The Crystal Structure of a Coxsackievirus B3-RD Variant and a Refined 9-Angstrom Cryo-Electron Microscopy Reconstruction of the Virus Complexed with Decay-Accelerating Factor (DAF) Provide a New Footprint of DAF on the Virus Surface

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The crystal structure of a coxsackievirus B3-RD variant and a refined 9-Angstrom cryo-electron microscopy reconstruction of the virus complexed with decay-accelerating factor (DAF) provide a new footprint of DAF on the virus surface.

Coxsackieviruses are significant human pathogens that cause myocarditis, menigitis, and pancreatitis and have been implicated in the development of juvenile diabetes (58, 60–64). Virulence determinants have been described throughout the genome (19, 20, 30, 51, 65), including the P1 region, which encodes the structural proteins (7, 10, 13, 25, 48, 49, 56). The capsid surface presents a topology of structural motifs that largely dictate receptor recognition and usage, directly affecting tropism and pathogenicity.

Group B coxsackieviruses (CVBs) belong to the genus Enterovirus of the family Picornaviridae. Picornaviruses are nonenveloped, positive-sense, single-stranded-RNA animal viruses with a capsid comprised of 60 protomers arranged to form an icosahedral shell ~300 Å in diameter with T = 1 (pseudo- T = 3) symmetry (ICTV classification) (8). In mature capsids, each protomer contains four structural proteins, VP-1, -2, -3, and -4. Structural studies have shown that capsids share common features, including a depression around the icosahedral 5-fold symmetry axes (called the “canyon”) and a hydrophobic cavity located underneath the floor of the canyon (called the “pocket”) (52). Biochemical and structural evidence indicates that the ligand within the pocket is a fatty acid (26, 55). For many picornaviruses, a receptor binds into the canyon, which suggests a possible explanation for why interactions with DAF do not appear to cause the conversion of native virions to A particles (16, 35, 46). However, a variety of evidence suggests that different DAF-binding viruses interact primarily with different SCR domains (3, 11, 23, 47, 54), suggesting that several modes of virus-DAF interactions are possible.

The first DAF-binding CVB3 isolate identified was obtained by the passage of a prototype strain, CVB3 strain Nancy (CVB3-Nancy), on rhabdomyosarcoma (RD) cells (50); the RD-adapted...
virus, CVB3-RD, binds DAF, whereas the original CVB3-Nancy does not (3). Previously, a 14-Å-resolution cryo-electron microscopy (cryo-EM) reconstruction of the CVB3-RD isolate complexed with DAF was interpreted with the crystal structure of a non-DAF-binding CVB3 strain (CVB3 strain M [PDB accession number 1COV]) (18, 37). This analysis showed clashes between DAF and the virus loops at the “puff” region of VP2 (loop EF; residues 2129 to 2180) (18, 37). Here, we present the crystal structure of the CVB3-RD variant virus solved to 2.74 Å and a cryo-EM reconstruction of CVB3-RD bound to DAF refined to 9-Å resolution. The higher-resolution cryo-EM map of the virus-receptor complex made it clear that in our previous reconstruction (18), we had incorrectly assigned the handedness of the virus, resulting in an incorrect interpretation of the contact sites between CVB3 and DAF. In the new structure, each DAF molecule was found to link two adjacent protomers of the virus capsid in a mode of DAF binding different from that described previously for echoviruses 7 and 12 (4, 22, 43, 45). Since the crystallographic symmetry (NCS) operators. During the first round of refinement, the residues that were different in the RD strain relative to the parental virus were mutated to their proper identity. After each refinement cycle, a single cycle of electron density Fourier map (2Fo – Fc and Fo – Fc, where Fc indicates the observed structure factors and Fc indicates the structure calculated from the model) averaging was carried out in CNS, with strict NCS operators, using the experimentally measured amplitudes and the improved phases. The program Coot was used for model building into averaged electron density maps between cycles of the refinement and averaging procedures (14).

