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Current Opinion in Virology

The amazing HK97 fold: versatile results of modest differences

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dsDNA Bacteriophages, some dsDNA archaeal viruses and the Herpesviruses share many features including a common capsid assembly pathway and coat protein fold. The coat proteins of these viruses, which have the HK97 fold, co-assemble with a free or attached scaffolding protein and other capsid proteins into a precursor capsid, known as a procapsid or prohead. The procapsid is a metastable state that increases in stability as a result of morphological changes that occur during the dsDNA packaging reaction. We review evidence from several systems indicating that proper contacts acquired in the assembly of the procapsid are critical to forming the correct morphology in the mature capsid.

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Current Opinion in Virology 2019, **36**:9–16

This review comes from a themed issue on **Virus structure and expression**

Edited by **Juliana Reis Cortines** and **Peter Prevelige Jr**

For a complete overview see the Issue and the Editorial

Available online 8th March 2019

https://doi.org/10.1016/j.coviro.2019.02.001

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Introduction

Viruses are thought to be ancient, and likely existed before the division of life into three domains [1]. Although they are made from extremely divergent proteins, often there are detectable lifestyle and structural similarities that suggest distant evolutionary relationships [2••]. Viruses use a range of virion designs to protect and deliver their genetic instructions to their host. Some virions have a membrane outer coat, some have a protein coat, while others use a combination of the two. Among those with a protein-only coat, some co-assemble the coat and nucleic acid to build a protein capsid, while the tailed dsDNA bacteriophages, some archaeal viruses, and the Herpesviruses build precursor procapsids and then later fill them with DNA using an ATP-driven motor. Curiously, the Herpesviruses, phages, and some archaeal viruses appear to be evolutionarily related: they all form icosahedral protein shells or capsids using a shared common assembly pathway and all use a variant of a single protein fold, the HK97 fold (Figure 1a) [3•,4•,5]. That this fold is found in viruses from all domains of life gives credence to the presence of viruses in evolution for billions of years. Proteins with the HK97 fold are capable of forming particles as small as the *Thermotoga maritima* encapsulins (T = 1, 24 nm diameter) [6], to the biggest thus far discovered, jumbophage G (T = 52, 150–180 nm diameter) (Figure 1b) [7•]. Thus, these particles are composed of 60 to more than 3000 monomers. How can this fold be so amazingly versatile?

