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Implications of the picornavirus capsid structure for polyprotein processing

(protein–nucleic acid catalysis/virus structure/polyprotein proteolysis/virion morphogenesis/maturation)

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ABSTRACT **Mature picornaviral proteins are derived by progressive, posttranslational cleavage of a precursor polyprotein. These cleavages play a role in the control of virus functions. Although the processed termini are separated by as much as 75 Å in the native virus capsid, the fold and arrangement of polypeptide chains in a protomer before proteolysis are likely to be similar to that found in the mature virus. The three-dimensional structures of rhinovirus and Mengo virus suggest that the cleavage sites within the protomeric precursor are in structurally flexible regions. The final proteolytic processing event, maturation of the virion peptide VP0 (also called peptide 1AB) appears to occur by an unusual autocatalytic serine protease-type mechanism possibly involving viral RNA basic groups that would serve as proton-abstractors during the cleavage reaction.**

Controlled limited proteolysis plays a major role in the regulation of many biological processes. Examples are activation of zymogens to enzymes and prohormones to hormones, assembly of cytoskeletal components, control of cellular differentiation, initiation of cascade mechanisms such as blood clotting, removal of signal peptides in the transport of proteins across membranes, and the recycling of cellular proteins. In a viral life cycle, proteolysis can be involved in the differential control of host versus viral biosynthesis, in posttranslational modification of a precursor polyprotein, and in directing assembly events. Most of these processes utilize one of four different types of enzymic activity: serine proteases, cysteine proteases, acid proteases, or metalloproteases. Both convergent and divergent evolution have been involved in producing the observed mechanisms. Here we present evidence suggesting a viral proteolytic mechanism in which both protein and nucleic acid components participate.

Picornaviruses (1) are of major economic and medical importance. The family contains a diverse variety of highly virulent human and animal pathogens, which traditionally are subdivided into four genera on the basis of physical properties of the virions. The subgroups include rhinoviruses (human and bovine), cardioviruses [e.g., Mengo, encephalomyocarditis (EMC) and Theiler viruses], enteroviruses (e.g., polio, hepatitis A, and coxsackie viruses), and aphthoviruses (foot-and-mouth disease viruses). In spite of the disparate afflictions caused by these agents, recent determinations of three-dimensional structures (2–4) and nucleotide sequences have shown that all picornaviruses share remarkable similarity in their particle structure and genome organization. Indeed, the similarity also extends to plant RNA viruses (2) and insect RNA viruses (J. E. Johnson, personal communication).

Picornavirus virions contain a single, positive-stranded RNA genome (of between 7000 and 8500 bases) enclosed in a protein capsid shell with an external diameter of around 300 Å. The capsids are composed of 60 copies of four nonidentical virion polypeptide chains (VP1, VP2, VP3, and VP4). The RNA codes for only one long open reading frame. Translation proceeds primarily from a single strong initiation site and produces a giant precursor polyprotein ($M_r \simeq 250{,}000$) that is divided into three regions: P1, P2, and P3. The polyprotein is processed in a series of proteolytic cleavage steps to yield mature virion capsid proteins as well as other noncapsid viral proteins (Fig. 1) (5). At least three types of proteolytic activities are involved in the process (12, 13).

The very first cleavage within a polyprotein takes place while the polypeptide is still nascent on a ribosome. For poliovirus this cleavage, between the P1 and P2 regions [i.e., between peptides 1D (VP1) and 2A] is produced autocatalytically by protein 2A (1, 12, 13). In cardioviruses and aphthoviruses, the primary (nascent) cleavage occurs between peptides 2A and 2B, and the responsible proteolytic agent has not yet been identified.

Most of the remaining cleavages within the picornaviral polyprotein are carried out by the viral protease 3C, which is a cysteine-type enzyme (13–21) with specificity for its own polyprotein substrate (22). The processing sites for the enzyme 3C in various picornaviruses are shown in Fig. 1. Most protease 3C-directed cleavages, deduced initially by comparison with poliovirus cleavages, occur between glutamic acid or glutamine and glycine (or serine) dipeptide pairs. The surrounding sequences (not shown) have a high frequency of helix-breaking residues, such as proline and threonine, but a specific peptide pattern is not apparent.

