Single amino acid changes in the virus capsid permit coxsackievirus B3 to bind decay accelerating factor

Running title: Critical sites of CB3 interaction with DAF

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ABSTRACT

Many coxsackievirus B isolates bind to human decay accelerating factor (DAF) as well as to the coxsackievirus and adenovirus receptor (CAR). The first described DAF-binding isolate, coxsackievirus B3 (CB3)-RD, was obtained during passage of the prototype strain CB3-Nancy on RD cells, which express DAF but very little CAR. CB3-RD binds to human DAF whereas CB3-Nancy does not. To determine the molecular basis for the specific interaction of CB3-RD with DAF, we produced cDNA clones encoding both CB3-RD and CB3-Nancy, and mutated each of the sites at which the RD and Nancy sequences diverged. We found that a single amino acid change, the replacement of a glutamate within VP3 (VP3-234E) with a glutamine residue (Q) conferred upon CB3-Nancy the capacity to bind DAF and to infect RD cells. Re-adaptation of molecularly cloned CB3-Nancy to RD cells selected for a new virus with the same VP3-234Q residue. In experiments with CB3H3, another virus isolate that does not bind measurably to DAF, adaptation to RD cells resulted in a DAF-binding isolate with a single amino acid change within VP2 (VP2-138 N to D). Both VP3-234Q and VP2-138D were required for binding of CB3-RD to DAF. In the structure of the CB3-RD-DAF complex determined by cryo-electron microscopy, both VP3-234Q and VP2-138D are located at the contact site between virus and DAF.
INTRODUCTION

Coxsackieviruses, members of the enterovirus genus within the picornavirus family, are common human pathogens (23). Group B coxsackieviruses (CB) are important causes of viral meningitis and myocarditis, and have been implicated as a possible trigger for the onset of childhood diabetes.

CB interact with at least two receptors. All isolates so far examined attach to the coxsackievirus and adenovirus receptor (CAR), a 46 kD transmembrane protein that also serves as a receptor for many adenoviruses (2, 21, 34). In addition, a large subset of CB1, 3, and 5 (including clinical isolates) also bind to decay accelerating factor (DAF, CD55), a GPI-anchored complement regulatory protein (3, 4, 29). Many other enteroviruses also bind to DAF (1, 16, 26, 30, 36). Because phylogenetically diverse enteroviruses interact with different domains within the DAF molecule, it has been suggested that viruses have evolved independently to bind DAF (26). The evolutionary pressures that gave rise to the DAF-binding phenotype, and the role for DAF attachment during infection in vivo, are uncertain.

In polarized epithelial cells CAR is a component of the tight junction (7), a structure at the apical edge of the basolateral membrane that regulates the paracellular flow of ions and macromolecules across intact epithelium (9). No CAR is present on the apical cell membrane, and within the tight junction CAR is inaccessible to virus; as a result, exposure of the apical surface to CB isolates that do not bind DAF results in little, if any, infection (7). In contrast, DAF is highly expressed on the apical membranes of polarized epithelial cells, and interaction with DAF permits DAF-binding CB to infect polarized epithelial cells (33). DAF functions both to permit virus attachment and to initiate kinase signals (8) essential for subsequent events in virus entry.
The first DAF-binding CB isolate to be identified was CB3-RD, originally obtained by Crowell and colleagues after blind passage of the prototype CB3-Nancy strain on RD rhabdomyosarcoma cells (27). Unlike the Nancy strain, CB3-RD readily infected RD cells, and bound to a 60-70 kD cellular protein (13, 24) that was subsequently identified as DAF (4).

