Coxsackievirus B3

Subjects: Virology

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Coxsackievirus B3 (CVB3) is a non-enveloped single-stranded RNA virus belonging to the genus Enterovirus of the picornavirus family.

Coxsackievirus B3 microRNA miR oncolytic virus cancer virus adaptation

1. Coxsackievirus B3 (CVB3) Structure, Genome and Protein Functions

CVB3 is a non-enveloped single-stranded RNA virus belonging to the genus *Enterovirus* of the picornavirus family. As with all members of the *picornaviridae*, CVB3 is characterized by an icosahedral capsid of approximately 30 nm diameter, which houses the positive-sense (+) RNA genome [1][2]. The capsid consists of twelve pentamers, each composed of five asymmetric units of the structural proteins VP1–VP4 (**Figure 1**A). VP1 to VP3 form the viral shell. VP4 lies at the inner surface of the viral shell making a connection between *N*-termini of the other capsid proteins and the viral RNA, thereby acting as a stabilizer of the capsid pentamers during virus assembly [3][4].

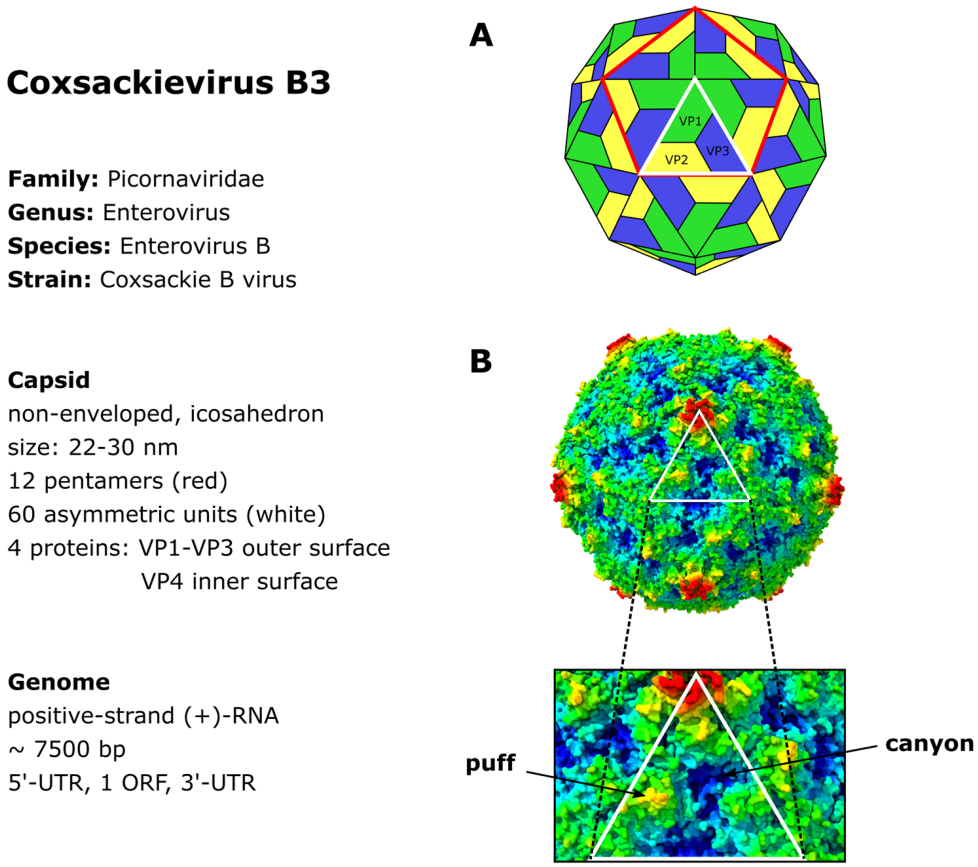


Figure 1. Structure of the CVB3 capsid. (**A**) A schematic model of the icosahedral CVB3 capsid structure composed of 60 asymmetric units, each composed of one VP1 (green), VP2 (yellow), VP3 (blue) and VP4. VP1, VP2 and VP3 form the capsid surface, while VP4 is located on the inside surface. The red outlined area depicts a pentamer, the white outlined triangle depicts an asymmetric unit. (**B**) *Upper panel*: The capsid surface structure colored radially. The radial surface structure was calculated and modelled with the bioinformatic software UCSF ChimeraX [2] based on the structural data of CVB3 RD from the RCSB protein databank (accession no. 4GB3). *Lower panel:* A magnified section of the asymmetric unit with the arrows highlighting the puff region and the canyon.