The final cleavage within picornaviral polyproteins is probably in VP0 (peptide 1AB) to give VP4 (1A) and VP2 (1B) and is not catalyzed by proteases 2A or 3C (2, 13). This reaction is observed *in vivo* during the final stages of virion morphogenesis and is probably concomitant with RNA association with the 12S–14S pentameric assembly units (23).

Proteolysis of the Protomer into VP0, VP3, and VP1 and Assembly into Pentamers

The three larger capsid proteins, VP1, VP2 (Fig. 2), and VP3, each fold into similar eight-stranded antiparallel β-sheet-barrels (2–4). The organization of the β-barrels within the capsid is shown in Fig. 3. A similar organization of eight-stranded antiparallel β-barrels exists in some plant (24, 25) and insect viruses, as well as in cowpea mosaic virus (J. E.

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Abbreviations: HRV, human rhinovirus; EMC, encephalomyocarditis; EMCV, EMC virus; HAV, hepatitis A virus; VP0, VP1, VP2, VP3, and VP4, virion peptides 0, 1, 2, 3, and 4.

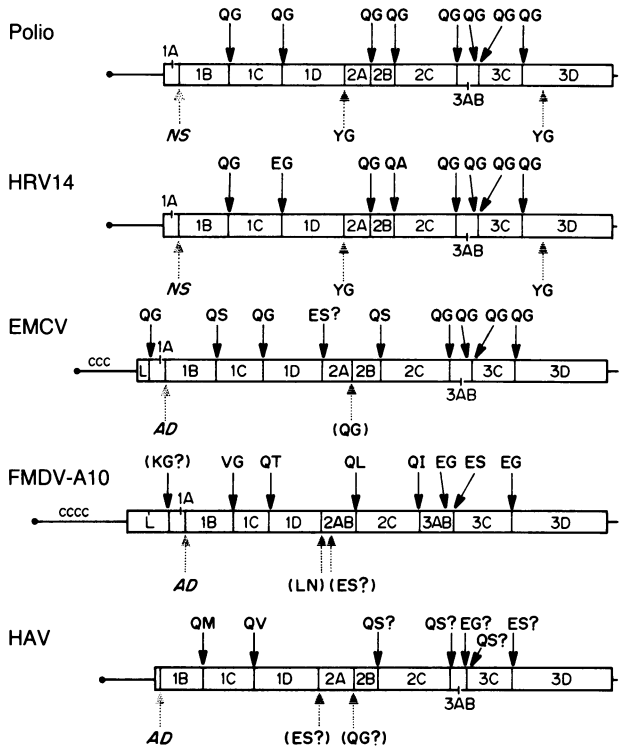

FIG. 1. Structure of the picornaviral genome for some picornaviruses. Peptide nomenclature is according to standard convention (5). The P1 region is composed of four peptides [1A (VP4), 1B (VP2), 1C (VP3), and 1D (VP1)], the P2 region is composed of 3 peptides (2A, 2B, and 2C), and the P3 region is composed of four peptides (3A, 3B, 3C, and 3D). Proteolytic cleavages of the polyproteins occur between amino acid pairs indicated in standard single-letter code (6–11). Cleavages produced by protease 3C are shown above each genome. Protease 3A (hatched arrowhead) and VP0 (1AB) (stippled arrowhead) cleavage sites are shown below the genome. Sites for which the proteolytic agent has not been specifically identified are enclosed in parentheses. Question marks denote sites where the sequence has not been established precisely. FMDV, foot-and-mouth disease virus; Polio, poliovirus.

