JOURNAL OF VIROLOGY, Nov. 1996, p. 8124–8127 Vol. 70, No. 11
0022-538X/96/$04.00+0

Foot-and-Mouth Disease Virus 2A Protease Mediates Cleavage in Attenuated Sabin 3 Poliovirus Vectors Engineered for Delivery of Foreign Antigens

NORA M. MATTION,* ELENA C. HARNISH, JOAN C. CROWLEY, AND PATRICIA A. REILLY

Wyeth-Lederle Vaccines and Pediatrics, Viral Vaccine Research, Pearl River, New York 10965

Received 26 April 1996/Accepted 22 July 1996

Poliovirus vectors are being studied as potential vaccine delivery systems, with foreign genetic sequences incorporated as part of the viral genome. The foreign sequences are expressed as part of the viral polyprotein. Addition of proteolytic cleavage sites at the junction of the foreign polypeptide and the viral proteins results in cleavage during polyprotein processing. The ability of foot-and-mouth disease virus (FMDV) 2A to mediate proteolytic cleavage in the context of poliovirus vectors was studied. The results demonstrate that FMDV 2A is able to generate cleavage of the foreign antigen from the viral polyprotein. A second cleavage event between the FMDV 2A peptide and the foreign protein was also observed.

Poliovirus is a human pathogen that belongs to the *Enterovirus* genus of the *Picornaviridae* family. Following infection of humans, poliovirus replicates in the gastrointestinal tract with the potential to develop viremia and to invade the central nervous system, resulting in paralysis. Attenuated strains of poliovirus (Sabin strains) have been developed that replicate well in the human intestine but do not infect the central nervous system (17). Three poliovirus serotypes (Sabin 1 to 3) are the basis of the oral poliovirus vaccine which has been effective in providing protection from paralytic poliomyelitis. The oral poliovirus vaccine results in generation of mucosal immunity as well as long-lasting systemic immunity (10–12).

Work in our laboratory has focused on the use of Sabin strains of poliovirus as live vectors for the delivery of foreign antigens. We have previously reported the expression of foreign antigens from Sabin 3 poliovirus vectors (4, 7–9). Foreign antigens are expressed as fusions with the viral polyprotein and are subsequently cleaved at an engineered 3C protease ($3C^{pro}$) cleavage site. Results of these studies demonstrate the ability of poliovirus to maintain foreign sequences in the genome and express the heterologous antigen during viral replication. It was also found that the size of the foreign sequence inserted into the poliovirus genome affected both the viability and genetic stability of the recombinant polioviruses. Recombinant polioviruses with foreign sequences of 300 nucleotides or less were found to be genetically stable over multiple passages in tissue culture (9). Cleavage of the foreign sequences from the polyprotein at the engineered $3C^{pro}$ cleavage site was found to be less efficient than normal viral cleavage events (9) and may be the rate-limiting step in replication of recombinant polioviruses. The efficiency of cleavage by poliovirus 3C or 3CD is dependent on both the linear sequence at the cleavage site (13) and the conformation adopted by the flanking polypeptide sequences (20). The presence of foreign sequences in the polyprotein of recombinant polioviruses would be expected to influence the secondary structure and therefore the ability to generate proteolytic cleavage.

The goal of the present work is to test the ability of the $2A^{pro}$ of foot-and-mouth disease virus (FMDV $2A^{pro}$) to mediate proteolytic cleavage in the context of recombinant poliovirus vectors. FMDV $2A^{pro}$ is a short polypeptide of 16 amino acids that cleaves the polyprotein of FMDV at the 2A/2B junction cotranslationally (16). This cleavage, which occurs at its carboxyl terminus at a glycine-proline amino acid pair, appears to be highly efficient in FMDV as precursors spanning the 2A/2B region are not detected. Other laboratories have reported that cleavage by FMDV $2A^{pro}$ is independent of the presence of other FMDV sequences and can generate cleavage in the presence of heterologous sequences (14, 15).