The capsid surface forms a depression, called the canyon, around the five-fold axis of symmetry of each pentamer [5] (**Figure 1**B). Underneath the bottom of the canyon there is a hydrophobic pocket hosting a C_{16} fatty acid which is referred to as the pocket factor and contributes to the stability of the viral capsid [6][7][8]. It is thought that the binding of the Coxsackievirus and Adenovirus Receptor (CAR) [9][10] to the pocket displaces the pocket factor, thereby destabilizing the capsid, triggering the uncoating and delivery of the viral RNA into the cells [7][8][11]. Another important structural feature of the capsid surface, the elevated hypervariable puff region, located at the southern rim of the canyon (Figure 1B), functions as a known antigenic site [5][12][13]. Furthermore, it is involved in the binding of the decay accelerating factor (DAF) which serves as a co-receptor of CVB3 [14][15].

The positive-sense (+) RNA genome of CVB3 has a length of approximately 7.5 kilobase pairs (kb). It comprises a single large open reading frame (ORF) flanked by a 742 nucleotide (nt) long 5′-untranslated region (5′-UTR) and an

about 100 nt long polyadenylated 3‘-UTR [1]. Particularly the long 5′-UTR builds a number of stem-loop structures, among them the cloverleaf (CL) and the internal ribosomal entry site (IRES) which play major roles in viral replication and protein synthesis [1][16][17][18][19]. The CL interacts with VPg (virus protein genome-linked, also known as 3B), which is covalently attached to the 5′-end of the positive-sense RNA, and with the 3′-UTR and other trans-acting proteins to form the replication complex during RNA synthesis [18][20][21][22][23][24]. The IRES conveys the CAP-independent interaction with the cellular ribosome for viral translation [17][20]. The ORF encodes a continuous polyprotein which is autocatalytically processed into the 4 structural (VP–VP4) and 7 non-structural proteins (2A–2C, 3A–3D), as well as 3 intermediate cleavage products (2BC, 3AB and 3CD) [20] (**Figure 2**).