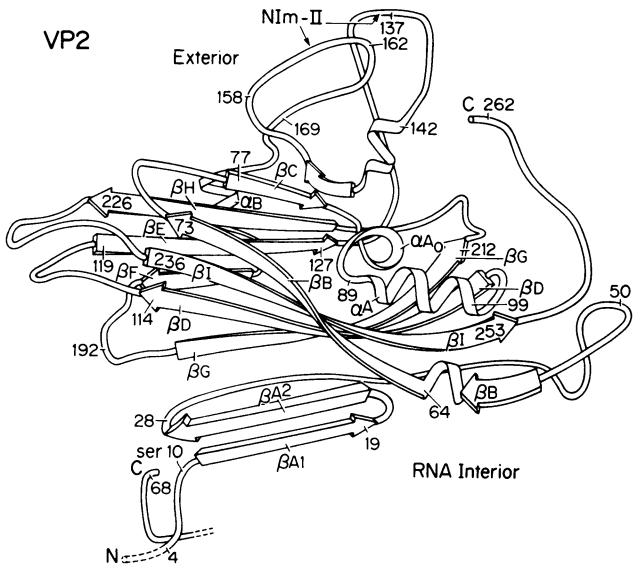

FIG. 2. Ribbon drawing of VP2 in human rhinovirus 14 (HRV14) showing proximity of Ser-10 to the carboxyl terminus of VP4. This serine is conserved in all known sequences of rhino-, entero-, and cardioviruses except for that of hepatitis A virus (HAV). The aphthoviruses and HAV have instead a serine corresponding to position 28 in VP2 of HRV14, which also is close to the carboxyl terminus of VP4. The secondary structural nomenclature is also shown. Similar nomenclature is used for each of the three larger structural proteins VP2, VP3, and VP1.

Johnson, private communication), where two β-barrels are connected covalently. The amino-terminal arms of VP0, VP3, and VP1 in picornaviruses are intimately intertwined within one protomeric unit and, therefore, are likely to correspond to a 6S assembly unit (Fig. 3) (26–29).

The pervasiveness and stability of the barrel arrangement strongly suggest that the three major barrel domains probably fold and organize themselves within a protomer prior to any posttranslational processing. Protease 3C-directed cleavage of region P1 into VP0, VP3, and VP1 occurs at a relatively slow rate, with a minimum half-life of 1–2 min per step (30). The cleavage sites are positioned in regions between the β-barrel domains. The probable manner in which the carboxyl end of VP0 would be associated with the amino end of VP3 and the carboxyl end of VP3 would be associated with the amino end of VP1 before proteolysis can readily be visualized (Fig. 4). After cleavage, the new ends could easily reposition themselves into the orientations observed in crystallized virions without disrupting the contacts between β-barrels.

It is not known how the 3C enzyme recognizes or selects appropriate cleavage sites within the polyprotein. Sequences specific for viral proteases occur not only at the actual cleavage points between VP0, VP3, and VP1 but also elsewhere in the capsid (and polyprotein) structure (Table 1). Contrary to expectation, many of these capsid sites are quite external to the protomeric fold. However, unlike authentic sites, the pseudo-sites within region P1 invariably occur in rigid portions of the β-barrel domains. Flexibility and surface probability predictions for other regions of the polyprotein support the idea that viral protease 3C requires structurally flexible contexts surrounding specific substrate sequences for catalysis. Presumably, the flexibility is necessary to permit the protein substrate to accommodate itself into the enzyme's active center. This is consistent with the observations that thermolysin undergoes limited proteolysis only at