Sequences encoding FMDV $2A^{pro}$ were incorporated into recombinant polioviruses in place of the engineered $3C^{pro}$ site in an attempt to increase the efficiency of cleavage of foreign sequences from the polyprotein during viral replication. More efficient cleavage may minimize the effects of the foreign protein on normal polyprotein maturation and therefore viral replication, resulting in increased stability of the inserted sequences.

Design and generation of recombinant Sabin 3 viruses containing FMDV $2A^{pro}$. To test the ability of FMDV $2A^{pro}$ to cleave foreign antigens from the poliovirus polyprotein, the sequence encoding FMDV $2A^{pro}$ was cloned into the plasmids pV1gD and pV2gD (8). These plasmids contain a full-length cDNA copy of the Sabin 3 genome (18) plus 225 nucleotides of herpes simplex virus type 2 glycoprotein D (gD), followed by sequence encoding an artificial $3C^{pro}$ cleavage site (9). The gD sequences were cloned at either the 5′ end of the poliovirus open reading frame (pV1gD) or at the junction between P1 and P2 (pV2gD).

Recombinant PCR was used to modify the poliovirus vectors by introducing sequence encoding 16 amino acids of FMDV $2A^{pro}$ plus a proline for cleavage at the glycine-proline junction 3′ to the sequence encoding gD. Three different vectors containing FMDV $2A^{pro}$ were designed, as shown schematically in Fig. 1. Two different cleavage sites were introduced following the foreign gene cloned at the 5′ end of the open reading frame, designated pV1F and pV1FC. The third vector, pV2FgD, contains the foreign sequence plus FMDV $2A^{pro}$ cloned between the precursors P1 and P2-P3. The rationale for designing two different cleavage sequences at the 5′ end of the open reading frame was based on the requirement to maintain a glycine for myristylation of the poliovirus polyprotein. During poliovirus replication, the polyprotein is myristylated at the

* Corresponding author. Present address: Biogenesis S.A., Ruta Panamericana, Km 38,2 (1619)-Garin, Argentina. Electronic mail address: nmattion@virol.wamani.apc.org.