Lindberg et al. (20) constructed a series of genomic chimeras between CB3-RD and a CB3-Nancy isolate (not the isolate from Crowell's lab, however), and identified a genome segment that conferred on the Nancy isolate the capacity to infect and kill RD cells efficiently—a phenotype that likely depends on, but is not necessarily synonymous with, DAF binding. Within this segment (which encodes VP2 and a part of VP3), the wild-type and RD isolates differed at 2 sites: in RD, a serine was substituted for threonine at position 151 within VP2 (VP2-151S), and a valine substituted for aspartate at VP2 position 108 (VP2-108V). However, other investigators have questioned whether VP2-151S and VP2-108V account for the RD phenotype (31). Furthermore, in the structure of the CB3-DAF complex determined by cryo-electron microscopy (cryoEM) (10), neither VP2-151S nor VP2-108V was found to be in contact with DAF.

To determine the molecular basis for the specific interaction of CB3-RD with DAF, we have produced cDNA clones encoding both CB3-RD and CB3-Nancy, from low-passage virus stocks obtained from Dr. Richard Crowell. We find that a single amino acid change, the replacement of a glutamate within VP3 (VP3-234E) with a glutamine residue derived from CB3-RD (VP3-234Q), confers upon CB3-Nancy the capacity to bind DAF and to infect RD cells. To investigate further the routes by which CB3 can adapt to bind DAF, we passed CB3-H3—an isolate that does not bind to DAF, although it already possesses VP3-234Q—on RD cells, and obtained a DAF-binding isolate with a single amino acid change within VP2 (VP2-138 N to D).
Both VP3-234Q and VP2-138D are required for virus attachment to DAF. Both residues are within the site of interaction with DAF, as determined by the published cryo-EM structure of the virus-receptor complex (10), as well as in a newly refined structure (J.D.Y., J.O.C, and S.H., in preparation). These results indicate that single amino acid changes permit CB3 to bind DAF and gain tropism for new cell types.
MATERIALS AND METHODS

Cells. HeLa cells were maintained in Modified Eagle's Medium supplemented with L-glutamine, penicillin, streptomycin, non-essential amino acids and 5% fetal bovine serum; RD cells were maintained in the same medium, but with 10% fetal serum. CHO-CAR (35), CHO-DAF (22), and CHO-pcDNA (35) cells were generated and maintained as described previously.

Infectious viral cDNA clones. To generate clones of CB3-RD and CB3-Nancy, HeLa cells were infected (1 PFU/cell) for 7 hours and total RNA was isolated with Trizol reagent (Invitrogen). Reverse transcription was performed with 5 μg of total RNA, 20 pmol of virus-specific primer incorporating an MluI restriction site (CB3-7381RMlu, 5’-GTACGCGTTTTTTTTTTTTTCGCACCGAATGCGGAGAATTTA-3’) and SuperScript III polymerase (Invitrogen). PCR amplification was then performed with rTH DNA polymerase XL (Applied Biosystems), using primer CB3-7381RMlu and an upstream primer incorporating an SbfI site (5’-GGAATTAACCTGCAGGTTAAAACAGCCTGTGGGTGGTTG-3’). The PCR program was as follows: 1 cycle, 94°C for 1 min; 35 cycles, 94°C for 15 sec followed by 68°C for 8 min; then 1 cycle, 72°C for 12 min. PCR products (approximately 7400 bp) were gel purified and digested with SbfI and MluI (New England Biolabs), then cloned into plasmid pSport1, downstream of the T7 promoter. Full length CB3-H3 cDNA was provided by Drs. Kirk Knowlton and Sally Huber (18). Specific mutations were introduced by splice overlap extension PCR (11).
**Generation of viruses from infectious viral cDNA clones.** For CB3-Nancy, CB3-RD, and their derivatives, viral cDNA was reverse transcribed using T7 polymerase (RiboMAX, Promega), viral RNA quality was assessed by gel electrophoresis, and virus was produced by transfection of RNA into HeLa cells using DMRIE-C (Invitrogen). Three to five days after transfection, virus was harvested, purified, and titered on HeLa cells as described previously (32). CB3-H3 and H3-VP2-138D were generated by transfection of HeLa cells with DNA, using Lipofectamine 2000 (Invitrogen), and virus was harvested four days later.