Cryo-EM reconstruction. CVB3-RD was incubated with full-length DAF molecules, as reported previously (18). Micrographs of the vitriﬁed virus-receptor complexes used in this study were reported previ-
ousely (18). Micrographs were digitized with a Zeiss Phodis microden-
sitometer at 7–9262 m intervals, averaged in boxes of 2 pixels by 2 pixels,
providing a final pixel size of 3.11 Å. Using Robem, 3,011 particles
were isolated from 36 of the highest-quality micrographs, with a selec-
tion area sized to 171 by 171 pixels, and preprocessed by using autoppp
in order to remove blemishes, linearize, normalize, and apodize (71).
To correct for contrast transfer function, defocus and astigmatism
values were assessed over the digitized images by using ctffind3 (36),
obtaining a range of 1.04 to 5.05 m for the defocus values and a ratio
for the major/minor axis average of 1.05 $\pm$ 0.04. The reconstruction
was initiated by using a random model (70). An amplitude contrast
factor of 7% was applied during the reconstruction. Reconstructions
initiated with several different starting models converged to the same
result with auto3dem imposing icosahedral symmetry. All resulting
maps had a resolution higher than 10 Å, estimated at a Fourier shell
correlation (FSC) value of 0.5. Using a final delta angle of 0.25° and
applying a temperature factor, $B$, of 400, the map improved to a 9-Å
resolution and was used for subsequent analyses.

Assessment of cryo-EM map handedness. Using the final 9-Å CVB3-
DAF complex reconstruction, a map representing the stereoisomer was gen-
erated by mirroring the complex map across the x-y plane to "flip" the hand.
Using Chimera, an 8-Å-resolution map calculated from the new crystal struc-
ture of CVB3-RD was fitted separately into each map, rendered at a density
threshold of 1 $\sigma$ (15, 42). Correct handedness was assessed by the quality of
the fit, by measuring correlation coefficients (CCs) calculated about the mean
data value using the Chimera protocol Fit in Map. With the correlation-
about-mean option, the correlation can range from $-1$ to 1, as defined by
Chimera. In addition, the newly solved crystal structure of the RD variant
virus was fitted into the flipped and unflipped complex maps with the DAF
density masked out by using the segger subroutine of Chimera to reveal the
surface features of the virus (15, 42) (data not shown). This segmentation
approach separated densities in the map according to local minima by using a
watershed algorithm (44). In both analyses, the map in which the hand had been flipped relative to the previously reported reconstruction of the complex of CVB3-RD and DAF, EMD-1412 (18), revealed a better agreement with the virus crystal structure.

**Structure fitting.** Using EMfit, the exact pixel size (2.94 Å) of the cryo-EM reconstruction was determined by scaling to a map calculated from the crystal structure (31, 53). A map calculated to 9 Å was also used to obtain a difference map by subtracting it from the virus-receptor complex map (53). The fitting of the eight different crystal forms of DAF SCR1 to SCR4 (PDB accession numbers 1OJV, 1OJW, and 1OJY) (32) into the difference map density was carried out by using Chimera. Briefly, the average map value at each fit atom position is maximized, and for each atom within the bounds of the reference map, the map value is found by trilinear interpolation from the eight corners of the enclosing data grid cell. Atoms outside the bounds of the map are not used for computing averages, and the best fit was determined by the highest relative average map value (42) (Table 2). Using the best fit of DAF, the buried surface between DAF and CVB3-RD was calculated by

![FIG 3](image-url)

**FIG 3** Determination of the handedness of the cryo-EM reconstruction. (A) An 8-Å-resolution map calculated from the crystal structure (white) was used to measure the correlation (CC) of both possible hands of the cryo-EM maps (42). The calculated map is placed within the new 9-Å cryo-EM map, depicted in blue mesh, at 1 with the handedness previously reported (18), which gave a CC of 0.14. The X-ray-calculated map placed into the cryo-EM reconstruction of the alternate hand is shown in green mesh at 1, producing a CC of 0.6. Not only does the poor correlation of the blue map indicate the wrong hand assignment, the map calculated from the crystal structure also protrudes significantly from the blue density. In contrast, the correct-handed green map fits better with the calculated map of CVB3-RD. (B) Close-up view of the 5-fold vertex showing the disagreement and agreement in directionality for the incorrect hand and the correct hand, in blue and green, respectively.