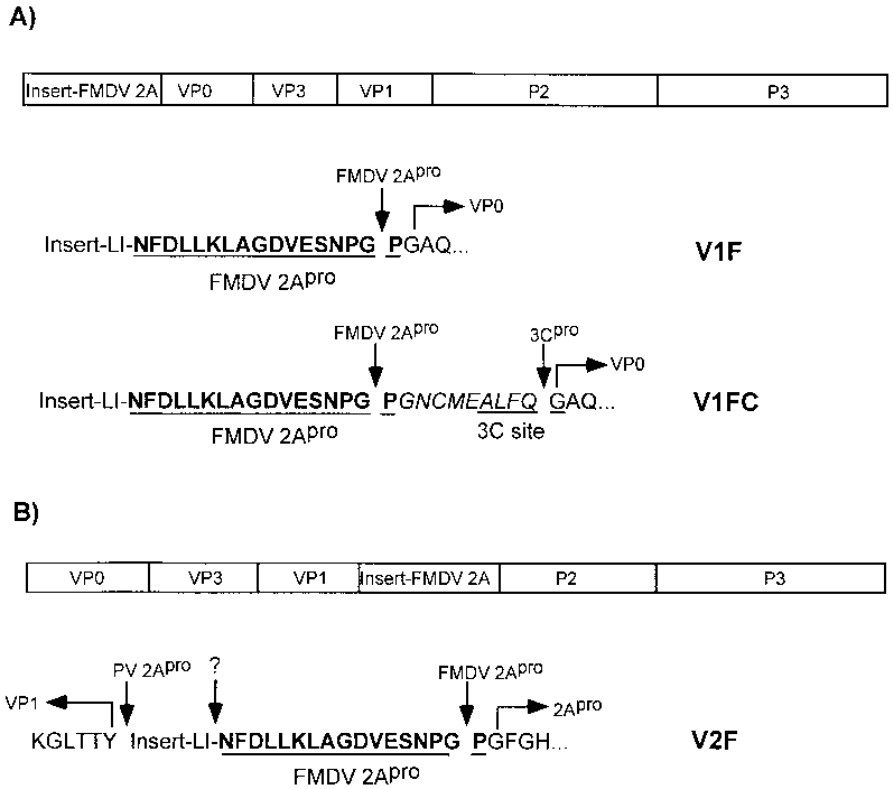

FIG. 1. Construction of Sabin 3 vectors carrying foreign sequences fused to the heterologous FMDV 2A protease. The vectors shown have been named V1F, V1FC, and V2F. The diagram shows the poliovirus polyprotein with insertions at the N terminus (A) or the P1-P2 junction (B), as well as the amino acid sequence of the regions of interest. The sequence of the FMDV $2A^{pro}$ polypeptide is in bold, and the amino acids added for the $3C^{pro}$ cleavage site are in italics. The vertical arrows indicate the expected cleavage sites. The question mark in panel B indicates an unexpected cleavage at the amino terminus of FMDV $2A^{pro}$ in V2F. PV $2A^{pro}$, poliovirus $2A^{pro}$.

amino-terminal glycine, which has been shown to be essential for virus viability (1, 2). In vectors with the foreign sequences fused to the amino terminus of the polyprotein, a glycine is exposed after cleavage at the 3C site and can be myristylated (8). Replacement of the 3C site with FMDV $2A^{pro}$ results in a proline at the amino terminus after cleavage, which is expected to prevent myristylation of the polyprotein. The vector, pV1FC, contains both FMDV $2A^{pro}$ and 3C cleavage sites in order to provide a glycine for myristylation. For the vector pV2F, it was expected that addition of a proline to the amino terminus of poliovirus 2A would not significantly affect poliovirus $2A^{pro}$ function. We have previously shown that recombinant polioviruses containing foreign sequences fused to the amino terminus of poliovirus 2A were viable (9).

Viable viruses were obtained from pV1FCgD and pV2FgD but not from pV1FgD after transfection of RNA transcripts into HeLa cells (19). This indicates that poliovirus $2A^{pro}$ with an additional amino-terminal proline is functional but that addition of a proline at the amino terminus of P1 is lethal. The stability of the foreign sequences in the Sabin 3 genome was tested by reverse transcription of the recombinant viral RNA, followed by PCR amplification using poliovirus primers flanking the gD insertion or gD-specific primers (8). Serial passages before and after plaque purification were performed, and the recombinant polioviruses were found to be stable for at least six passages after plaque purification.

We also wanted to test whether the poliovirus vectors containing the FMDV $2A^{pro}$ provide stable expression of a longer foreign sequence. The gene encoding the bacterial enzyme chloramphenicol acetyltransferase (CAT) was chosen for expression in the vectors because of the size (660 nucleotides) and the availability of sensitive assays to detect CAT expression. The sequences encoding gD (225 nucleotides) were replaced with the CAT gene by subcloning into the *Not*I and *Pac*I sites in the vectors pV1FC and pV2F. The resulting vectors were named pV1FC/CAT and pV2F/CAT, with the CAT gene cloned at the 5′ end of the poliovirus open reading frame or at the P1-P2 junction, respectively. Viable recombinant polioviruses were recovered from these constructs after transfection of RNA into HeLa cells.

Reverse transcription PCR was performed on RNA extracted from recombinant virions obtained after serial passages in order to evaluate the stability of the CAT gene. The full-length CAT gene was maintained in the Sabin 3 genome through the four passages tested (data not shown). In addition, cells infected with the recombinant viruses were assayed for CAT enzymatic activity. Both V1FC/CAT and V2F/CAT exhibited CAT activity after infection of HeLa cells for all passages tested, confirming the presence of the CAT gene in the viral genome. The recombinant Sabin 3 viruses carrying the CAT gene grew more slowly and to lower titers than recombinant polioviruses carrying the smaller gD fragment (data not shown). This supports the observation that the length of the inserted sequence impacts viral growth (8, 9).