**Radiolabeled virus binding.** CB3-RD and CB3-Nancy were radiolabeled with $[^{35}\text{S}]$-methionine/cysteine as described elsewhere (5). $^{35}$S-labeled virus was bound to confluent cell monolayers in 24-well plates for 1 hr at room temperature. Unbound virus was removed with three washes with binding buffer, cells were lysed with Solvable detergent (PerkinElmer, Waltham, MA), and cell-bound radioactivity was assessed.

**Immunofluorescence staining of virus-infected cells.** For immunofluorescence assays, RD cell monolayers in 8 well Lab-TEK chamber slides (Nalge Nunc international) were infected with viruses (1 PFU/ cell); after 72 hours, the monolayers were fixed and stained with monoclonal anti-enterovirus VP1 antibody (NCL-ENTERO, Leica) and FITC-labeled secondary antibody as described (8).

**Adaptation to RD cells.** CB3-Nancy and CB3-H3, derived from molecular clones, were passed 5 times on HeLa cells to permit variants to accumulate. Virus was then passed 4 times on RD cells, and the consensus sequence determined by direct sequencing of RT-PCR products. To
isolate individual virus variants within the RD cell-adapted population, HeLa cell monolayers in 96 well plates were infected with virus at approximately 0.3 infectious virions per monolayer. Monolayers were observed for appearance of cytopathic changes, and then virus from individual wells was expanded and sequenced.
RESULTS

CB3-Nancy and CB3-RD differ in 6 amino acids in the P1 region. To identify the genetic changes that accompanied the adaptation of CB3-Nancy to growth in RD cells, we first determined the nucleotide sequences of the two virus isolates. HeLa cells were infected with CB3-Nancy and CB3-RD, both from low passage stocks provided by Dr Richard Crowell, and RNA was isolated during the first infection cycle. Viral sequences were reverse-transcribed and amplified with PCR primers spanning the entire viral genome. To obtain the consensus sequence for each virus— independent of errors introduced by PCR and sequence variation within the viral quasispecies— PCR products were sequenced directly, without cloning into plasmid vectors.

CB3-Nancy and CB3-RD were found to differ at 13 nucleotide positions (Table I). 2 nucleotide differences were found within the 5'-untranslated region (UTR), and 1 within the 3'-UTR. Within the protein coding region, 10 nucleotide differences were observed. These result in 6 predicted changes at the amino acid level, all within the P1 region that encodes the capsid proteins VP1-VP4. To generate infectious cDNA clones encoding CB3-Nancy and -RD, we amplified full-length cDNA copies of the two genomes, and inserted them into a plasmid vector, downstream of a T7 promoter. When full-length RNAs were transcribed in vitro, and transfected into HeLa cells, viruses were produced that maintained the phenotypes of the original virus stocks: cloned CB3-RD readily infected RD cells, as determined by a fluorescent focus assay, and radiolabeled CB3-RD bound to human DAF expressed on CHO-DAF cells; in contrast, cloned CB3-Nancy infected HeLa cells, but not RD cells, and showed no measurable binding to human DAF (Figures 2 and 3).

The sequences of the cloned CB3-Nancy and -RD showed 8 additional nucleotide differences that had not been observed in the consensus sequences (Table I); these may have
resulted from PCR errors, or they may reflect sequence diversity among the many individual viral genomes that arise during replication. With one exception, these nucleotide differences had no effect on the predicted protein sequence: in the CB3-RD cDNA clone, a guanine residue at position 4044 results in a single amino acid change within the non-structural protein 2C, which is unlikely to affect virus interaction with DAF.