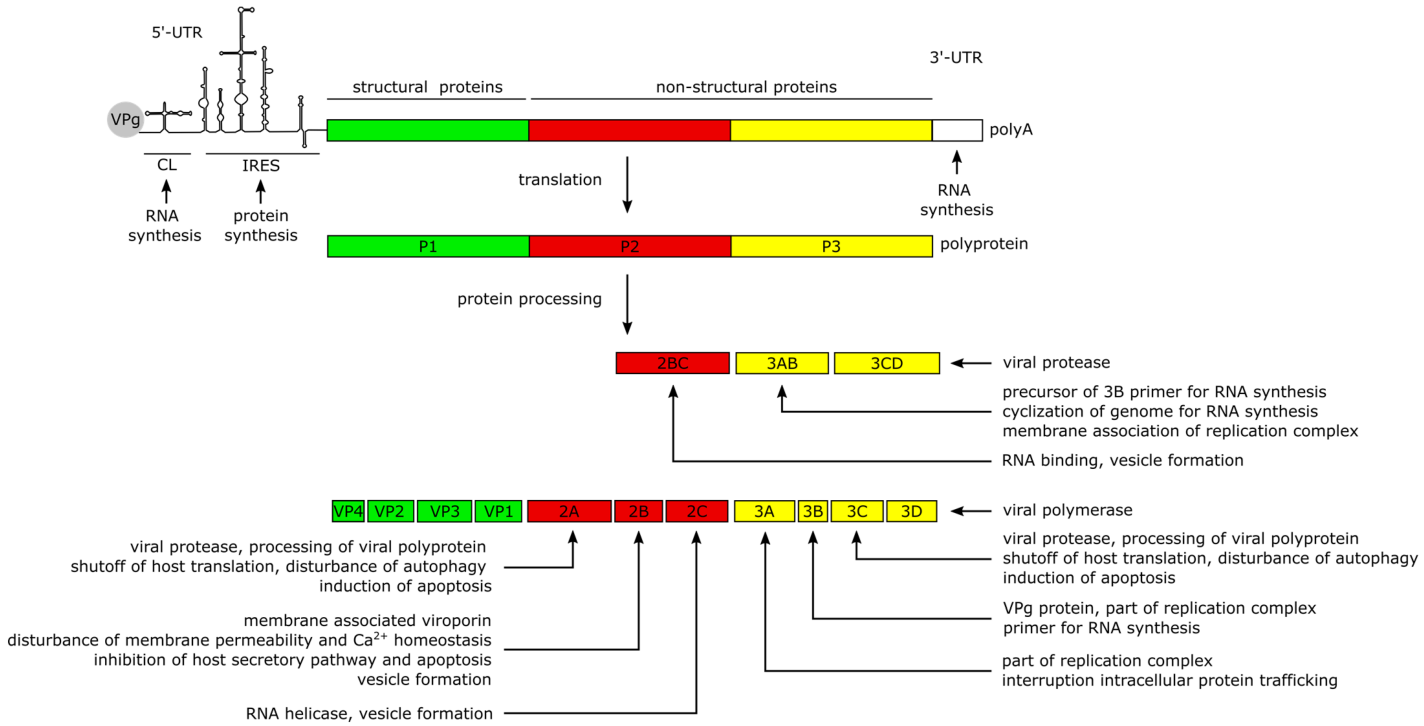


Figure 2. Structure of the CVB3 genome and the functions of the viral proteins. The ~7.5 kb viral genome comprises a 5′-UTR with the cloverleaf (CL) structure and the internal ribosomal entry site (IRES), modelled according to Bailey et al. [19], an open reading frame and a 3′-UTR with a poly(A) tail. The small VPg protein binds to the 5′-end of the genome. The open reading frame is translated into a polyprotein which is autocatalytically processed into the structural (green) and non-structural (red and yellow) proteins. During the processing of the non-structural proteins, three precursor proteins (2BC, 3AB, 3CD) with distinct functions in the viral life cycle are formed, which are processed further into the seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, 3D).

The non-structural proteins function to promote viral protein synthesis, replication, release and spread by interacting with the RNA genome and polyproteins, while also interfering with cellular processes. Most of the manipulation of host cell processes and virus-induced pathogenesis can be traced to the activities of viral proteases 2A and 3C. Besides the proteolytic processing of the polyprotein into the 11 structural and non-structural proteins, the proteases are involved in the shutdown of host cell translation and transcription, disruption of the cytoskeleton, induction of apoptosis and attenuation of the innate immune response. The blockage of translation is

mainly carried out by cleavage of host factors like the eukaryotic initiation factor 4G [25], the poly(A)-binding protein [26] and the Death-Associated Protein 5, as they are important mediators of cap-dependent and IRES-dependent translation initiation in the cell [1][27][28]. To prevent premature viral clearance from the cell, the proteases also cleave the immune adaptor molecules and pro-apoptotic factors, named Mitochondrial Antiviral Signaling Protein (MAVS) and Toll/IL-1 Receptor Domain-containing Adaptor Inducing Interferon-β (TRIF), which leads to an attenuated type I interferon response and apoptotic signaling during the early stages of CVB3 infection [29]. In addition, Protease 2A cleaves the cytoskeletal protein Dystrophin, an event shown to be important for the pathogenesis of CV-induced cardiomyopathies [30]. Another key feature of the 2A and 3C proteases is their ability to induce apoptosis through caspase-8-mediated activation of caspase-3 and to activate the intrinsic mitochondria-mediated apoptosis pathway during the late phase of viral infection [25].