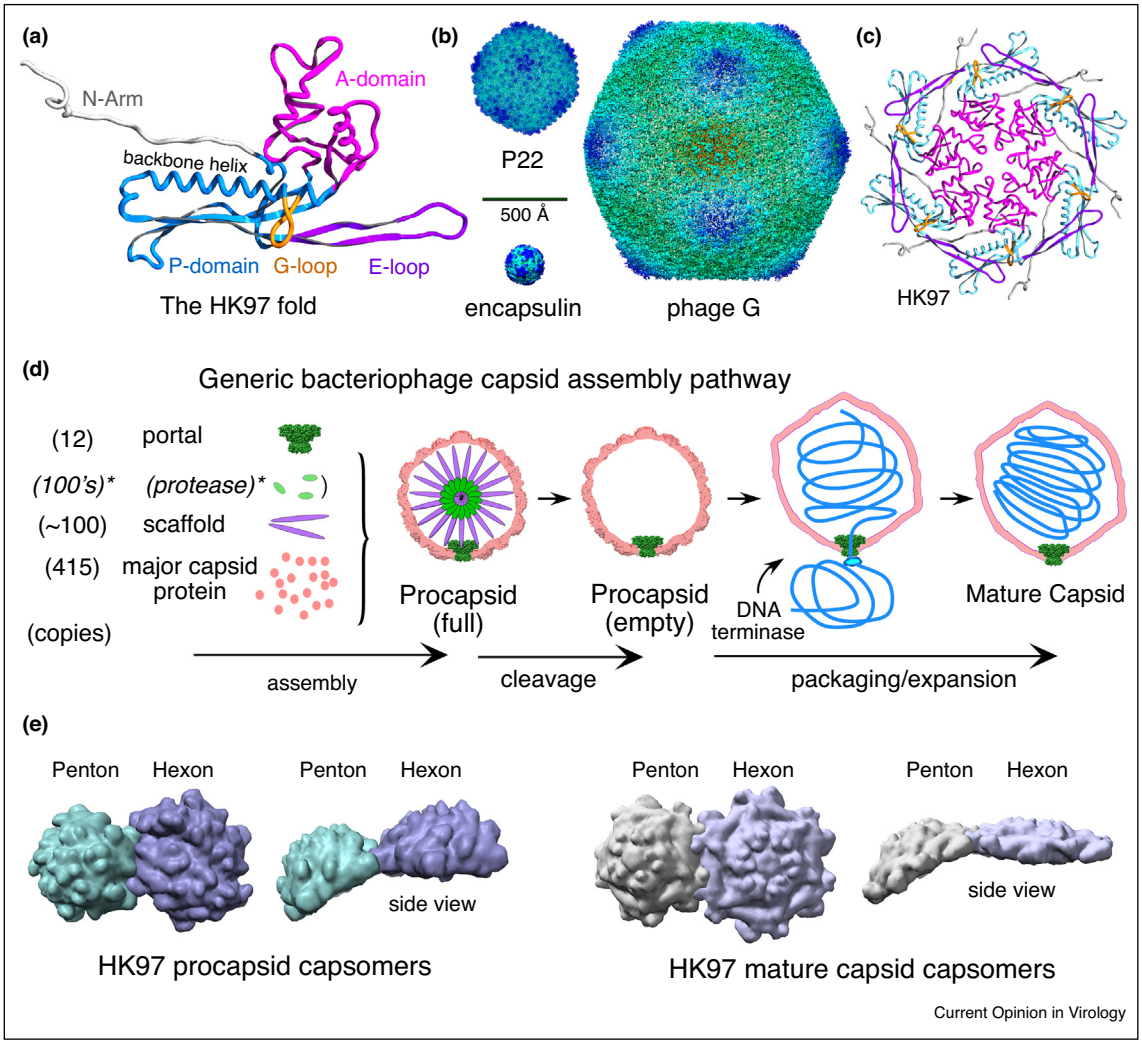
Icosahedral virus basics

Caspar and Klug [8] showed how coat proteins in pentameric and hexameric arrangements (pentons and hexons, or capsomers, Figure 1c shows the arrangement of subunits in a phage HK97 hexamer) can build icosahedral capsids of different sizes. The twenty faces that define an icosahedron are composed of ~flat protein hexamers, while the 12 vertices (corners) are pyramids made of five copies of the same or similar protein. The smallest capsid is composed of 60 subunits in 12 pentamers. Larger capsids are formed by moving the pentons apart and connecting them with a group of hexons that form the icosahedral faces. These concepts are easy to understand when equilateral triangles of paper or plastic are used, but the real viruses we wish to discuss are composed of proteins subunits made using one of the many variants of the unusually shaped HK97 fold (Figure 2).

Features of the HK97 fold (Figure 1a)

The conserved elements are 1) **A-domain** (axial domain), 2) the **P-domain** (peripheral domain), **E-loop** (extended Loop), and **N-arm** (N-terminal arm), but there are additional 'bells and whistles' which appear in some, but not all capsid proteins (Figure 2). These include the **I-domain** (insertion domain, in P22 this is grafted onto the A-domain and contains the D-loop, Figure 2d and e), and the **G-loop** (gycine-rich loop) in HK97 and many others. The triangular **A-domain** makes up the central wedge-shaped sectors of hexons and pentons and has a β-sheet-rich core surrounded by prominent A-helices along the edges that interact with adjacent A-domains. The **P-domain** forms the periphery of hexons and pentons and is composed of a long A-helix (backbone or spine helix) nestled against a multi-stranded β-sheet with prominent loops at one end. The flexible **E-loop** is a