![FIG 4](image-url)

**FIG 4** (A) Refined fit of DAF (yellow ribbon) into the difference density, displayed at 1.0σ. (B) Capsid-DAF interaction rendered to show capsid surface topology. Five protomers of the virus capsid were colored by radius from the center of the virus, with the key shown at the right. DAF is shown as a highlighted yellow ribbon that binds to a depression in the virus capsid via SCR2 and crosses the virus canyon to interact beneath the puff via SCR3. Residues known to be necessary for DAF binding, residues 2138 and 3234, are highlighted in magenta (41). The Cα-to-Cα distance between residues 2138 and 3234 is 17.6 Å. (C) Each DAF bridges two protomers of the virus capsid, delineated in blue and green. The residues that define the region of DAF that binds convertases are highlighted in red on the surface-rendered DAF molecule (yellow).
using the CCP4 program Surface, with the probe set to a 3.5-Å diameter (9, 29). Residues in the virus-receptor interface were identified by using the CCP4 program Contact (69).

**Protein structure accession numbers.** PDB accession numbers for data bank deposition are as follows: 4GB3 for the CVB3-RD crystal structure, 5475 for the cryo-EM reconstruction of CVB3-RD complexed with DAF (SCR1-SCR2-SCR3-SCR4), and 3J24 for the structure of DAF fitted into the cryo-EM reconstructions of the complex.

**RESULTS**

**CVB3-RD crystal structure.** Frozen CVB3-RD crystals diffracted X rays coherently to a 2.5-Å interplanar spacing. The structure of the capsid protein was determined to 2.74 Å (Fig. 1A). As reported previously for the structure of CVB3-M (PDB accession number 1COV) (37), residues 2001 to 2007 and 4012 to 4024 were disordered and could not be built. However, four additional residues could be placed into the N terminus of VP1; whereas the CVB3-M structure is missing residues 1001 to 1012, the RD structure begins at residue 1008. The density corresponding to the pocket factor that is characteristic of picornaviruses is clearly visible. This ligand is modeled with a 16-carbon fatty acid palmitate molecule in the same orientation as that described previously for CVB3-M (37).
FIG 6 The virus surface represented as a quilt of amino acids, shown as a projection, with the icosahedral asymmetric unit indicated by the triangular boundary. Virus residues representing two adjacent protomers are shown in light blue and green, with DAF contacts shown in yellow, orange, and red, corresponding to the SCR (SCR2, -3, and -4, respectively) that is predicted to interact with the virus surface. Rather than showing sequence-equivalent residues plotted previously (18, 45), this figure shows the CVB3-RD–DAF contacts on the CVB3-RD virus roadmap, the E7–DAF contacts on the E7 roadmap (PDB accession number 2X5I) (45), and the E12–DAF contacts on the E11 roadmap (accession number 1H8T) (43, 57).
density corresponding to a calcium ion seen in the CVB3-M structure is present at the 3-fold icosahedral symmetry axes, although the peak is not as strong as what was reported previously for the structure of the M strain (37). CVB3-RD VP1, -2, and -3 adopt the canonical β-sandwich motif seen in many icosahedral viruses, with eight antiparallel β-strands forming two sheets, BIDG and CHEF (Fig. 1A) (52). VP3 also contains a β-cylinder structure comprised of the symmetry-related N termini forming a pore, located on the icosahedral 5-fold axis, that is highly conserved in all picornavirus structures.

Of the six total amino acid differences between parental strain Nancy and the variant RD strains, four map to the surface of CVB3-RD. Of the remaining two, residue 1092 (L to I) maps to the top of the pocket and is in contact with the pocket factor, and residue 2013 (A to V) maps to the protomer-protomer interface at the icosahedral 3-fold axes. In all six locations, the side chains of the substitutions could be placed without ambiguity (Fig. 1B and C). There were no significant changes in local or global structure between CVB3-M and CVB3-RD. The RD crystal structure is nearly identical to the structure of CVB3-M, with an overall root mean square deviation (RMSD) of 0.4 Å or lower for each VP (Fig. 1).