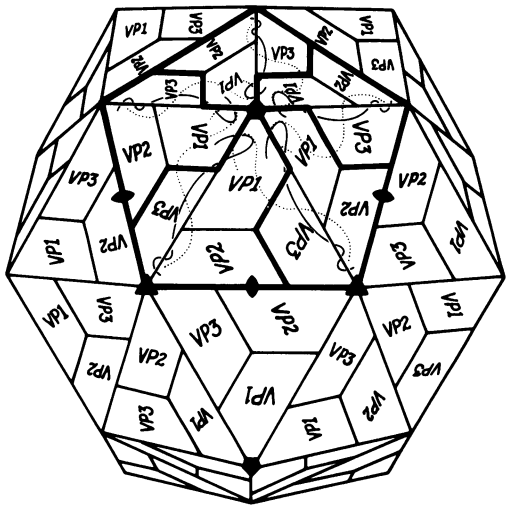

FIG. 3. Diagrammatic representation of the organization of the three larger, external capsid proteins, VP1, VP2, and VP3, of picornaviruses in an icosahedron. Thicker lines designate the association of VP0, VP3, and VP1 into a protomeric 6S assembly unit and the association of five pentamers into a 12S–14S assembly unit. Shown also is the course of five of the internal VP4 peptides for HRV14 (continuous line) and for Mengo virus (dotted line). While VP4 follows a quite different course in Mengo virus as opposed to rhino- and polioviruses, the carboxyl terminus of VP4 is near the amino end of VP2. These proteins apparently are cleaved from one another in an autocatalytic process during virion maturation.

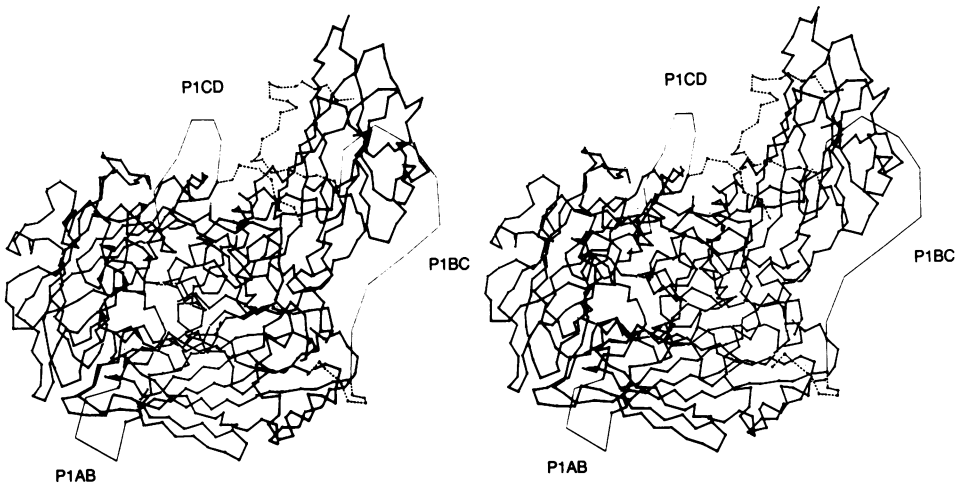

FIG. 4. Stereographic view of the C_α backbone of one HRV14 protomer. Hypothetical linkages of VP1, VP2, VP3, and VP4 are indicated by thinner lines labeled P1AB, P1BC, and P1CD as they might occur in the precursor polyprotein prior to posttranslational cleavage. Dashed portions correspond to terminal segments of the native structure that might have rearranged subsequent to processing.

external mobile segments (31) and also that many protein cores remain undigested unless the protein is fully denatured before digestion.

The processing of picornaviral protomers into VP0, VP3, and VP1 is a necessary prerequisite to pentamer formation (14). The amino termini of VP3, released by cleavage, associate with each other in a parallel five-stranded β-cylinder about the icosahedral 5-fold axes. These interactions may promote and stabilize the formation of 12S–14S pentamers (26–29). The amino termini of the small protein VP4 (the amino end of VP0) also form a concentric cylinder about the VP3 β-cylinder. Therefore, it is apparent that the amino termini of VP0, VP3, and VP1 guide the assembly of the particle.

The proteolytic cascade plays a major role in many phases of the picornaviral life cycle, including virion morphogenesis. For example, the precursor protomeric structure P1 must be cleaved (14) into its components VP0, VP3, and VP1 in a 5S–6S protomer before assembly can proceed via 12S–14S pentamers and possibly the procapsid. It is unknown how the structural precursors and RNA finally form the virion. However, during this last step, VP0 peptides are cleaved into VP2 and VP4, thus completing the final stages of both processing and assembly (1, 27, 32). The VP0 digestion takes place deep within a maturing particle, inaccessible to exogenous proteases, including proteases 3C and 2A.