The Viroporin 2B and its precursor 2BC build homo- and heteromultimers, which integrate into the membranes of the Golgi apparatus and the endoplasmic reticulum (ER) [31][32][33]. The resulting pore formation leads to a leakage of Ca^{2+} into the cytoplasm [31][32][34], disturbing pro-apoptotic signaling during the early stages of infection [32][35][36] thereby preventing a rapid clearance of the virus. Furthermore, the membrane interaction of 2B is thought to induce the formation of vesicles which are important for viral replication and release [34][35][37]. In addition, the 2C protein possesses a RNA helicase function in enteroviruses [38] which could also be confirmed for CVB3 [39][40].

The proteins 3A, 3AB and 3D interact with the viral genome to form the replication complex [21]. The 3B protein serves as the primer for the transcription of the viral genome [41]. The binding of 3AB is thought to activate the protease activity of CL-bound 3CD precursor to release the 3D polymerase and mediate the cyclization of the genome by interacting with the 3′-UTR during (−) RNA synthesis [22][42][43].

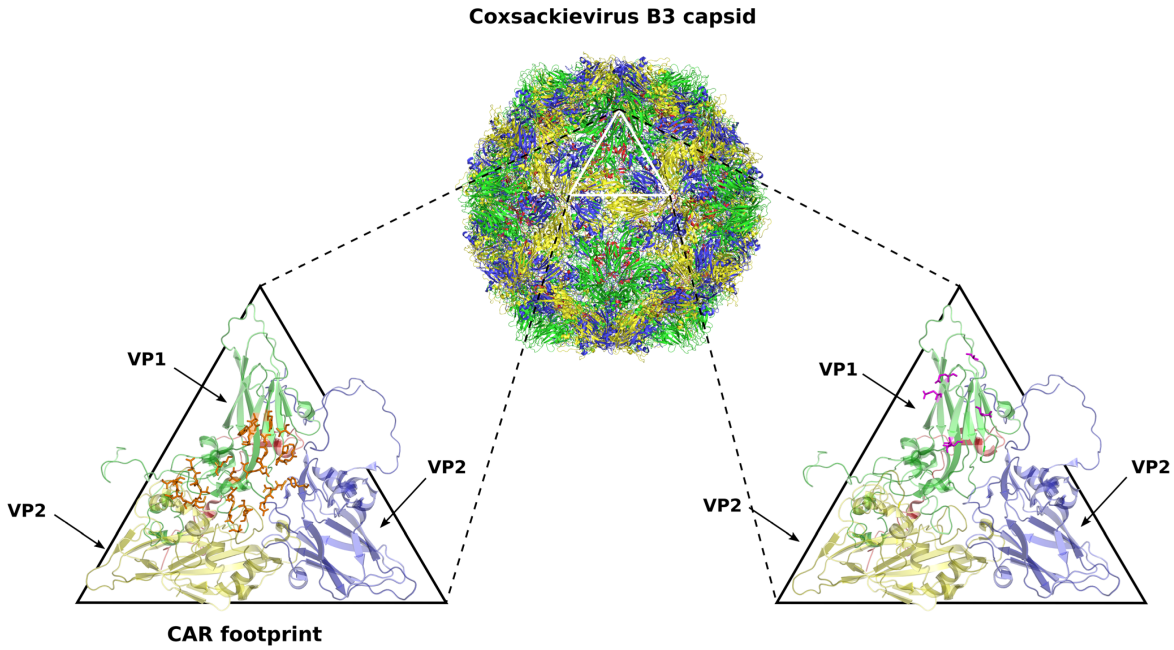
2. CVB3 Infections in Humans and in Experimentally Infected Mice

CVB3 usually induces mild self-limiting disease with flu-like symptoms in humans. Under certain circumstances, which involve genetic and individual predispositions, severe disease can result. Most frequently, patients suffer from aseptic meningitis, encephalitis, and myocarditis [44][45], whereas pancreatitis [46][47] and hepatitis [48] are less frequently observed. Individuals of all ages and either sex can be infected with CVB3 [49], but infants are particularly at risk. CVB3 infection in infants can lead to severe systemic disease and death by hepatic, cardiac or multi-organ failure [50][51][52].