**VP3-234Q is essential for CB3-RD attachment to DAF.** The consensus sequences revealed 6 amino acid differences between the capsid proteins of CB3-RD and CB3-Nancy. To determine which of these differences accounts for the different avidity of the two viruses for DAF, we introduced mutations into the CB3-RD molecular clone to replace each of the divergent RD amino acids with the corresponding Nancy residues (Figure 2A, constructs 1-10). Mutant viruses were generated by transfection of HeLa cells with viral RNAs transcribed in vitro, and each virus was radiolabeled by growth in $^{35}$S-cysteine and -methionine. All of the mutant viruses bound detectably to HeLa and CHO-CAR cells (Figure 2B); less than 0.1% of input virus bound to control CHO-pcDNA cells.

Mutation en bloc of the three divergent residues within VP3 (231I, 232T, and 234Q; construct 3) ablated virus binding to DAF expressed on CHO-DAF cells, but mutation of the divergent residues within VP2 (construct 1) or VP1 (construct 4) had no effect. Mutation of VP3-234Q ablated virus attachment to DAF (construct 7); in contrast, mutation of the other divergent VP3 residues, either singly or in combination (constructs 5, 6, and 8) had no effect. These results suggested that VP3-234Q is essential for CB3-RD attachment to DAF. Introduction of this single residue into CB3-Nancy (construct 11) permitted attachment to DAF, thus confirming its importance for DAF interaction.
VP3-234Q is essential for CB3-RD infection of RD cells. The tropism of CB3-RD for RD cells has been related to its avidity for DAF, although the relationship between DAF binding and RD cell infection has been questioned by some investigators (31). Each of the radiolabeled mutant viruses that had bound to CHO-DAF cells also bound to RD cells (Figure 3A); the lower levels of binding to RD cells most likely reflects the low expression of DAF on these cells (12). When RD cells were exposed to mutant viruses, infection by each of the DAF-binding viruses was detectable by immunofluorescence staining for VP1 (Figure 3B); infection was not detected with any of the viruses that did not bind DAF. Infection of RD cells by CB3-Nancy with VP3-234Q (construct 11) indicates that this residue, which is essential for attachment to DAF, is also a critical determinant of tropism for RD cells.

Passing cloned CB3-Nancy on RD cells reselects for VP3-234Q. CB3-RD was originally selected by passing CB3-Nancy on RD cells. To determine whether a mutation to VP3-234Q was the sole path by which CB3-Nancy could acquire avidity for DAF and tropism for RD cells, we passed CB3-Nancy, derived from the molecular clone, on RD cells. After 4 passages we obtained a new virus stock (which we called CB3-Nancy-RD) that readily infected RD cells (Figure 4A) and which also bound to DAF (Figure 4B). Direct sequencing of RT-PCR products spanning the capsid region revealed a single substitution (G to C) at nucleotide 2438, resulting in the same mutation of VP3-234 from E to Q that we had observed for the original CB3-RD isolate.

To determine whether less common mutations—not evident from the CB3-Nancy-RD consensus sequence—also permit adaptation of CB3-Nancy to RD cells, we isolated clonal virus
populations by limiting dilution from the CB3-Nancy-RD stock, and directly sequenced the region surrounding nt 2438. VP3-234Q was seen in 45 of the 47 cloned viruses examined; the 2 remaining clones did not bind to DAF and did not infect RD cells. Thus, mutation to VP3-234Q is the dominant— if not the sole— route by which CB3-Nancy acquires RD cell tropism and avidity for DAF.

**VP2-138D is also important for CB3 interaction with DAF.** We had previously observed (unpublished) that CB3-H3, a cardiovirulent virus isolate originally obtained from the heart of an infected mouse (14), did not bind measurably to DAF. The nucleotide sequence of CB3-H3 (18) reveals that this virus possesses the VP3-234Q residue important for CB3-RD attachment with DAF, consistent with the idea that other capsid residues are also essential for the interaction. We generated CB3-H3 from a molecular clone and passed it in RD cells to obtain a variant (CB3-H3-RD) that infected RD cells (Figure 5A) and bound to DAF (Figure 5B). Direct sequencing of the CB3-H3-RD capsid region revealed that the adapted virus had undergone a single amino acid change within VP2 (nt 1361, A to G; VP2-138 N to D).