Figure 1



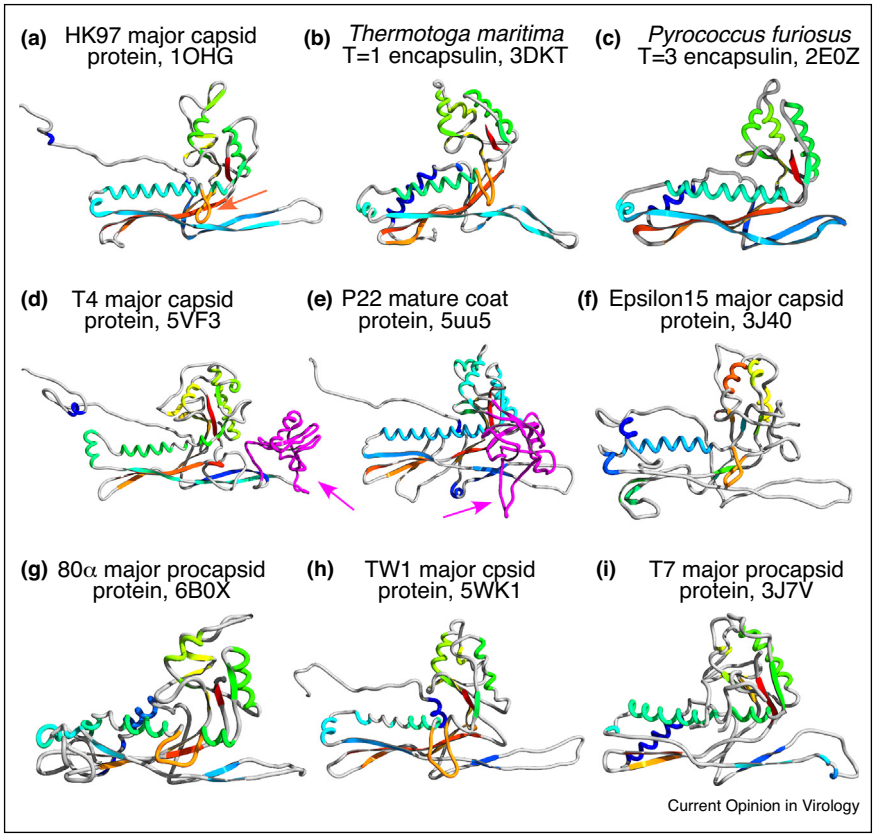
Components and features of capsids.
(a) The HK97 fold with important common features labeled and color-coded as indicated in the figure. The ribbon diagram is from the mature HK97 capsid chain A (PDB 1OHG). **(b)** The size range of the HK97 fold. Shown are images of surface rendered cryo-EM density maps from the T = 1 encapsulin of *Thermotoga maritima* [6], the P22 mature capsid [19], and *Bacillus megaterium* jumbophage G [7•]. **(c)** A hexon from the HK97 mature capsid colored as in (a). **(d)** A generic pathway for capsid assembly that applies, in general features, to nearly all double-stranded DNA tailed phages and Herpesviruses. *Note that while a capsid maturation protease is a common feature, there are many bacteriophages that do not utilize one. **(e)** Examples of the shape changes of capsomers that occur when procapsids convert to mature procapsids. Surface rendered images are shown of the hexons and pentons of phage HK97 from X-ray structures (PDB ID 3E8K (procapsid) and 1OGH (mature)). Structural models in figures were visualized using Chimera [47] or SPDBV [48].

long 2-strand β-sheet hairpin that protrudes from the P-domain and makes contacts with other subunits. In an isolated subunit, the **N-arm** appears to protrude from the subunit body, but it associates intimately with other domains and changes its associations during maturation.

Procapsids are essential

In addition to the ability of HK97-fold capsid proteins to adapt to generate many particle sizes, the first product of the assembly reaction is not the final virion but a metastable precursor capsid, known as a procapsid, prehead, or prohead depending on the system. A generic capsid assembly pathway is shown in Figure 1d. Procapsids convert to mature capsids via large scale conformational changes during DNA packaging (Figure 1d) [9••]. Assembly of procapsids is driven by a scaffolding protein or an equivalent scaffold domain ('delta domain') that is covalently attached to the major capsid protein. Scaffolding proteins are discarded after assembly is complete and leave through ports in the capsid, either intact or after proteolysis [10,11]. Procapsids are round, lumpy, and thick-shelled (Figure 3), while usually mature capsids

