Krausslich et al. (18), as well as similar experiments reported here, demonstrate that translation of poliovirus RNAs containing an intact 2A coding sequence results in the activation of p220-specific proteinase activity. In our study, some of the poliovirus RNA transcripts produced products that induced p220 cleavage only with poor efficiency, even when the RNAs were actively translated (e.g., SPOV-5602; Fig. 2 and 3, lanes e). These RNAs produce aberrant 2A-containing proteins owing to the deletion of 3C coding sequences which would normally process the 2A/2B junction. These results suggest that a properly processed 2A protein, rather than a larger 2A-containing precursor protein, is responsible for the activation of p220-specific proteinase.

A comparison of the computer-aligned 2A coding sequences of several representative picornaviruses revealed than an 18-amino-acid sequence that is conserved within the 3C proteinase proteins of all picornaviruses is also present in the 2A proteinase sequences of the enteroviruses and rhinoviruses. Although currently classified as an enterovirus, HAV does not contain this conserved 2A sequence, and it has only very limited homology with other enteroviruses in other parts of the genome (5). Neither the cardiovirus nor the aphthovirus 2A sequences have conserved this putative cysteine proteinase active site. When members of the different picornavirus genera were screened for the ability to induce p220 cleavage activity, it was found that both enteroviruses and rhinoviruses, but not cardioviruses, induced p220 cleavage. HAV is excluded from this discussion, since its protracted growth cycle usually results in persistent infection, without inhibition of host cell protein synthesis. HAV-infected cells have not been examined for p220 cleavage, but it is assumed that it does not occur. The single exception to the correlation between the conserved cysteine proteinase active-site sequences in 2A and p220 cleavage

induction is FMDV, which does cleave p220 but lacks the conserved proteinase sequence in 2A. FMDV infection does result in efficient inhibition of host cell protein synthesis (14). At least three FMDV proteinase activities have been described (37, 39), including 3C, an unidentified activity that cleaves the viral polyprotein at the 2A/2B junction, and the leader peptide, which autocatalytically cleaves itself from the capsid protein precursor. Recent studies implicate the leader protease as the viral mediator of p220 cleavage in FMDV-infected cells (6). None of the poliovirus 2A sequences, including the putative cysteine proteinase active site, appear to be conserved in the FMDV leader peptide. Thus, induction of p220 cleavage activity by the FMDV leader protein may occur by a different biochemical mechanism than is used by the enteroviruses and rhinoviruses. Nevertheless, it seems likely that activation of the latent cellular p220-specific proteinase, in all three cases, involves a proteolytic reaction that then initiates a two-step protease cascade.

The alignment of 2A sequences revealed the presence of a region of amino acid homology which is present in cardioviruses and aphthoviruses but missing in the other two genera. This region is near but distinct from the putative cysteine proteinase active site. Another short region appears to be conserved among all picornaviruses except the cardioviruses and HAV. These observations raise the possibility that there may be other, as yet unidentified functions for the 2A protein.

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