Expression and cleavage of the foreign antigen from Sabin 3-FMDV $2A^{pro}$ vectors. Expression of the foreign polypeptide from the recombinant polioviruses containing gD was detected by Western immunoblots developed with an anti-gD monoclo-

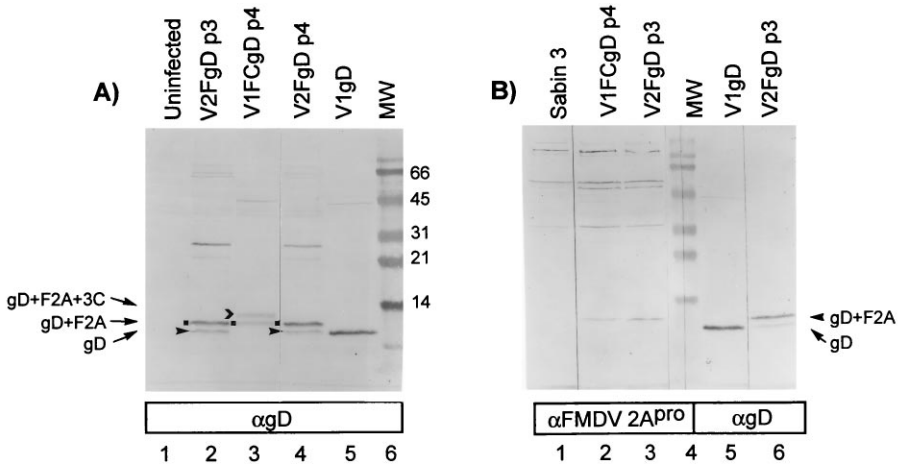

FIG. 2. Expression of herpes simplex virus type 2 (HSV-2) gD fused to FMDV $2A^{pro}$ in Sabin 3 poliovirus vectors. HeLa cells were infected with recombinant Sabin 3 viruses carrying an HSV-2 gD fragment fused to FMDV $2A^{pro}$ either at the 5′ end of the poliovirus polyprotein (V1FC) or at the P1-P2 junction (V2F). V1gD is a Sabin 3 vector carrying the same gD insert fused to a poliovirus $3C^{pro}$ cleavage site (8). Western blots were developed with an anti-gD monoclonal antibody (DL6) or with an anti-FMDV $2A^{pro}$ antibody, as indicated. MW, prestained molecular weight markers. The bands of interest are indicated on the blot in panel A as follows: >, gD+FMDV $2A^{pro}$+3C site; ■, gD+FMDV $2A^{pro}$; ➤, gD.

nal antibody, DL6 (5). The occurrence of proteolytic cleavage of the gD sequences from the polyprotein precursors is determined by the size of the bands containing gD. If cleavage mediated by FMDV $2A^{pro}$ is very efficient, a band corresponding to the migration position of gD plus FMDV 2A at 10 kDa would be observed. HeLa cells were infected with the recombinant viruses, V1FCgD and V2FgD, at a multiplicity of 10 and harvested at 8 h postinfection. In the Western blot, two bands containing gD sequences were observed for both viruses, corresponding to the expected molecular size of the gD fragment (~10 kDa) (Fig. 2A). The band corresponding to the position of peptide gD+FMDV 2A (10 kDa) was observed for both viruses (indicated by ■; Fig. 2A, lanes 2 to 4). A smaller band was also detected in lysates infected with V2FgD that comigrates with the gD fragment expressed from the virus V1gD, which lacks the FMDV $2A^{pro}$ sequence. This suggests the occurrence of a novel cleavage event in which the FMDV $2A^{pro}$ sequence is removed from the gD sequence. A larger precursor was also observed for V2FgD, corresponding to the position of peptide gD+FMDV 2A+PV2A at ~ 26 kDa, indicating that cleavage by FMDV $2A^{pro}$ is not completely efficient.