Figure 2



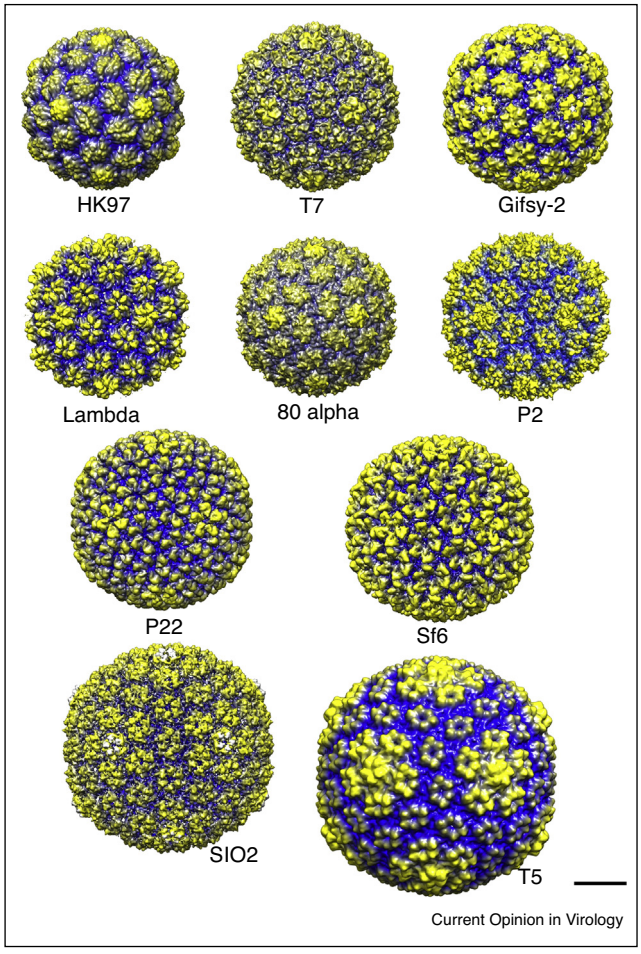
HK97 fold gallery.
Nine examples of the HK97 fold determined by X-ray crystallography or cryo-electron microscopy with secondary structure colored from N-terminus to C-terminus in blue to red hues. Protruding G-loops are colored orange, I-domains (Insertion domains) are colored magenta. Panels, in order: **(a)** phage HK97 mature capsid protein [43]; **(b)** *Thermotoga maritima* encapsulin [6]; **(c)** *Pyrococcus furiosus* encapsulin (previously 'PFV' for *Pyrococcus fuiosus* virus-like particle [39]); **(d)** phage T4 mature major capsid protein gp23* [49]; **(e)** P22 coat protein [19]; **(f)** epsilon 15 mature capsid protein [50]; **(g)** 80alpha procapsid major capsid protein [42]; **(h)** phage TW1 major capsid protein [18]; **(i)** phage T7 major capsid protein from the procapsid [41].

are angular, thin-shelled and usually larger, which is why maturation is often referred to as 'expansion'. All capsomers in procapsids have dome-like shapes that give procapsids the lumpy appearance that is lost during maturation. The domed hexons in proheads are asymmetric (elongated, 'skewed,' or oval) and morph into a nearly flat regular hexagons in mature capsids (Figure 1e). Pentons also change conformation, but retain the pyramid shape needed at vertices. All procapsids examined to date have asymmetric hexons, suggesting that they are an intrinsic and essential feature (Figure 3). The asymmetric hexon shape is a result of the breaking of the normal contacts found in the mature capsids between adjacent A-domains to allow a shift or twist of the subunits along that boundary [12•]. We propose that assembly is controlled at the prohead stage, and it is at this stage that interactions determine capsid size and shape — interactions that may be missing, and, therefore, not discoverable, in the mature capsid [12•,13•]. Below we highlight some interactions that we deem important to formation of proper capsids, concentrating on the A-domain and E-loop.

A-domain

The A-domain fills the centers of the hexons and pentons, and undergoes important changes during capsid maturation [14,15]. The skewed and dome-shaped hexons become flat and hexagonal when the subunits tilt and slide along the A-domain:A-domain interfaces so that all of those interactions are about the same [12•,16]. There are other changes as well that involve the A-domain's pointed tip or A-loop [17] and an attached segment that is tucked under the tip and usually contains an alpha-helix, which is very short in HK97, and longer in phage TW1 [18], P22 [19] and others. In P22 this segment is like a flap because it unfurls from its location in the interior of the procapsid to attain a fully formed and stable secondary structure that fills the centers of the hexons [20,21,22•]. In P22, this A-domain refolding is much larger than seen for