In mice, the pancreas and heart are the main target organs of CVB3, but in contrast to humans, the pancreas is the most susceptible organ in mice [47][53][54]. CVB3 infections of the pancreas results in acute pancreatitis with advancing destruction of the exocrine part of the organ. Myocardial infection leads to direct acute and chronic inflammation, impaired cardiac contractility and heart failure [47][55]. The degree of infection, inflammatory processes and tissue damage, however, depends on several factors, such as the virulence of the virus strain, genetic background of the mice, age, sex and route of virus administration [47][53][56][57][58][59].

3. CVB3 Receptors and Its Importance for CVB3 Targeting of Cancer

Occurrence of viral receptors on the cell surface is a key feature that contributes to virus tropism. Hence, the expression of CVB3 receptors on cancer cells is vital for the successful treatment of cancer with oncolytic CVB3. The main receptor for CVB3 binding and uptake is CAR [10][60][61] (**Figure 3** and **Figure 4**), a transmembrane protein which is involved in cell adhesion and inflammation [62]. In addition to CAR, several CVB3 strains, such as RD and HA, use DAF, which is involved in the regulation of complement activation and cell signaling. DAF functions as co-receptor for CVB3 attachment to the host cell surface [63][64]. The binding of DAF alone, however, is not sufficient to mediate viral entry into the cell and subsequent lytic infection [8][64]. Thus, cancer cells that express DAF but not CAR are not vulnerable to oncolytic CVB3.



accession no.	CVB3 strain	VP1					
		78	80	91	92	152	223
JX312064.1	Nancy	E	E	V	L	D	A
AF231765.1	PD	K	A	A	I	E	S

Figure 3. Amino acids of the CVB3 capsid involved in CAR and heparan sulfate binding. *Upper image*: The capsid is shown along with the tertiary structure of the capsid proteins VP1 (green), VP2 (yellow), VP3 (blue) and VP4 (red). The triangle outlines one asymmetric unit. The structure is modelled with the bioinfomatic software PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) based on the structural data of CVB3 RD from RCSB protein databank (accession no. 4GB3). *Lower left magnified image*: the amino acids involved in CAR binding (according to He et al. [65] and Organtini et al. [5]) are shown in orange. *Lower right magnified image*: the amino acids within one asymmetric unit of CVB3 PD strain (NCBI accession no. AF231765.1) that differ to the CVB3 Nancy strain (NCBI accession no. JX312064.1) are shown in magenta. Their position within VP1 is indicated in the table. The amino acids which differ in the sequence of PD compared to Nancy are involved in binding of the virus to *N*- and 6-*O*-sulfated heparan sulfates.

