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MINIREVIEW

Picornavirus Nonstructural Proteins: Emerging Roles in Virus Replication and Inhibition of Host Cell Functions

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INTRODUCTION: THE THEME

Picornaviruses are small, plus-strand RNA viruses with a single-stranded genomic RNA of ~7,500 to 8,000 nucleotides, which is replicated in the cytoplasm of infected cells (33, 45). They divide into four genera: the aphthoviruses (foot-and-mouth disease virus [FMDV]), the cardioviruses (e.g., encephalomyocarditis virus [EMCV]), the hepatitis A isolates, and the renteroviruses. The latter are usually regarded as two groups, the rhinoviruses (e.g., human rhinoviruses) and the enteroviruses (e.g., poliovirus). The replication and genome organization of viruses in these genera appear to be basically similar (Fig. 1), but the genome organization of the renteroviruses differs in some respects from that of the aphthoviruses and cardioviruses (Fig. 1; also see figures in references 45 and 51).

To complete a round of infection, a number of complex activities must be performed and coordinated. These include replication of the plus-strand RNA via minus-strand intermediates, translation, proteolytic processing, inhibition of host cell transcription/translation, virion assembly, and cell lysis. These activities must be directed by the 26 or more viral polypeptides detected in virus-infected cells, although most of these polypeptides are intermediates in the proteolytic processing of the polyprotein precursor with no known function (45). Depending on the genus, about 11 to 13 fully processed viral polypeptides are produced in infected cells (Fig. 1). Of these, about seven to nine proteins are nonstructural (i.e., noncapsid) proteins. The theme of this review is to discuss the emerging multiple roles of these nonstructural proteins both in virus replication and in the associated inhibition of cellular functions. Evidence will also be presented that certain host cell proteins are important for virus replication by acting in cooperation with particular viral nonstructural proteins.

L (Lab AND Lb) AND 2A PROTEINS

In the FMDV strains and the cardioviruses, the leader or L region maps in front of the capsid precursor region, 1A to 1D (L is absent in the renteroviruses) (Fig. 1). The L protein of FMDV occurs in two distinct forms, termed Lab (23 kDa) and Lb (16 kDa), which are the result of ribosomes initiating translation at two AUG codons 84 bases apart (7). Both Lab and Lb of FMDV are (thiol?) proteases with multiple activities; they cleave themselves (in *cis* or in *trans*) from the amino terminus of the capsid protein precursor between a K-G dipeptide, thereby initiating the maturation of the adjacent capsid proteins (Fig. 1) (39, 45). The two forms of L also initiate the cleavage of the p220 subunit of the ribosomal initiation factor eIF-4F protein complex, which appears to be partly responsible for the inhibition of cellular 5′ cap-dependent translation without affecting translation of the uncapped viral mRNA (39).

In the renteroviruses, the 2A region codes for a protease ($2A^{pro}$) which is autocatalytically released from the nascent polyprotein by rapid cotranslational cleavage at its own amino terminus at (Y, T, H, F, A, or V)-G dipeptide pairs (Fig. 1) (26, 45, 62). Cleavage is a prerequisite for further proteolytic processing of the capsid precursor region. A wealth of evidence shows that a second function of $2A^{pro}$ is to initiate the cleavage of the p220 subunit of eIF-4F (8, 18, 26, 62), but $2A^{pro}$ itself is not directly responsible (37). Thus, $2A^{pro}$ of renteroviruses appears to be functionally analogous to the Lab and Lb proteins of FMDV.

