Picornaviruses Are No Longer Black Boxes

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Most biologists are forced to think about the problems that intrigue them without benefit of a high-resolution picture of the object of their investigations. They are forced into stratagems that can produce revelation even when the central figure is a black box. Those investigators who care about the study of poliovirus and its relatives have been removed from this purgatory by the efforts of two groups who have solved the high-resolution structure of such viruses by means of x-ray crystallography. Their efforts have produced beautiful pictures that will forevermore be the basis of thinking how the viruses are assembled, how they are held together, and how they are taken apart to infect cells.

The viruses whose structures have been "solved" at 2.9 to 3.0 Å are poliovirus, type I, Mahoney strain and human rhinovirus (common cold virus) type 14. Hogle, Chow, and Filman report the poliovirus structure in full color in *Science*, page 1358; Rossmann and 12 coauthors have a report on the rhinovirus structure with equally revealing precision in *Nature* (*1*). Both viruses are picornaviruses—a huge group of animal viruses with RNA genomes and protein coats—which include human hepatitis A, many enteroviruses, and foot-and-mouth disease virus.

The big news from this work is that the structures are old hat: these animal viruses have structures very reminiscent of the RNA plant viral structures solved a number of years ago. To appreciate the significance of this statement requires a bit of background.

In the early 1960's, Donald Caspar and Aaron Klug set the basis for thinking about viral structure by elaborating the principles of icosahedral symmetry as applied to proteinaceous particles (*2*). They pointed out that to build strictly symmetric particles, each particle could have no more than 60 subunits. They suggested that 180 or even more subunits could be arrayed to produce such a particle by slight deformations of a single basic structure so that the polypeptide subunits would be as quasi-equivalent in structure although not strictly identical.

The first viruses whose structures were solved were plant viruses with 180 identical protein subunits: southern bean mosaic and tomato bushy stunt viruses (*3*). The principles of quasi-equivalence were satisfied although reinterpreted: the single polypeptide was found in three different but related configurations. In each configuration the main body of each polypeptide consisted of an eight-stranded beta barrel (a closed barrel formed by beta-sheet interactions).

Picornaviruses posed an apparently different problem (*4, 5*). Their icosahedral virions consist of 60 copies of a subunit made of four polypeptides that have no apparent sequence homology to one another. Thus, it could easily be imagined that the subunit structure of picornaviruses would be unrelated to that of the icosahedral plant viruses. Herein lies the surprise: for both poliovirus and rhinovirus, the individual polypeptides of the virion were found to be strikingly similar in configuration to those of tomato bushy stunt virus and southern bean mosaic virus. The main body of each of the three major picornavirus subunits is a beta-barrel almost undistinguishable from those of the plant viral polypeptides. The polypeptides also have individual features that allow them to play independent roles in the virion structure. Whether these virion polypeptides evolved from each other or all evolved from a common cellular protein is not clear, but the structures are too similar to believe that they arose by convergent evolution.

For someone like me who has worked on poliovirus for 25 years, the first glimpse of the virus' structure brings to mind a host of puzzles that have been posed over the years but only now might be answered. An incomplete catalog, including new questions raised by the structural evidence, would be:

What is the value of the proteolytic cleavages that produce the four virion proteins? Picornaviruses make all of their proteins by cleavage from a single "polyprotein" precursor. The four virion proteins are made in three stages: first a segment containing them is cleaved from the nascent polyprotein, then that polypeptide is cleaved into three pieces (VP0, VP1, and VP3) that presumably hold together as a single unit, and finally, as the very last step of morphogenesis, about 58 of the 60 units in the virion undergo a cleavage of VP0 to form VP2 plus VP4. Why need the virion proteins be separated from one another when they form a compact unit anyway? For the initial cleavages the answer seems reasonably clear. The ends that are joined in the polyprotein are not contiguous in the final structure so that the cleavages to generate VP0, VP1, and VP3 allow the three proteins to take on independent, untethered positions in the virion.

The cleavage to generate VP4 is a special case. VP4, along with the NH_2-terminus of VP3, forms a highly ordered "β-annulus" interior to the VP1 units that are aggregated at fivefold axes of the virion. The VP4 interactions with VP3 and VP1 can presumably take place without cleaving VP4 from VP0 because the cleaved ends do not move far apart. Why then the cleavage? Although no answer presents itself from the structure, the likely answer is that the cleavage allows some new contacts to form so that the particle becomes locked together. Before cleavage (in the "provirion"), the particle is easily disrupted by detergent; after cleavage, at least for poliovirus, the particle is one of the most difficult macromolecular aggregates to disrupt—not even sodium dodecyl sulfate can disaggregate it. Both the Hogle group and the Rossmann group suggest that the final cleavage must be carried out by a virion-associated enzyme activity because of the internal localization of VP4. Rossmann *et al.* even suggest that RNA may play a catalytic role, explaining why only full and not empty particles undergo the cleavage.

Why are poliovirus and rhinovirus virions so different in stability and permeability? The defining characteristics of a rhinovirus include acid liability, but poliovirus is very acid-stable. Wherein lies the difference? At the present level of resolution, no answer is suggested. Also, rhinovirions are permeable to Cs^+ ions while poliovirions are impermeable. The poliovirion must be more tightly organized than the rhinovirion, but again the difference is not evident. One clue might

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come from Rossmann *et al.*, who suggest that VP2 in rhinovirus is loosely associated with the others, producing holes in the structure.