Structural Analysis of VP0 Cleavage

The structures of HRV14 (2), poliovirus (Mahoney type 1) (3), and Mengo virus (4) all show the close proximity of Ser-10 (HRV14) or Ser-11 (Mengo virus) to one of the cleaved ends of VP0 (the amino end of VP2 or carboxyl end of VP4). The course of VP4 is substantially different in Mengo virus as opposed to HRV14 and poliovirus (Fig. 3), yet the cleaved ends between VP4 and VP2 are adjacent in both types of structures. Ser-10, in HRV14, forms a hydrogen bond with the carboxyl terminus of VP4. The detailed structure in Mengo virus is not as clear.

The structural proximity of the carboxyl end of VP4 and amino end of VP2 as well as the conservation of Ser-10 suggest that the serine acts as a nucleophile for an autocatalytic cleavage of VP0 into VP4 and VP2 (2). By analogy with other proteases (33–36), it would also be necessary to have a proton-abstracting base to activate the nucleophile for catalysis (Fig. 5). However, none of the available virus

Table 1. Dipeptides in protomer that are not cleaved by the appropriate 3C enzyme

Virus	3C specificity	Occurrence* (HRV14 equivalent position)	Secondary structure†
HRV14	Gln-Ala	1149 in HRV14	In βE
		1182 in HRV1A, HRV2, HRV49	In βG
Polio, HRV14, EMCV, Mengo	Gln-Gly	1207 in HRV49	In FMDV loop
		2119 in polio 1, 2, 3; coxsackie B3; HRV2, HRV14	In βE
FMDV	Gln-Ile	1108 in FMDV O	Loops I and II in Mengo at end of βC
		3192 in FMDV A	In βH
FMDV	Gln-Leu	1231 in FMDV A	Second corner βH to βI
		1283 in FMDV A, O	Carboxyl-terminal arm
		3035 in FMDV O	On entering β-barrel
		4035 in FMDV A, O (Mengo virus equivalent)	Ordered part of VP4 in Mengo
HAV	Gln-Met	3102 in HAV	In αA
EMCV, Mengo, HAV	Gln-Ser	2026 in EMCV, Mengo, HAV	In βA_2
FMDV	Gln-Thr	1108 in FMDV A	Between βC and αA; between loops I and II
HRV14, FMDV, HAV	Glu-Gly	1141 in FMDV O	Near NImIB; third corner down in Mengo
		2136 in HRV14	NImII in puff

Polio, poliovirus; EMCV, encephalomyocarditis virus; Mengo, Mengo virus; FMDV, foot-and-mouth disease virus. Note: It is assumed that protease 3C specificity observed in poliovirus is relevant in all HRV.

*The first digit of sequence numbers represents the viral protein (VP1, -2, -3, or -4).

†The secondary structural nomenclature is shown in Fig. 2. The positioning of FMDV and HAV is inferred from sequence alignments.