$2A^{pro}$ may represent a novel class of protease; inhibitor studies indicated that poliovirus $2A^{pro}$ contains an active-site thiol group, yet the proteolytic activity of poliovirus and rhinovirus 2A proteases is inhibited by elastase-specific inhibitors (40). In addition, site-directed mutagenesis and modelling suggest that $2A^{pro}$ is structurally related to the small subclass of trypsin-like serine proteases (6, 26, 62). Mutations in the proposed poliovirus $2A^{pro}$ catalytic triad of His-20, Asp-38, or Cys-109 which significantly reduced proteolytic activity also abolished p220 cleavage, indicating that the putative cellular factor which cleaves p220 is probably proteolytically activated by $2A^{pro}$ (26, 62). The p220-specific cleavage activity has been found to copurify with a polypeptide of ≈55 to 60 kDa associated with initiation factor eIF-3 (59). eIF-3 itself has no activity, but must be present for the 55- to 60-kDa protein to activate cleavage of p220 (59). Proteolysis of p220 may be necessary but not sufficient for complete inhibition of cellular translation after poliovirus infection (12, 18, 53). Expression of just the poliovirus $2A^{pro}$ coding region in mammalian cells led to a marked reduction in RNA polymerase II transcription and, to a lesser extent, in DNA replication and translation (18). Although it was unclear whether these effects of $2A^{pro}$ were direct or indirect, the results imply the existence of further activities of $2A^{pro}$ and indicate how mechanisms other than p220 cleavage might contribute to the complete shutoff of host protein synthesis in virus-infected cells (18). Curiously, the $2A^{pro}$ region of FMDV is only 16 amino acids in length, yet primary scission still occurs at the carboxy terminus at the 2A-2B junction (51). No host or viral protease has been implicated in this scission, and the balance of evidence favors the astonishing possibility that the sequence at the 2A-2B junction (consensus NPGP) is unstable and literally breaks itself without being enzymatically cleaved (45, 51). In the cardioviruses (EMCV), primary cleavage similarly occurs at the 2A-2B junction, and microsequencing revealed that scission occurs at the Gly-Pro pair within the NPGP sequence (47).