Figure 3



Procapsid gallery.
Ten examples of procapsid structures from the Protein Data Base (PDB) or Electron microscopy database (EMDB) rendered at low resolution using Chimera [47] so that the shapes of the procapsid hexons can be discerned. Procapsids are round (not angular) and have lumpy capsomers with a domed and asymmetric shape, as noted in the text. The procapsids are radially cued with yellow being the furthest from and blue being closest to the center of each particle. The scale bar represents 20 nm. Shown are the procapsids of phages HK97 (PDB 3E8K, [17]); T7 (EMD-1321, [51]); Gifsy-2 (EMD-1691, [52]); Lambda (EMD-1507, [53]); 80alpha (EMD-7030, PDB 6B0X; [42]); P2 (EMD-5406, [54]); P22 (EMD-5149, [15]); Sf6 (EMD-5724, [55]); SIO2 (EMD-5383, [56]); and T5 (not deposited, [57]).

other phages because the procapsid is assembled with holes in the center of the hexons that are used for scaffolding protein exit during DNA packaging and maturation. Prevelige's group found that this part of P22's A-domain is highly sensitive to amino acid insertions and could tolerate the addition of three, but not four alanines [23]. The thermophilic phage P74-26, which infects *Thermus thermophilus*, has evolved the largest T = 7 capsid thus far identified to contain its 83.3 kb genome. Encapsidating this larger genome (a typical T = 7 phage has a ~48 kb genome) is accomplished by increasing the size of the A-domain, and lengthening the E-loop (see below for the importance of the E-loop), along with a number of other changes [24]. In phage P74-26, the A-loop is extended by 6 Å by the addition of a new helix and the A-domain is widened as well.

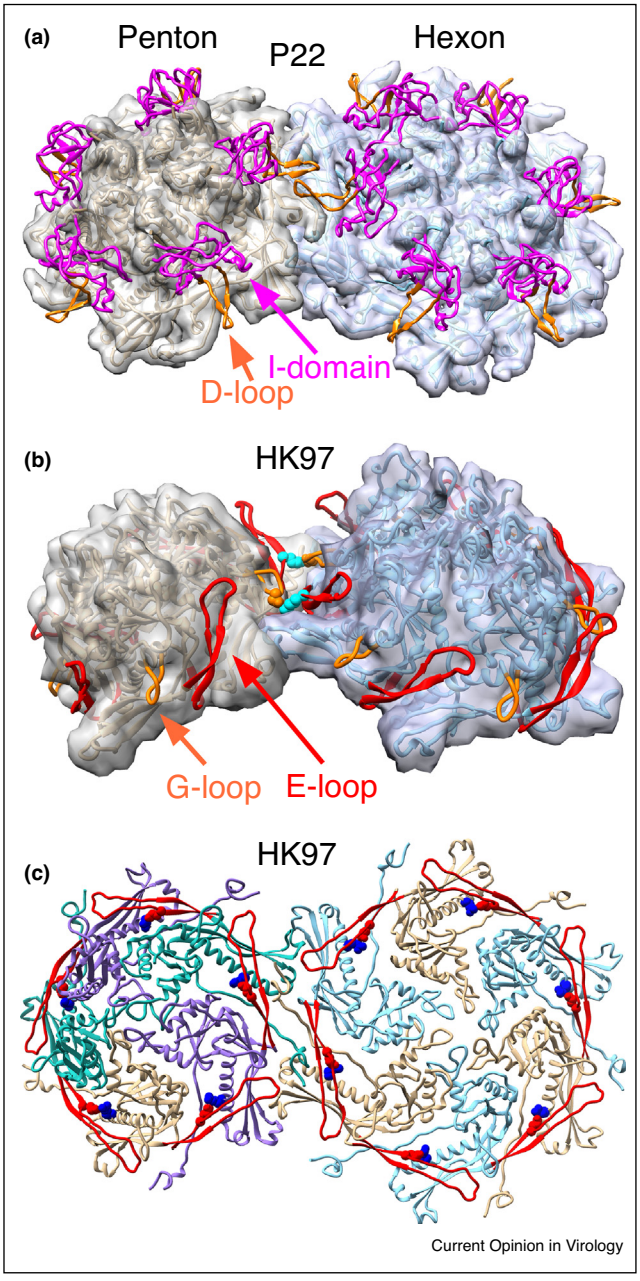
Mutants in the A-domain can lead to alternate capsid forms in many phages, indicating that the A-domain also is important for proper assembly. The A-domain has a conserved feature in its core that we call the β-hinge, which is a 5 stranded β-sheet that connects all of the other domains in the protein [25,26•]. In P22, the β-hinge is important for coat protein folding, in addition to proper capsid morphology. One of the 18 P22 coat protein temperature-sensitive-folding (*tsf*) mutants is in the β-hinge, as well as all three of the *tsf* global suppressor substitutions D163G, T166I and F170L that alleviate folding defects from *tsf* mutations all over coat protein, signifying the importance of the β-hinge in P22 coat protein folding [25,27]. The global suppressor substitutions alone have no phenotype, but double and triple combinations are cold-sensitive [28]. F170L coat protein, even though it is able to support phage growth, makes short tubes of coat protein *in vivo* and *in vitro* [28]. When this residue is changed to alanine or lysine, a *ts* phenotype results and long regular tubes of hexameric coat protein are assembled *in vitro*, highlighting the importance of the β-hinge in P22 assembly as well as folding [29,30]. The F170L tubes have the skewed hexons seen in procapsids. F170A tubes have the symmetric hexons of mature capsids, but likely assemble with skewed procapsid hexons and become symmetric during purification [30]. The F170 substitutions appear to decrease conformational flexibility in the A-domain, as probed by accessibility to protease digestion in procapsids. We hypothesize this leads to the assembly tubes of only hexons because penton formation is inhibited by steric crowding in the center of pentons, but not in hexons [29,30].