Introduction of VP2-138D into cloned CB3-H3 (Fig. 5C) resulted in a virus with the capacity to bind DAF, confirming the importance of this residue for DAF interaction. Like CB3-H3-RD, both CB3-Nancy and -RD have a D residue at VP2-138. We replaced this residue in the RD clone with the VP2-138N observed in CB3-H3. The resulting virus showed a dramatic loss in its avidity for DAF (Figure 5C), indicating that VP2-138D, like VP3-234Q, is important for the interaction of CB3 with DAF.
Mutation of VP2-151S does not affect virus attachment to DAF. As discussed above, it had previously been reported that sequence differences at VP2-108 and VP2-151 accounted for the differences in RD cell tropism observed for CB3-Nancy and CB3-RD (20). In particular, replacement of VP2-151T with S was believed to be important for RD cell tropism; substitution of V for D at VP2-108, which is not exposed on the virus surface, was thought less likely to be involved. We introduced into the CB3-RD backbone mutations replacing VP2-151S with T, and VP2-108V with D, both together and singly. CB3-RD with VP2-151T bound to DAF as efficiently as did virus with VP2-151S (Figure 5D), indicating that VP2-151S is not required for virus interaction with DAF. No virus with VP2-108D was obtained, and we suspect that the capsid structure does not tolerate aspartate at this position.

VP3-234Q and VP2-138D are in close contact with DAF in the virus-receptor complex. The 3-D reconstruction of CB3-RD complexed with the extracellular domain of DAF, previously obtained by cryo-electron microscopy (10), has now been refined to 9Å resolution (J.D.Y, J.O.C, and S.H., in preparation). As shown in the new structure, the predominant interactions between virus and receptor occur between the "puff region," formed by residues in VP2, and the second short consensus repeat (SCR2) domain of DAF; the junction between SCRs 2 and 3 is also in close proximity to the virus surface, and several residues within capsid proteins VP1 and VP3 make additional contacts with DAF. Both VP3-234Q and VP2-138D make direct contacts with DAF, on opposite sides of the SCR2 domain (Figure 6). VP3-234Q is likely to interact with DAF residue Thr70, and VP2-138D with DAF residue Val89. The two interactions effectively anchor SCR2 to the virus surface.
DISCUSSION

Although all group B coxsackieviruses use CAR as a receptor for attachment and infection, isolates differ in their capacity to bind DAF. The results presented here demonstrate that single amino acid changes allow non-binding CB3 isolates to interact with DAF, and thus broaden their tropism. Mutation of VP3-234E to Q permitted CB3-Nancy to bind DAF and infect RD cells. With CB3-H3, in which the identity of VP3 residue 234 was already Q, an additional mutation of VP2-138N to D had a similar effect. Mutation of either residue in CB3-RD resulted in a loss of avidity for DAF, indicating that both are important for virus interaction with this receptor. Consistent with these results, both residues are located at the site of contact between the virus and DAF, as determined by the cryo-EM structure.

The observation that VP3-234Q and VP2-138D interact directly with SCR2 is consistent with earlier data indicating that SCR2—but not SCRs 1, 3, and 4—is indispensable for virus attachment (4). Although we identified two critical capsid residues, the new structure of the CB3-DAF complex shows at least 15 capsid residues in close proximity to SCR2, some of which are also likely to contribute to the avidity of the virus-receptor interaction. These residues, which are largely conserved among the virus isolates we examined here, may provide a surface that accommodates DAF in a way that is either stabilized by VP3-234Q and VP2-138D, or destabilized by VP3-234E and VP3-138N. Neither of the amino acid changes we identified is likely to perturb the overall conformation of the virus surface, but it is interesting that both involve charge changes. Upon examining the electrostatic surface of DAF, we find that VP2-138D points into a positively charged pocket (Figure 6 C); replacement of the negatively charged aspartate residue with asparagine may destabilize this interaction. The positively charged amide
moiety of VP3-234Q is in contact with a negatively charged patch on the SCR2 surface; replacement of the glutamine with a negatively charged glutamate may also have a destabilizing effect (Figure 6D).