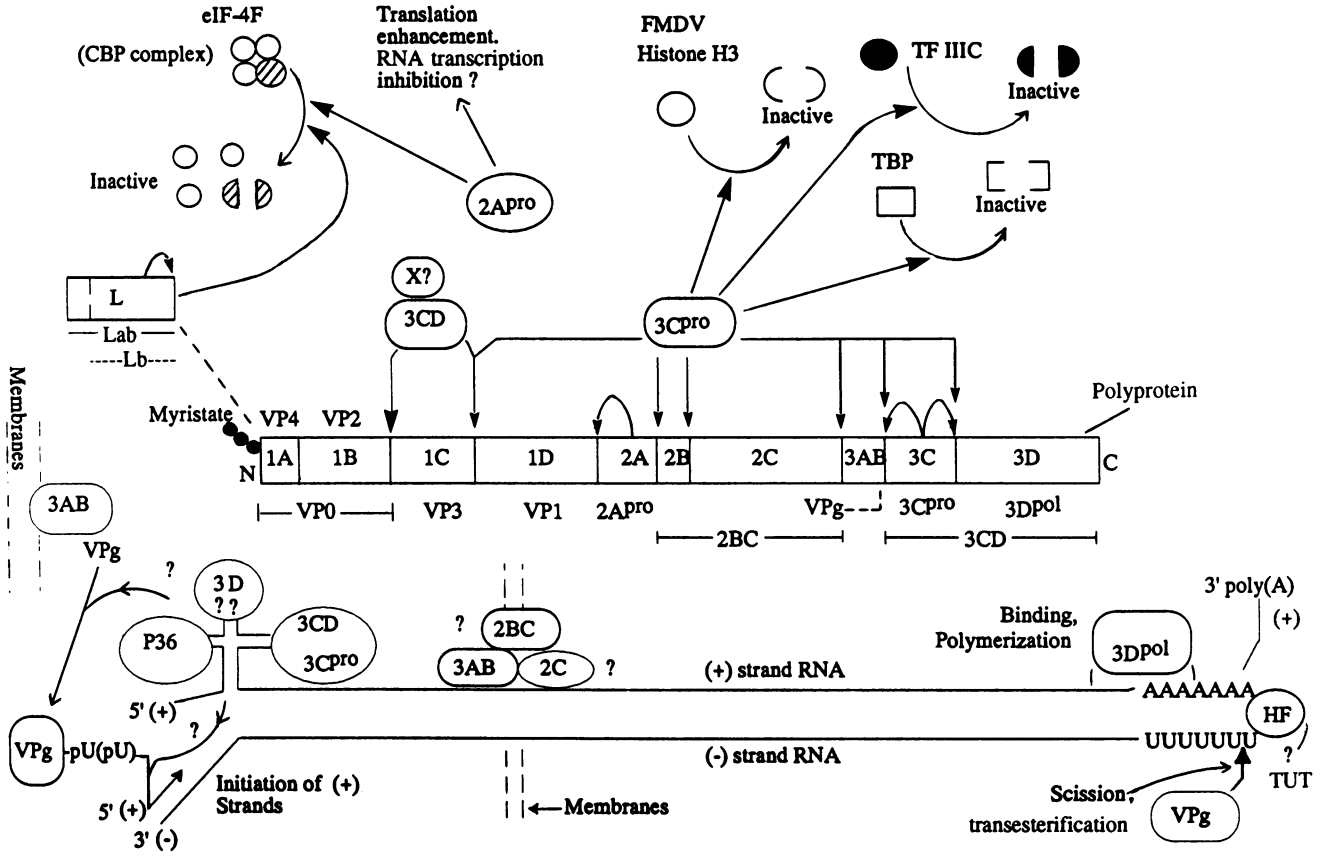

FIG. 1. Diagrammatic summary (composite) of activities of picornavirus nonstructural proteins and particular host proteins involved in virus replication, proteolysis, and inhibition of host cell functions (not to scale; see text for details). The organization of the polyprotein (from regions 1A to 3D) is based on that of the enteroviruses (e.g., poliovirus) and the rhinoviruses. Major differences between these viruses and other picornaviruses include the following. Only FMDVs and cardioviruses have an L protein leader (shown) and their 2A regions do not cleave at the 1D-2A junction; only FMDVs have three distinct VPg coding regions (see Fig. 3 of reference 45). For clarity, not all intermediates and possible cleavages in polyprotein processing are shown (45). TF IIIC, transcription factor IIIC; CBP, cap-binding protein; X?, putative 3CD-stimulating protein; P36, ribosome-associated host protein; HF, host factor; VP0 to VP4, capsid proteins; eIF-4F, eukaryotic initiation factor 4F.

Yet another role of poliovirus $2A^{pro}$ may be as a specific enhancer of poliovirus mRNA translation early in infection by an unknown mechanism involving the 5′ noncoding region (5′-NCR) (24). A likely consequence of this specificity is the efficient translation of poliovirus mRNAs early in the infectious cycle, when host mRNAs can still compete with viral mRNAs for the cellular protein synthesis machinery.

2B, 2C, AND THEIR PRECURSORS

The functions of proteins coded for by the 2B and 2C regions are poorly understood. Mutagenesis of either 2B or 2C of poliovirus led to a strong defect in viral RNA synthesis (29, 36). 2C and the precursor 2BC are both contained exclusively within a replication complex on virus-induced vesicles formed on membranes originating from the rough endoplasmic reticulum (10), and the presence of both the vesicles and 2C is a prerequisite for viral RNA synthesis (54). The precursor 2BC may be involved in the membrane proliferation leading to vesicle formation (9), and there is evidence that a proportion of 2C (and/or the precursor 2BC) binds to the viral RNA and attaches it to the vesicular membranes (10). Because 2C and the hydrophobic precursor 3AB associate very strongly, it is possible that 2C may be bound to membranes via 3AB (33). This would anchor the viral RNA in order to provide the correct spatial organization necessary for its replication. The precise role of 2C in viral RNA synthesis is unclear, but mutagenesis experiments and computer alignments that identified 2C as a potential nucleoside triphosphate binding protein led to the idea that 2C may be a helicase involved in viral RNA strand separation during replication (19, 56).

3A, 3AB, AND 3B (VPg)

3B is usually referred to as VPg, a small peptide of 22 amino acids (in poliovirus) covalently linked via tyrosine to the 5′ termini of all full-length and nascent viral plus- and minus-strand RNAs (21). Poliovirus VPg is removed by a cellular unlinking enzyme (leaving 5′ pU) from those viral RNAs destined to become mRNAs. An attractive hypothesis is that 5′-linked VPg serves as an encapsidation signal, leaving the mRNAs free for translation without obstruction from the replication machinery (33).