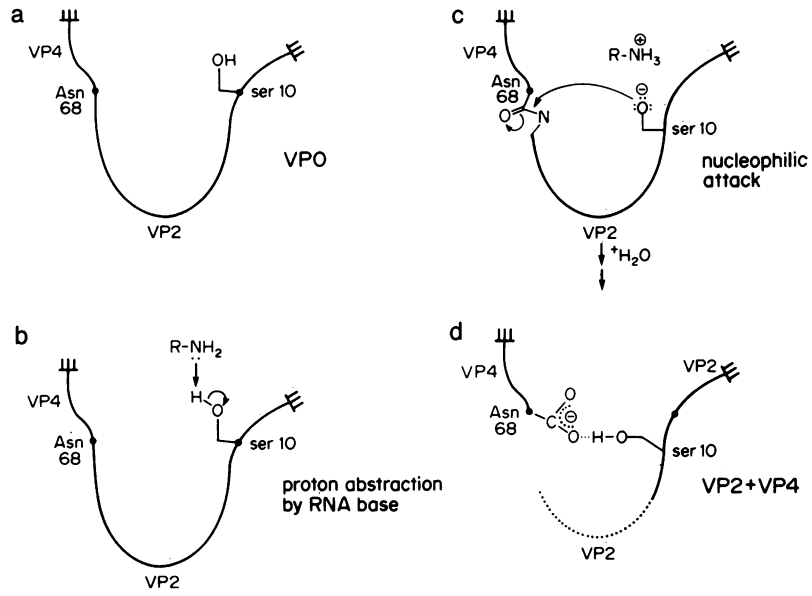

FIG. 5. Proposed autocatalytic maturation cleavage of VP0 capsid protein. (*a*) Partial structure of VP0 before cleavage. (*b*) Abstraction of a proton from HRV14 Ser-10 (Ser-11 in Mengo virus). (*c*) Nucleophilic attack on the neighboring peptide bond. (*d*) Partial structure of cleaved VP2 and VP4 as observed in HRV14. The electron density in Mengo virus is not so clear in this neighborhood. The Mengo virus map shows density near the catalytic serine, which corresponds either to the four carboxyl-terminal residues of VP4 or the four amino-terminal residues of VP2.

structures have any suitable base in the vicinity of the cleavage site. As VP0 processing *in vivo* takes place deep within a maturing particle, inaccessible to external agents, it was proposed (2) that a RNA base (the amino groups in adenine, guanidine, and cytosine) or polyamines found in picornaviruses (37) might serve this purpose. The encapsidation of RNA during the assembly process would initiate VP0 cleavage. This hypothesis also provides a possible explanation for the observation that mature virions invariably contain a few uncleaved VP0 chains (1). As the virion maturation progresses, the RNA structure could be expected to become less flexible, and the likelihood of the proton abstraction from the serine becomes correspondingly less. When the majority of cleavages have already occurred and the virion assembly is nearly complete, the nucleotide bases may not be able to move sufficiently to reach the remaining serines.

Conservation of the catalytic serine among rhino-, entero- (except HAV), and cardioviruses supports the essential role of this serine. Although this serine is not conserved in aphthoviruses and HAV, nevertheless these viruses have a serine corresponding to position 28 of VP2 in HRV14 that is spatially close to Ser-10 (Fig. 2). Arg-12 is conserved in VP2 for all sequenced picornaviruses. Its function could be to bind to the phosphates of an RNA base, thereby giving some stability and orientation to the base during catalysis. Similarly the conservation of Glu-5 and Asp-11 in VP2 suggests that they also may play a role in the catalytic mechanism—perhaps to activate the substrate peptide or even to act as general bases in the carboxylate form.

The proposed mechanism is unusual in that the catalysis is produced by a combination of protein and nucleic acid functionalities. Thus, the active site of an enzyme has been described that contains both protein and nucleic acid (38). Whether VP0 and its associated RNA can be described as an enzyme is questionable, as the autocatalytic cleavage also alters the final state of this "enzyme." This cleavage is irreversible and thereby dictates the direction of the viral assembly.

Whereas the conservation of the mechanism supports divergence of picornaviruses from a common ancestor, it also represents convergent evolution to a catalytic mechanism that is similar in outline to other serine proteolytic mechanisms. Not only is the structural framework for this autodigestion completely different from other serine proteases, but even the identity of the activating base would have changed from a histidine to a propitiously placed nucleotide base. Interestingly, the serine proteases have previously provided one of the clearest cases for convergent molecular evolution (33–36).