How is the RNA held in the particle? Plant viral proteins have many basic residues that interact with the viral RNA. These occur in NH_2-terminal segments that do not take on symmetric positions in the particle and are therefore invisible in the x-ray structure. In the picornavirus structures, VP1 and VP2 have short unresolved NH_2-terminal segments but they are not basic (if anything, they are hydrophobic). Thus, there is no evidence suggesting close interaction of protein and RNA in the virion. The virion protein might help RNA compaction, but not by neutralizing charge. The reason for this fundamental difference in plant and animal virion structures may relate to the uncoating process. How plant virus penetrate cells is not clear, but the mechanism could involve extensive proteolysis for the separation of the protein from the RNA. For picornavirus, penetration involves a capsid transition induced by the receptor, a process that may require independence of the RNA and protein. Somehow, viral RNA's penetrate the lipid bilayers surrounding cells. For animal and plant virus, the mechanisms are likely to be very different.

How is the virus assembled? We have long known that there are a number of protein substructures related to picornavirus virion assembly: these include a 14*S* pentameric structure, a 74*S* shell structure lacking RNA (procapsid), and an immature form of the virion called provirion (*5*). The structural evidence makes the fundamental importance of the pentamer quite clear: in the virion, the interactions around the fivefold axes are very intimate, implying that pentameric units should be particularly stable substructures.

The role of the RNA-less procapsid in virion morphogenesis has been debated for years. The issue is that, kinetically, the shell behaves as an intermediate but it has been hard to imagine that the RNA could be threaded or melted into a preformed protein shell. It could be, however, that free pentamers easily form and dissociate from a shell, but to make a virion they must organize around an RNA molecule. The procapsid would then be a reservoir of subunits rather than an intermediate. The virion structure does not settle the issue but the apparent independence of RNA and protein structure fits better to a model in which the virion polymerizes around a compact RNA core. If this is true, procapsid would be a poorly named reservoir. Its ease of formation would then only be testament to the strength of protein-protein interactions.

If the virion forms around an RNA ball, how is the RNA recognized? There could be a specific RNA-protein recognition step but there is another possibility. Each virion RNA molecule has an oligopeptide of about 20 amino acids, VPg, bound at its 5′ end. It has been a puzzle whether VPg is a vestigial remnant of the RNA synthesis machinery or an important determinant of morphogenesis or both. Perhaps virion proteins can bind to VPg, initiating virion formation. There is only one copy of VPg per virion, and therefore it is not surprising that the x-ray structure—depending as it does on symmetry—does not include it.

There is another nonrepeated aspect of the virion structure not resolved by the x-ray analysis. Approximately two protein subunits do not undergo the final proteolytic cleavage step in virion morphogenesis which generates VP2 and VP4. (Plant viruses and icosahedral phage particles also contain about one copy of a unique polypeptide so that, perhaps, no virions are strictly icosahedrally symmetric.) It may be that one or a few VP0 molecules are bound to VPg and therefore are not cleaved.

What imparts receptor specificity to the virion? Picornavirus can be very specific as to the cells they infect and most of that specificity involves restriction in the kinds of receptors recognized by a given virion. Poliovirus is the classic case: it binds to receptor found only on certain primate cells. The similarity of polio- and rhinovirions argues that the receptor specificity is encoded in the details of surface architecture, not in gross feature. Both Hogle and Rossmann makes some guesses where the specificity might lie but I would think that more data are required before definitive answers will appear.

How are the particles disassembled? The interaction of picornaviruses with surface receptors, in a temperature-dependent reaction, causes the virion to alter its configuration drastically, releasing all copies of VP4 and making the viral RNA accessible to ribonuclease. The receptor must somehow find a key site in the virion to effect this transition. The cellular receptor certainly does not exist solely to help the cell become infected by the virus—it must have a crucial role in cell physiology. What is the role and is it somehow analogous to the viral uncoating reaction?

How does antibody neutralize viral infectivity? Many viral mutants that escape the neutralizing effect of antiviral antibody have been isolated. They map into three or four clusters of sites that occur on loops of protein that extend from the beta barrels. Thus the neutralization escape mutants are probably changes at the sites of antibody binding and do not act by "hiding" sites. But if three or four sites can bind neutralizing antibody, then neutralization is not a consequence of an interaction of antibody with some critical virion structure. It is rather probably a consequence of either physical interference with receptor interaction or, more likely, a locking together of virion structure by cross-links induced through the multivalency of the antibody. A puzzle posed by the structural evidence is that peptides representing internal residues of the virion can induce neutralizing antibody. This unlikely observation requires clarification.

The structure of picornaviruses has come to us at a very propitious time. Over the last few years, numerous picornavirus genomes have been cloned and sequenced. Cloned DNA has turned out to be infectious (*6*), allowing the production of site-specific mutants of various types. My laboratory has constructed both deletion and insertion mutants in various parts of the genome that exhibit temperature-sensitive, cold-sensitive, host-range, and plaque size alterations (*7*). Thus there is the opportunity to correlate mutational analysis with detailed structural knowledge.

This is a great time for animal virology. The last few years have seen many genomes cloned and sequenced, many virion structures solved, many new analytic techniques developed. Key questions about animal viruses remain to be answered. These include how do they replicate, how do they enter cells, and how is morphogenesis accomplished. A marriage of the new capabilities and the old questions should produce clear answers.

References and Notes

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