Different approaches have shown that VPg plays a role in the initiation of viral RNA synthesis (4, 21, 33, 49). In addition, some mutations in poliovirus VPg gave replication-competent RNA but no virions, suggesting that a late function (encapsidation?) is impaired in these mutants (49). There are two models of viral RNA chain initiation on the basis of available evidence. In the first model (Fig. 1), VPg is uridylylated to give

VPg-pU or VPg-pU-pU and the nucleotidyl protein acts as a primer of RNA synthesis (33, 54). A molecular genetic approach demonstrated that a defect in uridylylation of poliovirus VPg was associated with the viral RNA polymerase $3D^{pol}$ (Fig. 1), suggesting that $3D^{pol}$ may be involved in the synthesis of VPg-pU(-pU) (58). However, attempts to demonstrate that $3D^{pol}$ has in vitro uridylylation activity have not been successful. Uridylylation is strictly dependent on the presence of intact membranes (54), so how can a hydrophilic peptide like VPg exist in a hydrophobic environment? The abundant hydrophobic precursor of VPg, 3AB, is the most likely candidate both as an anchor and a donor for VPg in the membrane-associated poliovirus RNA-synthesizing complex (22) (Fig. 1). The observation that mutations in poliovirus 3A affected uridylylation of VPg and selectively inhibited the initiation of plus-strand viral RNAs supports this idea, and implies that the initiation of minus strands occurs by a different mechanism (22).

The synthesis of poliovirus minus strands in vitro requires the viral RNA polymerase ($3D^{pol}$) and depends on a host factor from uninfected cells. Three different host factors have been described: a terminal uridylyltransferase (TUT), a protein kinase, and an endonuclease (3, 27, 42). The roles of the kinase and endonuclease are obscure, but the observations that TUT stimulated the initiation of poliovirus RNA synthesis in vitro (3) and that newly synthesized minus-strand RNA was covalently linked at one end to the plus-strand RNA template (60) led to the template-priming model. Thus, the second mechanism of RNA chain initiation (Fig. 1) predicts that minus-strand RNA is formed by (i) uridylylation of the 3′ poly(A) of plus strands by TUT, (ii) hybridization of the resultant oligo(U) to the adjacent poly(A) tail, and (iii) initiation of minus-strand RNA chains at the 3′ terminus of the oligo(U) by a template-priming mechanism (21). Thus, VPg is not required for minus-strand initiation in vitro, but how does it become linked to the 5′ termini of minus strands in vivo? Adding synthetic poliovirus VPg to the in vitro host factor-dependent synthesis of minus strands resulted in its covalent linkage via tyrosine specifically to the oligo(U) at the 5′ termini of minus strands (21). Remarkably, the covalent attachment of VPg appears to occur by a self-catalyzed transesterification reaction in which only the replication intermediate synthesized on poliovirion RNA and VPg is required (21, 57). It seems possible that initiation of minus strands and VPg attachment are closely linked events, since anti-VPg antibodies inhibited de novo synthesis of viral RNA (4).

3C, 3CD, AND 3D

The 3C regions of all picornaviruses code for a protease ($3C^{pro}$) with a critical function as the enzyme responsible for the majority of maturation cleavages in the precursor polyprotein (13, 25, 28, 32) (Fig. 1). Proteolysis by $3C^{pro}$ occurs in a complex and incompletely understood cascade of *cis* and *trans* cleavages at mainly Q-G, Q-S, Q-A, and Q-N pairs (except in FMDV strains for which cleavage sites are more diverse, e.g., E-G, V-G, C-N, L-N, and Q-G) (45).