**Cryo-EM reconstruction of the CVB3-RD–DAF complex.** The cryo-EM reconstruction of the CVB3-RD variant complexed with all four SCRs of DAF reached a 9.0-Å resolution, calculated at an FSC value of 0.5 (Fig. 2A and C). The resolution provided detailed surface features, making it possible to assign the absolute hand of the map by comparing the directionality of structural features with the fitted crystal structure of the virus (Fig. 3). In order to quantify the fit and confirm the correct hand, a calculated map made from the newly solved crystal structure of the RD variant virus was fitted into the cryo-EM complex map, generating a correlation coefficient (CC) of 0.6 (15, 42) (Fig. 3). The same fitting routine was done by using a previously reported map of the opposite hand (18), which generated a significantly worse score of 0.14 (42), thus confirming the correct handedness of the cryo-EM structure presented here (Fig. 2 and 3). The magnitude of the receptor density relative to that of the capsid was nearly equal (Fig. 2B), indicating that the complex is close to full occupancy of the 60 DAF-binding sites. The radial distribution of the density differentiated into three regions, the RNA core, the virus capsid, and the bound receptor (Fig. 2D).

**CVB3-RD interactions with DAF.** To construct a pseudo-atomic model of the complex, the CVB3-RD crystal structure was placed into the cryo-EM density map of the complex by superimposing the icosahedral symmetry elements. The DAF crystal structure was placed into the corresponding DAF density by using all nonhydrogen atoms (see Materials and Methods). The general shape of DAF, specifically the distinct kink between SCR1 and SCR2, readily determined the overall orientation. Eight monomers from three different DAF crystal structures (PDB accession numbers 1OJW, 1OJW, and 1OJW) (32) were fitted independently into the difference map. Molecule B from the structure reported under PDB accession number 1OJW (32) fit best into the DAF density, quantified by using relative average map value scores (see Materials and Methods) (Table 2 and Fig. 4A) (42). Atoms that
potentially participate in interactions between CVB3-RD and DAF were identified by determining which atoms on the virus and receptor surfaces were within 3.6 Å of one another and had the proper geometry to form bonds (Table 3). The identified virus surface residues define a footprint of DAF on the capsid. There is 1,081 Å² of total buried surface area in the interface of the virus-receptor complex (Table 4). Each bound DAF molecule links two adjacent viral capsid protomers by binding to the northern side of one puff (Fig. 4C, green) and the southern side of the puff in the neighboring subunit (Fig. 4C, blue). SCR1 of DAF rises above the surface of the virus, and no contacts were identified. Portions of SCR1 have a weaker density, likely due to a flexibility in the hinge that connects SCR1 to SCR2. Most virus-receptor interactions occur between SCR2 and the northern region of the viral puff of the first of the two protomers (Fig. 4A), contributing 75% of the total buried surface area (832 Å² of the total 1,081 Å²) (Table 4). From SCR4 toward the C terminus of DAF, there is a small volume of uninterpreted density localized to the icosahedral 3-fold vertices that was reported previously (18). This density is likely filled by the C-terminal six-His tags from each of the three DAF molecules.

DAF interactions with convertase. Three DAF residues, R69, R96, and L171, have been shown to be critical for native DAF function. The region of the surface of DAF defined by the location of R69 and R96 is especially critical for binding to and hastening the decay of convertases in the classical and alternative complement pathways (27) (Fig. 4C). This functional face of DAF does not overlap the footprint that identifies DAF binding to CVB3-RD and is oriented away from the surface of the virus in the structure of the complex.

CVB3 DAF binding compared to the previously identified echovirus mode of binding. Virus-DAF complex structures have been solved for echovirus 7 (E7) (22, 45) and echovirus 12 (E12) (4, 43). In the case of both echoviruses, the mode of binding is the same, with SCR2/3 nearly crossing the 2-fold axis, but the interactions are not identical (43). The new CVB3-DAF map demonstrates a second mode of DAF binding with SCR2/3 across the canyon, crossing to bind the puff near the 5-fold axis. There are