Consistent with our conclusions, other investigators have recently suggested that VP3-234Q is important for CB3-RD interaction with DAF (although this was not tested by mutagenesis) (6). However, our results do not support the conclusion of Lindberg and colleagues (20) that VP2-151S and/or VP2-108V account for the tropism of CB3-RD for RD cells, and they do not suggest that these residues are important for interaction with DAF. These residues are present in both the CB3-Nancy and -RD isolates we studied and thus cannot be responsible for the differences in tropism we observed. We found that mutation of VP2-151S had no effect on virus interaction with DAF, and were unable to generate virus with VP2-108D. It is interesting that the viruses studied by Lindberg et al. (one with tropism for RD cells, and one without) both possessed the VP2-138D residue we found to be important for DAF interaction, and that— consistent with our observations— the RD-tropic virus had undergone a replacement of VP3-234E with Q, in addition to the changes at VP2-108 and VP2-151.

In addition to CB3-RD and CB3-H3-RD, a number of other CB3 isolates— CB3-PD and PD1 (28); CB3-HA (25); and CB3-Nancy/New (31)— have been shown to bind DAF. Although an isolate initially cloned by Kandolf and colleagues (15, 17) was reported to lack avidity for DAF (25), we (unpublished observations) and others (31) have found that this virus binds DAF quite well. Sequences of all the DAF-binding isolates show conservation of VP2-138D. VP3-234Q is conserved among all the isolates but Nancy/New, which has another uncharged residue, leucine, at this position.
The observation that single amino acid changes permit significant shifts in receptor tropism may have significance for understanding the pathogenesis of viral infection *in vivo*. As CB3 initiates infection in the GI tract, enters the bloodstream, and spreads to target organs, it is likely to encounter diverse cell types that vary greatly in expression and localization of CAR, DAF, and other potential receptor molecules. DAF-binding variants within a virus population may enjoy a selective growth advantage at some sites, such as the intestinal lumen, where DAF is readily accessible but CAR is not; we previously found that passage of CB3-Nancy on polarized intestinal epithelial cells selected for DAF-binding variants (33). At other sites, DAF-binding viruses may be at a disadvantage. Because of error-prone RNA replication, picornavirus exist as quasispecies, with a broad genomic diversity; experiments with poliovirus have demonstrated that virus populations experience multiple evolutionary bottlenecks during infection and spread through the host (19). Given the ease with which CB3 undergoes changes in DAF tropism during in vitro culture, it would not be surprising if similar changes occur as viruses encounter anatomic barriers in vivo.
ACKNOWLEDGEMENTS

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TABLE 1

Comparison of CB3-Nancy and CB3-RD nucleotide and amino acid sequences

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FIGURE LEGENDS

Figure 1. Amino acid sequence differences between CB3-Nancy and CB3-RD. Regions where the sequences of CB3-Nancy and -RD diverge are shown. Conserved amino acids are marked with hyphens. Sequences differences are in bold.

Figure 2. Mapping amino acid residues required for CB3-RD interaction with DAF. (A) Mutant constructs were made by overlap extension PCR from full-length cDNA clones encoding CB3-RD (in gray) and CB3-Nancy (in white). Mutations are shown with stars: black stars represent amino acid residues from CB3-RD; open stars indicate amino acid residues from CB3-Nancy. (B) CHO cells stably transfected with DAF (CHO-hDAF) and CAR (CHO-hCAR) were incubated with radiolabeled viruses at room temperature for 1 hr to measure binding to specific receptors. Data are presented as percentage of input virus bound to cells ± SD for triplicate samples. Mutants of CB3-RD in which VP3-234Q was replaced by 234E (constructs 2, 3, 7, 9, 10) lost the capacity to bind DAF. A mutant of CB3-Nancy in which VP3-234E was replaced by 234Q (construct 11) acquired the capacity to bind DAF.