In FMDV, $3C^{pro}$ also induces the specific cleavage of histone H3 in vivo (20, 55). Because the deleted part of histone H3 is in the amino-terminal region corresponding to the presumed regulatory domain of transcriptionally active chromatin, it seems likely that this specific cleavage is related to the severe inhibition of host cell RNA transcription observed in FMDV-infected cells. It is unclear whether histone H3 cleavage is direct or mediated by a host protease (20). Although $3C^{pro}$-mediated histone modification has not been observed in poliovirus-infected cells, severe inhibition of host cell RNA transcription by all three classes of RNA polymerase (pol I, pol II, and pol III) is observed (15, 16, 50). There is persuasive evidence that poliovirus $3C^{pro}$ specifically and directly cleaves the TATA-binding protein (TBP) subunit of transcription factor IID both in vivo and in vitro, resulting in the inactivation of pol II transcription (16). TBP is also involved in transcription mediated by RNA pol III, and thus the inactivation of TBP by poliovirus $3C^{pro}$ could also contribute to the inhibition of pol III-mediated transcription (16). In addition, poliovirus $3C^{pro}$ completes the proteolysis of an active form of transcription factor IIIC to an inactive form by about 5 h postinfection, suggesting that this is an important mechanism for the shutoff of host transcription by pol III (15). Poliovirus $3C^{pro}$ is further implicated in the destruction of a transcription factor complex essential for pol I transcription (50). Thus, $3C^{pro}$ may be centrally involved in inhibiting transcription by the three major classes of RNA polymerase (15, 16, 50).

$3C^{pro}$ is sensitive to thiol-blocking reagents, and evidence from mutagenesis (25, 32, 34, 35) and modelling (6, 23) suggests that $3C^{pro}$ is structurally and functionally related to the large subclass of trypsin-like serine proteases, with a catalytic triad of His-40, Asp/Glu-71 (or Asp-85?), and Cys-147 (the active site nucleophile). Relatedness to serine proteases was further supported by experiments in which human rhinovirus 14 $3C^{pro}$ was inactivated as a result of amino acid substitutions in areas structurally homologous to the substrate pocket of trypsin (6, 13). Certain substitution mutations in the putative catalytic triad of poliovirus $3C^{pro}$ had differential effects on cleavage at different $3C^{pro}$-sensitive sites, which complicated the task of identifying the members of the catalytic triad (25, 31, 32). Nevertheless, a consensus has emerged that the catalytic triad of poliovirus and human rhinovirus 3C proteases is probably composed of His-40, Glu-71, and Cys-147 (equivalent to Cys-146 in human rhinoviruses) (25, 32, 35).

$3C^{pro}$ of EMCV together with its precursors 3ABCD, 3ABC, and 3CD is capable of processing the EMCV polyprotein at all sites except 1A-1B and 2A-2B (Fig. 1) (45, 47). In contrast, $3C^{pro}$ of the renteroviruses is unable to perform the VP0-VP3 cleavage, which is carried out by 3CD (30, 61). Unlike $3C^{pro}$ cleavages, the 3CD cleavage at the VP0-VP3 site in poliovirus requires myristoylation of the amino terminus of the polyprotein (38), and an unknown cellular cofactor facilitates efficient 3CD cleavage at the poliovirus VP0-VP3 site and to a lesser extent at the VP3-VP1 site (11). It should be kept in mind that 3CD may also play a role (with or without $3C^{pro}$) in the modification of transcription factor complexes associated with the inhibition of cellular pol I, pol II, and pol III transcription by poliovirus (15, 16, 50).

The 3D regions of all picornaviruses (33) code for an RNA-dependent RNA polymerase ($3D^{pol}$) which has regions of homology with all known DNA and RNA polymerases (52). In fact, mutagenesis experiments revealed a conserved structural framework in EMCV $3D^{pol}$ and *Escherichia coli* DNA polymerase I and suggested which amino acids in $3D^{pol}$ may be important in catalysis and template binding (52). Native or recombinant $3D^{pol}$ of picornaviruses is capable of copying plus strands in vitro provided that an oligo(U) primer is present (21, 43, 52), but one or another of the host factors (e.g., TUT mentioned above) can circumvent the need for such a primer (3, 21, 27, 33, 42). The oligo(U)- or host factor-dependent poliovirus RNA syntheses will accept any polyadenylated RNA as template (33), indicating that one or more host (or viral) components which confer specificity on poliovirus $3D^{pol}$ for initiating viral RNA replication in vivo are missing in the in vitro reaction. The only other activity known to be associated with native $3D^{pol}$ is an RNA helicase activity (protein 2C?)

from EMCV-infected cells, which was lost when the enzyme was more extensively purified (19). The significance of this observation is still not clear, because poliovirus $3D^{pol}$ itself has intrinsic RNA duplex unwinding activity, a property not shared by all polymerases (14).