Other interesting phenotypes are associated with A-domain mutations in phages T4 and lambda. Phage T4 has two coat proteins that each have the HK97 fold: protein gp23 forms hexons, while gp24 makes the pentons needed to form the vertices [31]. There are mutants in gp23 that bypass the need for gp24 at the fivefold vertices, with the mutant gp23 taking the penton position [32]. Many of these mutants are found in gp23's A-domain [31]. In phage lambda, three of the five of the mutants that lead to assembly of thick tubes are found in the A-domain [33,34]. These observations indicate the A-domain is critical for proper assembly of coat proteins with the HK97 fold.

A-domain embellishments

In the P22-like phages, an extra domain is inserted between sheets β1 and β3 of the β-hinge called the

Figure 4



Capsid protein interactions that are important for assembly. Inter-capsomer and intra-capsomer interactions discussed in the text are illustrated in close-up views. **(a)** The site of inter-capsomer interactions between phage P22 coat protein D-loops. This shows the interaction in the mature capsid [19], but similar interactions occur in procapsids during assembly [22•,38•,58]. **(b)** Interactions between the E-loops (in red) and G-loops (in orange) of adjacent capsomers in HK97 procapsids (PDB ID 3e8k). These interactions (mediated by residues K178 (in cyan) on the E-loop and D231 (orange) on the G-loop) have been shown to be important for controlling the assembly of procapsids, but are not present in the mature capsid [13•]. **(c)** Interactions made by HK97 E-loops (shown as red ribbons) within capsomers. Residue E153 (in red) on the E-loop interacts with R210 (in blue) on the backbone helix of the adjacent subunit. These interactions are essential for assembly of the HK97 major capsid protein into procapsids [12•]. The figure shows the mature capsid (PDB ID 1OHG), but the interactions are present at all stages of HK97 assembly.

Insertion domain (I-domain) [15,22•] (Figure 2e). The 123 residue I-domain is a six-stranded β-barrel that forms P22 coat protein's folding nucleus and contributes half of its overall thermodynamic stability [35,36]. Loops connecting some of the I-domain β-strands have functionally significant roles. Mutations in the I-domain's S-loop (for size determination) at position 285 lead to the assembly of smaller than normal (petite) capsids with T = 4 rather than the normal T = 7 geometry. The S-loop is also involved in incorporation of the portal protein complex [22•,37•]. The petite capsids get smaller with increasing bulkiness of the residue 285 side chain, suggesting a mechanism involving contacts with another domain. Another loop of P22's I-domain, the D-loop, also appears to be involved in regulating assembly (Figure 4a). The D-loop makes crucial salt bridges across the twofold axes of symmetry in both in capsids and procapsids. Mutations of critical residues at the tip of the D-loop lead to aberrant partial procapsids or irregular tubes, showing the importance of these contacts for proper capsid assembly [38•]. Though not an A-domain embellishment, phage HK97 G-loop makes similar important interactions across the twofold axes of symmetry or the HK97 procapsid and mutations in the G-loop result in similar misassembly products (Figure 4b) [13•]. P22's I-domain D-loop and HK97's coat protein G-loop occupy spatially analogous positions on the exteriors of their respective coat protein subunits (Figures 2a and e, 4) although the G-loop is inserted into the spine helix rather than the A-domain, suggesting that they mediate potentially analogous inter-capsomer interactions that result in similar phenotypes when perturbed. Though little is known about the function of the elongated variant of the G-loop (or β-tongue) that is seen in lambda [33] and CFT073 coat proteins (PDB:3BQW), we can speculate that it may have a similar function in proper capsid assembly. These observations show that interactions that reach across twofold axes of symmetry between adjacent capsomers that are mediated by loops protruding from the outer surface of the coat protein subunits are an important and common feature of the HK97 fold.