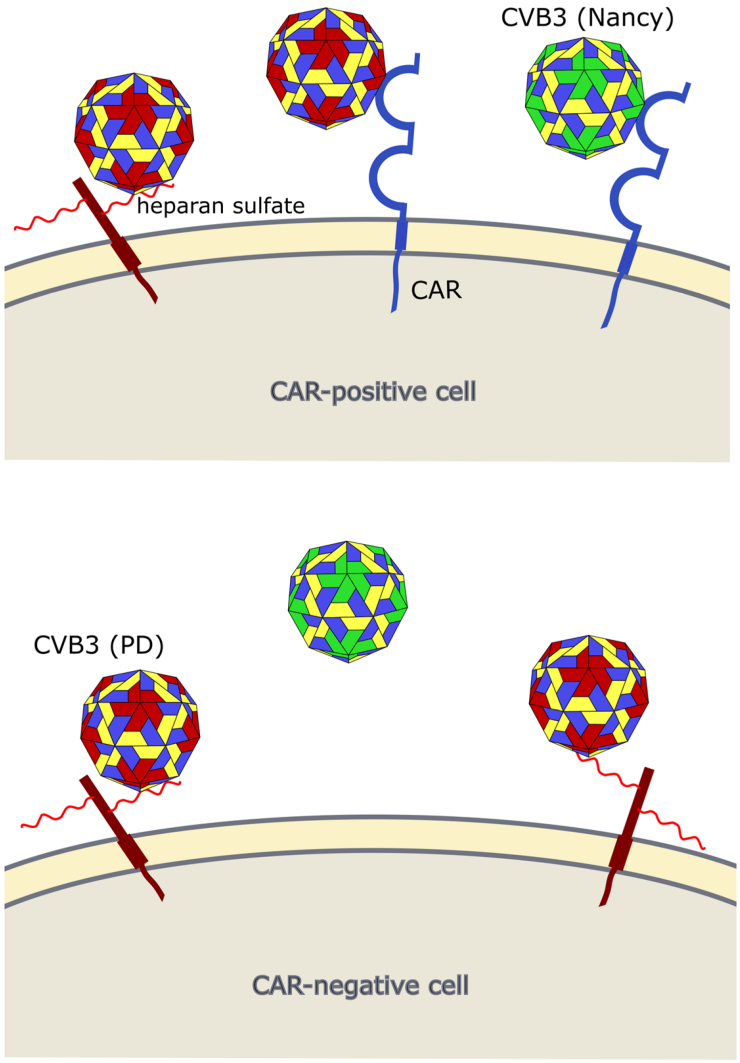


Figure 4. Interaction of CVB3 with cellular receptors. The prototype CVB3 strain Nancy uses CAR for cell entry, whereas the CVB3 variant PD can infect cells via *N*- and 6-*O*-sulfated heparan sulfates and CAR. *Upper panel*: CAR-positive cells can be infected with PD and the Nancy strain. *Lower panel*: CAR-negative cells cannot be infected with the Nancy strain, but with PD when *N*- and 6-*O*-sulfated heparan sulfates are expressed on the cell surface.

CAR is expressed in many tissues, including heart, lung, liver, testis, pancreas and kidney [66][67]. It is highly expressed during fetal development and in young individuals, while it is downregulated in adults [68]. In cancer, CAR is differentially expressed. Compared to normal tissues in lung cancer, cervical cancer, endometrial cancer, ovarian cancer and urinary bladder cancer, for example, CAR appears to be upregulated, whereas in colon and prostate cancers, as well as subtypes of renal cell cancers it is strongly downregulated [69].

Two studies, investigating lung [70] and endometrial cancer [71], found a good correlation between sensitivity of the cancer cell line to oncolytic CVB3 and their CAR and DAF expression levels. In another study, however, there was no clear correlation between abundance of CAR and susceptibility of colorectal carcinoma cell lines to oncolytic

CVB3 [72], which may mean that under certain conditions post-entry mechanisms may be of particular importance for cytolytic activity of oncolytic CVB3.

In addition to CAR, it has been shown that CVB3 can use heparan sulfates to enter cancer cells. Thus far, however, this has only been shown for the CVB3 variant PD, which uses *N*- and 6-*O*-sulfated heparan sulfates to infect cells [72][73][74] (**Figure 3** and **Figure 4**). Heparan sulfates are linear polysaccharides, which consist of repeating disaccharides bound to a core protein which links them to the cell surface. Based on the analysis of the expression of the heparan sulfate D-glucosaminyl 6-*O*-sulfotransferase-2 (HS6ST2), which catalyzes the transfer of sulfate groups to the C-6 (exocyclic carbon) of the glucosamine residue in heparan sulfate proteoglycans, the stomach, liver, adrenal gland, bronchus, breast, ovary, uterus, kidney and skin contain *N*- and 6-*O*-sulfated heparan sulfates. In other organs, such as the lung, pancreas, heart, spleen, prostate and colon, HS6STS expression could not be detected [75]. HS6ST2 is also differentially expressed in cancer. This enzyme is downregulated in ovarian cancer [76] but overexpressed in colorectal, gastric and pancreatic cancer [75][77][78].

A recent study from researcher group confirmed the importance of *N*- and 6-*O*-sulfated heparan sulfates for infection of cancer cells with the CVB3 variant PD. In fact, there was a positive correlation between expression HS6ST2 and the sensitivity of colorectal cancer cell lines to the PD strain of CVB3 [72].

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