Native poliovirus $3D^{pol}$ did not show significant template specificity for poliovirus RNA in vitro (44). However, recombinant EMCV $3D^{pol}$ bound specifically to the 3′-NCR and 3′ poly(A) tail of EMCV RNA in vitro, but surprisingly failed to bind to the 3′-NCR lacking poly(A) (17). This suggests that the 3′-NCR and poly(A) tail together play an important role in viral RNA template selection by $3D^{pol}$ (17), and may explain why deadenylated poliovirus RNA, or cDNA clones of EMCV RNA lacking 3′ poly(A), are noninfectious (46).

Initiation of plus strands in a defined in vitro system by poliovirus $3D^{pol}$ either does not occur or is extremely inefficient (33). One reason is likely to be that poliovirus plus-strand initiation depends on the formation of a ribonucleoprotein (RNP) complex on the plus strand, comprising 3CD, $3C^{pro}$, $3D^{pol}$ (?), and a ribosome-associated cellular protein (p36) bound to about 90 nucleotides of the 5′ end folded into a cloverleaf-like structure (1, 2). This structure is dispensable for the initiation of poliovirus minus strands, suggesting that the complex is important only for plus-strand initiation. It is unclear whether poliovirus 3CD, $3C^{pro}$, and $3D^{pol}$ all participate in complex formation, although it has been demonstrated that purified, recombinant human rhinovirus 14 $3C^{pro}$ binds in vitro specifically to the 5′ terminal 126 nucleotides of the 5′-NCR of the viral RNA in the absence of 3CD and $3D^{pol}$ (35). Overall, the results support an intriguing speculative model of membrane-bound plus-strand initiation in *trans* involving the following steps (Fig. 1). (i) $3D^{pol}$ (and an associated protein[s]?), having just completed the synthesis of a minus strand, provides the signal for assembly of the RNP complex on the 5′ end of the neighboring plus strand. (ii) The complex catalyzes the formation of VPg-pU(-pU) via 3AB. (iii) The complex then catalyzes the initiation of a new plus strand in *trans* by VPg-pU(-pU) at the 3′ end of the neighboring minus strand newly exposed by the formation of the complex (1, 2). The source of $3D^{pol}$ for plus-strand synthesis could be either $3D^{pol}$ or 3CD already present in the RNP complex. A remarkably similar mechanism for the initiation of viral plus strands has been proposed for the replication of a plant virus, brome mosaic virus (48).

CONCLUSIONS AND PERSPECTIVES

Evidence has accumulated that certain picornavirus nonstructural proteins such as Lab and Lb, $2A^{pro}$, $3C^{pro}$, and $3D^{pol}$ have two or more distinct functions. For example, $3C^{pro}$ is a protease and an RNA-binding protein, whereas $3D^{pol}$ is an RNA polymerase and is involved in proteolysis in the form of 3CD. Moreover, $2A^{pro}$ and $3C^{pro}$ not only participate in virus replication but also appear to inhibit essential host functions (RNA transcription and protein synthesis). In contrast, the identities of the host cell proteins which cooperate with the viral nonstructural proteins in virus replication (e.g., ribosome-associated P36, host factor, 3CD-stimulating protein, and 2A-activated protease) are unknown and their precise roles are poorly understood. Picornavirus replication is complex and is dependent on the presence of cellular membranes. Despite this, an important goal must be to recreate virus replication in defined in vitro systems in order to better understand the roles of the viral nonstructural proteins, including their interactions with host components. The net synthesis of poliovirions from genomic RNA in crude cell extracts is an important and exciting step toward this goal (5, 41).

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