What does the E-loop do?

The E-loop is shown as an isolated feature in subunit illustrations, but it is never actually without binding partners. In procapsids and capsids it always has multiple interactions that may include parts of 1) the same subunit, 2) the adjacent subunit in the same capsomer, and 3) subunits in adjacent capsomers. We give examples below and describe how some have demonstrated roles in regulating assembly.

In the non-viral T = 1 and T = 3 encapsulins from *T. maritima* [6] and *Pyrococcus furiosus* [39] and in all instances of the HK97 fold examined so far in viruses (all T = 4 or larger), the E-loops make *intra-capsomer* connections to the adjacent subunits' P-domain or A-domain, as can be seen in HK97 (Figures 1c and 4 c), T4, phi29 [40], P22 [15], T7 [41], HSV-1 [4•] 80alpha [42], and many others. Given the prevalence of this interaction, we would suggest that it is universal. In HK97, a salt bridge between the E-loop and the spine helix of the adjacent subunit is a key part of that essential connection: breaking it leads to the formation of sheets and tubes of flattened hexons similar to those in mature capsids (Figure 4c) [12•]. This suggests that these E-loop connections tether adjacent subunits to each so that hexons can take on the domed, asymmetric shape needed to build procapsids. The exact nature of these intra-capsomer connections may not be important: salt bridges may mediate some connections, as tested in HK97 and suggested for others [12•,19]. Indeed, T4 uses an extra domain inserted at the end of the E-loop to make analogous intra-capsomer connections (Figure 2d) [31].

E-loops also make many *inter-capsomer* contacts which are likely to play roles in assembly. In HK97 procapsids, E-loops from each capsomer contact the G-loop on the 'hillside' of the adjacent capsomer (Figure 4b). Those interactions occur only in procapsids, but are essential and appear to set the dihedral angle between adjacent capsomers [13•] since disrupting them either prevents normal assembly or leads to the formation of tubes or small incomplete shells. The all-penton T = 1 encapsulins from *T. maritima* [6] use E-loop:E-loop interactions exclusively to bind adjacent capsomers to each other. In other procapsids, including T7's [41], the E-loops on adjacent capsomers contact each other across the canyon of the inter-capsomer interface, or around the local-threefold symmetric junctions. We suggest that some of these interactions play roles in setting inter-capsomer angles (as they do in HK97 [13•]) and thus are likely to have a role in determining capsid size. The E-loop often extends far enough to make distant interactions with adjacent capsomers near its tip, whose roles are generally unknown, including in HK97 [43], T7 [41] and P22 [19]. One example is a lysine residue near the tip of the HK97 E-loop that inserts into a socket between subunits during expansion and forms a covalent link to the interior of an adjacent capsomer [5,44–46]. An unusual E-loop connection is found in P22, where it appears to be bound to the underside of the I-domain of the same subunit, but we do not yet know if that interaction is important.

Thus, what the E-loop does is *make connections*, some of which we can tentatively assign functions: *intra-capsomer* connections that appear needed for holding capsomers in the correct conformation for procapsid assembly, and *inter-capsomer* connections that participate in and can regulate the higher order assembly into capsid shells.

Conclusion

Here, we have highlighted some of the features and interactions that appear to be widely conserved and play important roles in assembling viral capsids from subunits having the HK97 fold. We propose that the size and shape of capsid particles are determined at the procapsid stage through inter-capsomer and intra-capsomer interactions that we have begun to reveal by studying the locations and phenotypes of mutants that divert capsid assembly into abnormal pathways.

Acknowledgements

This work was supported by the United States National Institutes of Health (NIH) National Institute of General Medical Sciences grants GM076661 to CMT, and GM47795 to RLD. We thank Alexis Huet for his assistance in making the figures.

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