Figure 3. Mapping amino acid residues required for interaction with RD cells. (A) Radiolabeled virus binding, as determined in preceding figures. (B) Infection. RD cell monolayers were incubated with viruses (10 PFU/cell) at 37°C for 72 hrs, then stained with anti-enterovirus VP1 mAb to measure virus infection. All DAF-binding virus strains infected RD cells, whereas non-DAF binding strains did not.
Figure 4. Adaptation of cloned CB3-Nancy to growth in RD cells selects for DAF binding. (A) Infection of RD cells by the readapted virus, Nancy-RD. (B) The readapted virus binds to DAF.

Figure 5. VP2-138D is also important for virus interaction with DAF. (A) Adaptation of CB3-H3 to grow in RD cells. RD cells were exposed to CH3-H3 or the adapted variant CB3-H3-RD, then stained at 72 hrs with anti-VP1 antibody to detect infection. (B) RD-adapted CB3-H3 binds to DAF. CHO cells expressing DAF or CAR, control CHO-pcDNA cells, and HeLa cells were incubated with radiolabeled viruses, and virus binding was measured as described in Materials and Methods. (C) In H3-VP2-138D, VP2-138D was introduced into cloned H3; this virus gained the capacity to bind DAF. In RD-VP2-138N, VP2-138D in the RD clone was replaced with VP2-138N; this virus lost the capacity to bind DAF. (D) Replacement of VP2-151S with T does not affect binding of CB3-RD to DAF.

Figure 6. Critical capsid residues are in contact with DAF. (A) Cryo-EM reconstruction of DAF bound to CVB3-RD at 9 Å resolution displayed at 1sigma. Density further than 160 Å from the center of the virus is shown in blue, an asymmetric unit is bounded by the black triangle. (B) Surface representation of CVB3-RD protomers surrounding one five-fold icosahedral axis of the virus with DAF shown in yellow ribbon. The DAF molecule attaches at the three-fold vertex by way of the C-terminal His-tag; SCRs 4 and 3 stretch across the virus surface of the red protomer (in standard orientation), crossing the canyon, and SCR2 interacts with the puff region of the blue (neighboring) protomer. SCR1 makes no contacts, rising above the virus surface. Residues VP2-138D and VP3-234Q are highlighted in green. (C) Close up view of residue VP2-138D (in
green, stick rendering) with DAF colored by electrostatic potential. The light blue coloring of DAF indicates an overall slightly positive charge in the region nearest to VP2-138D. (D) Close up view of residue VP3-234Q (in green, stick rendering with oxygen and nitrogen colored red and blue, respectively) with DAF surface-rendered and colored by electrostatic potential; the side-chain configuration shown is derived from the crystal structure of CB3-RD, and may differ in virus-DAF complex. The light red coloring of DAF indicates a negative charge in the region nearest to VP2-234Q.
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**VP2 1–50**
Nancy: SPTVEECGYSDRARSITLGNSTITTQECANVVGYGVPULLKDSEATAE
RD: ────────────────────────V─────────────────────────────

13

**VP2 121–170**
Nancy: CLLVCVPEAEMGCAIADNDTPSAGELGGDSAKEFADKPVASGSNLVQR
RD: ─────────────────────────────────V──────────────────────

144

**VP3 191–238**
Nancy: TCWYQTNIVVPADAQQSSCYIMCFVSACNDFSVRLLKODTFSTSQENFFQ
RD: ────I──Q────IT────────────────────────────────

234

**VP1 61–110**
Nancy: ESIENFLCRSACVFTEYENSGAKRYAEWTFRQAQQLRRKLEFFTYY
RD: ──────────────────────────────────────────────────