The pattern of bands containing gD from the virus V1FCgD is different from that of V2FgD. The doublet observed corresponds to the position of peptides gD+FMDV 2A at 10 kDa and gD+FMDV 2A+3C at 11 kDa. The presence of these two bands indicates that cleavage is occurring at both protease sites independently. It is interesting to note that there is no band corresponding to gD alone, as was seen with V2FgD.

In order to test the hypothesis that the FMDV $2A^{pro}$ polypeptide was cleaved from gD, antiserum to FMDV $2A^{pro}$ was generated in rabbits immunized with a synthetic peptide corresponding to the sequence of FMDV $2A^{pro}$. The antiserum, TB2513, showed a good specific reactivity by enzyme-linked immunosorbent assay to the peptide antigen (data not shown) and was used in Western blots against lysates of cells infected with the viruses V1FCgD and V2FgD. The results show that the FMDV $2A^{pro}$ antiserum cross-reacts with the band corresponding to the position of peptide gD+FMDV 2A from both viruses (Fig. 2B). The larger band expressed from V1FCgD which is predicted to be peptide gD+FMDV 2A+3C did not cross-react with the antiserum (Fig. 2B, lane 2). A possible explanation for the absence of the slower-migrating band in these Western blots is that the antigenic epitopes in FMDV 2A are not reactive to the antibody in the fusion polypeptide gD+FMDV 2A+3C.

Characterization of recombinant polioviruses expressing a foreign antigen plus FMDV $2A^{pro}$ has demonstrated the ability of FMDV $2A^{pro}$ to mediate cleavage in the context of the poliovirus polyprotein. The efficiency of cleavage of the FMDV $2A^{pro}$ in the poliovirus genetic background was not as high as expected, however. In FMDV, 2A-mediated cleavage is a cotranslational event. The results of our experiments show that FMDV 2A cleavage is not complete. This is demonstrated by the presence of larger precursors, gD+FMDV 2A+3C for V1FCgD and gD+FMDV 2A+PV2A for V2FgD. Analysis of cleavage products from V1FCgD indicates approximately equal amounts of cleavage occurring at the FMDV 2A and 3C sites. FMDV 2A cleavage, in the virus V2FgD, at the junction of the foreign protein and poliovirus 2A does appear to be more efficient than previously observed with vectors containing a 3C recognition site at this location (4, 9), as the small gD fragment was not detected previously. The ability of FMDV $2A^{pro}$ to mediate cleavage with high efficiency in foreign contexts has been reported. For example, expression of a CAT-2A-GUS fusion protein resulted in approximately 80% cleavage to CAT-2A and GUS, with 20% remaining uncleaved (15). High cleavage efficiency was also reported for the fusion protein CAT-2A-influenza virus NA; however, it was not quantified (14).

The presence of the band corresponding to gD (9 kDa) observed in V2FgD was not expected and may result from a novel cleavage event. The protease involved and the exact cleavage site have not been identified; however, this cleavage event could result from either virus-encoded proteases (poliovirus 3C, poliovirus 2A, or FMDV 2A) or a cellular enzyme. During replication of FMDV, the $2A^{pro}$ polypeptide cleaves its carboxyl terminus, giving rise to the capsid precursor P1-2A that is further processed by FMDV $3C^{pro}$ at an LL/N junction to generate free capsid precursor P1 (16). In the case of the gD-FMDV $2A^{pro}$ fusion polypeptide, the junction between gD and FMDV $2A^{pro}$ is an isoleucine-asparagine pair. While the

enzyme involved in cleavage has not been determined, poliovirus 3C or 2A could possibly be responsible. In the poliovirus polyprotein, poliovirus $3C^{pro}$ cleaves specifically at Q/G junctions. However, 3C has been reported to be involved in cleavage of cellular proteins, where the exact cleavage site is unknown (3, 6). It is interesting to speculate that poliovirus $3C^{pro}$ may be able to cleave at a different amino acid pair; however, further work is necessary to test this hypothesis.

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