FIG 7 CVB3-RD and echovirus sequence-equivalent residues from a clustaw alignment shown to compare DAF interactions (43, 45). The puff region of the virus consists of VP2 residues 129 to 180, outlined by a gray box. The four residues defined in the DAF footprint that are common to DAF binding in all three viruses are boxed. The two residues essential for DAF binding to CVB3-RD are circled (41). The domains of DAF SCR1 to SCR4 were assigned to residues 1 to 62, 63 to 127, 128 to 189, and 190 to 253, respectively, from data reported under Uniprot accession number P08174-2 (DAF 1) (PDB accession number 1OJV) (32). There are no DAF residues in common that interact with all three viruses.
fewer virus-DAF interactions than reported previously (18, 45) and less buried surface. No DAF residue makes contact with all three viruses. None of the three viruses interact with SCR1. Six SCR2 residues interact with both CVB3 and E7, making contact predominately with VP1 and VP3 residues on the viral surface. However, SCR2 interacts above the puff in CVB3 and below the puff in E7; there are no SCR2 interactions with equivalent virus residues (Fig. 5 to 7). E12 does not interact with SCR2. As previously noted (45), the echoviruses are similar in their SCR3 interactions but with only 2 shared DAF contacts. The new footprint on CVB3 shows that E12 shares two DAF contacts with CVB3. SCR3 interacts with VP2 residues in all three viruses (Fig. 5 and 6). SCR4 binds VP3 residues in the echoviruses, but no interactions were defined for CVB3.

An alignment of CVB3 contact residues with the sequence-equivalent residues for the two echoviruses (Fig. 7) (22, 43, 45) shows four DAF-binding virus residues in common, residues 1271, 2142, 2166, and 3063. However, the site of DAF binding for residue 1271 is located in the blue protomer (Fig. 5) of both echoviruses and binds SCR4 of DAF, whereas residue 1271, which interacts with DAF in CVB3, maps to the neighboring protomer (Fig. 5, green), where it interacts with DAF SCR2. The largest commonality in DAF interactions occurs at the lower puff region, where residues 2142 and 2166 map within all three DAF-binding footprints; this small patch on each virus surface residues abrogates DAF binding. At the virus-DAF interface, contacts with SCR2 account for 75% of the buried surface area, consistent with the observation that the deletion of SCR2 prevents virus attachment to DAF, whereas the deletion of other individual SCRs does not (3). The second contact area takes place in a neighboring protomer between the southern puff region and DAF SCR3. Thus, DAF crosses over the canyon to interact with two adjacent protomers. By crossing the canyon, DAF may interfere with virus attachment to CAR, which is known to bind within the canyon (21); indeed, we have found that excess soluble DAF inhibits the attachment of CVB3-RD to CHO cells expressing human CAR (J. M. Bergelson, unpublished observation). At the 3-fold vertex, the C-terminal six-His tags of three symmetry-related DAF molecules meet. This interaction effectively "staples" the DAF molecules to the virus surface. It is conceivable that interactions between the His tags might change the conformation of DAF within the complex, creating a false impression of contacts with SCR3. However, when the crystal structure of DAF was placed directly into the corresponding density in the EM model, no manipulation of hinge angles between SCRs was required, confirming that the DAF molecule is in a native state and has not undergone any conformational changes. Therefore, the His tag interactions likely did not alter the normal mode of DAF binding to the surface of the virus.

The predicted contacts of SCR3 and SCR4 had a combined buried surface area of 249 Å² (Table 4), which represents less than 25% of the total DAF molecule binding predicted from the structure. No contacts were predicted for SCR4; thus, the buried surface of SCR4 is inaccessible but is not an area of direct interaction. Additional interactions with SCR3 defined here may not be essential to DAF binding, as these interactions potentially take place according to the background of the specific CVB3 and depend on the identity of residues in the southern puff region. This is the only site where there is some commonality among all three viruses. Perhaps, the essential CVB3-SCR2 interactions evolved after E7/12 and CVB3 diverged from each other in a split from a common ancestor (17). Regardless of when the trait was acquired, the binding of DAF is a trait that persists and likely confers an advantage to the virus.

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S.H. designed research; J.D.Y. processed crystal data; J.O.C. processed and analyzed the cryo-EM data, including the assignment of absolute handedness; J.P. and J.M.B. provided sequence data; and J.O.C., J.D.Y., and S.H. wrote the paper.

REFERENCES


mutants with altered receptor requirements for infection. J. Virol. 76: 7694−7704.