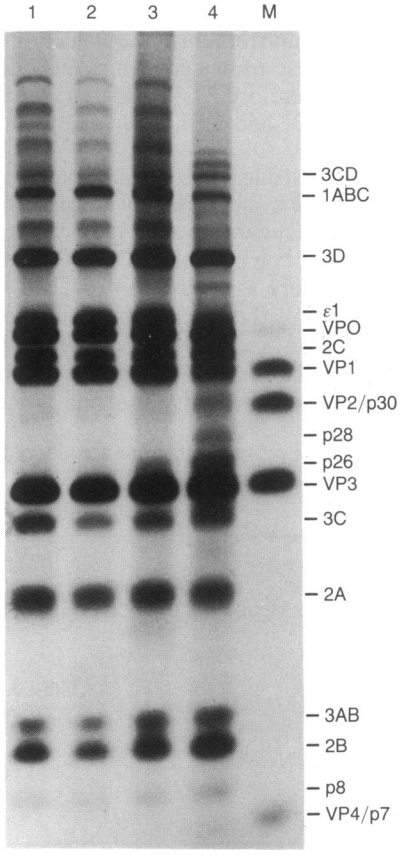

FIG. 6. Hydrazine-induced cleavage of VP0 *in vitro*. EMCV RNA was translated in [^{35}S]methionine-labeled reticulocyte extracts (7, 19). The samples were incubated for 5 hr at 30°C. Incubation was continued after addition of 0.3 mg of cycloheximide and 130 μg of pancreatic RNase per ml. Aliquots were taken, which were increased in volume by 7% by adding water (lane 1), methylhydrazine (lane 3), or hydrazine (lane 4), while the aliquot in lane 2 was brought to a concentration of 35 mM in NaOH. The mixtures were overlaid with paraffin oil and incubated at 30°C for an additional 15 hr. Samples were precipitated with acetone and fractionated by polyacrylamide gel electrophoresis, and the radiolabeled bands were visualized by autoradiography. Lane M contained marker peptides from radiolabeled EMC virions.

Cleavage of VP0 in a Cell Free System

Addition of certain small diamino compounds to a rabbit reticulocyte cell-free translation system programmed with EMC virus (EMCV) RNA (15, 16, 19) results in the apparent cleavage of VP0 into VP4 and VP2. A typical experiment is shown in Fig. 6 in which lanes 1–4 depict EMCV proteins after treatment with water, NaOH (35 mM), methylhydrazine (7%), or hydrazine (7%), respectively. Relative to the control (water), the NaOH and methylhydrazine samples are unchanged. However, the hydrazine-treated sample shows obvious alterations. Bands representing capsid precursor proteins (e.g., L-P1-2A, P1-2A, P1, 1ABC, VP0, and ε1) were diminished in intensity after hydrazine treatment, while bands from the P2 and P3 regions of the polyprotein (2A, 2B, 2C, 3AB, 3C, 3D, and 3CD) were generally unaffected. [Protein ε1 is a charge-altered form of VP0 particular to *in vitro* translation samples (15).] Among the new protein bands produced by the treatment, two peptides (p30 and p7) had electrophoretic mobilities comparable to VP2 and VP4. Preliminary characterization (gel migration, tryptic profiles, and carboxypeptidase analysis) of the p30 and p7 peptides were consistent with their identification as VP2 and VP4, respectively.

Hydrazine and ethylenediamine were the only tested compounds to induce this *in vitro* effect. Monoamine reagents such as methylamine, ethylamine, propylamine, hydroxylamine, and semicarbazide produced unaltered peptide patterns, like that shown for methylhydrazine. Presumably the dibasic compounds hydrazine and ethylenediamine played the role of proton abstracting components of the virion in VPO cleavage.

The Function of VP0 Cleavage: Proteolytic Control of Infectivity

Picornaviruses undergo VP0 cleavage during virion maturation. Apparently intact VP0 is necessary for correct assembly of the protomers, whereas processing of VP0 is necessary for the final assembly of the virion. Particles which are eluted from cell membrane after "tight binding" or have been heat treated are missing VP4 and sometimes also VP2 (32). It is possible that the cleavage of VP0 is a prerequisite for uncoating in that it renders VP4 mobile.

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