The Structure of Echovirus Type 12 Bound to a Two-domain Fragment of Its Cellular Attachment Protein Decay-accelerating Factor (CD 55)*

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Echovirus type 12 (EV12), an Enterovirus of the Picornaviridae family, uses the complement regulator decay-accelerating factor (DAF, CD55) as a cellular receptor. We have calculated a three-dimensional reconstruction of EV12 bound to a fragment of DAF consisting of short consensus repeat domains 3 and 4 from cryo-negatve stain electron microscopy data (EMD code 1057). This shows that, as for an earlier reconstruction of the related echovirus type 7 bound to DAF, attachment is not within the viral canyon but occurs close to the 2-fold symmetry axes. Despite this general similarity our reconstruction reveals a receptor interaction that is quite different from that observed for EV7. Fitting of the crystallographic co-ordinates for DAF34 and EV11 into the reconstruction shows a close agreement between the crystal structure of the receptor fragment and the density for the virus-bound receptor, allowing unambiguous positioning of the receptor with respect to the virion (PDB code 1UPN). Our finding that the mode of virus-receptor interaction in EV12 is distinct from that seen for EV7 raises interesting questions regarding the evolution and biological significance of the DAF binding phenotype in these viruses.

Echoviruses along with the coxsackieviruses and polioviruses comprise the Enterovirus genus within the Picornaviridae family. Echovirus infection is usually mild, although these viruses are sometimes associated with severe disease such as aseptic meningitis, encephalitis, hemorrhagic conjunctivitis, and myocarditis. The Picornaviridae family also includes a number of other important human and animal pathogens including rhinoviruses and foot and mouth disease virus.

Picornaviruses are small (~300 Å in diameter), non-enveloped icosahedral viruses that have a single-stranded positive-sense RNA genome of between 7.0 and 8.5 kilobases. The genome encodes a single polyprotein, which is co- and post-translationally processed by viral proteases to yield the capsid and non-structural proteins required for virus replication. The capsid assembles as a pseudo T = 3 icosahedral shell from four protein species, VP1-VP4. VP1–3 occupy the three quasi-equivalent positions in the icosahedral lattice, whereas VP4 is located at the inner surface of the capsid (1). The rhinoviruses and enteroviruses share a common and distinctive morphology consisting of a raised, star-shaped platform at the pentameric apices of the capsid. Surrounding this is a narrow cleft, termed the “canyon” (2).

For many picornaviruses (the polioviruses, the major receptor group rhinoviruses, Coxsackie A virus type 21, and Coxsackie B virus type 3) it has been shown that the interaction with their cellular receptors (all of which are members of the Ig-superfamily of proteins) occurs in the canyon (3–7). Binding of these viruses to soluble monomeric receptor protein in vitro triggers an irreversible conformational change in the virus particle that manifests as a change in the sedimentation characteristics of the virus (from the 160 S mature virion to the 135 S A-particle) (8). Similar changes in sedimentation coefficient are seen when the virus interacts with the receptor at the cell surface, and such transformations are thought to be essential for successful entry and infection (9, 10). The structural rearrangements that lead to this change in sedimentation include loss of VP4 and externalization of the N terminus of VP1, which is normally located at the inner surface of the capsid surrounding the pentameric apices in the mature virion. The extruded terminus of VP1, which is hydrophobic, becomes membrane-associated and is thought to form a pore through which the genome enters the cell cytoplasm (11).

Within the Rhinovirus and Enterovirus genera other types of receptor interactions have also been identified. The minor receptor group rhinoviruses, for example, bind the very low density lipoprotein receptor (12). This interaction does not lead to the irreversible conformational changes outlined above, and recent studies demonstrate that this receptor binds to the virion around the 5-fold symmetry axes, not in the canyon (13). The lack of virus uncoating upon receptor binding suggests the existence of another, as yet unknown receptor or co-factor that induces uncoating.

Many enteroviruses have been shown to bind decay-accelerating factor (DAF1, CD55), a member of the regulator of com-

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component activity protein family (14–18). DAF is a 70-kDa glycosylphosphatidylinositol anchored protein, present on the surface of the majority of serum-exposed cells, which functions to protect them from complement-mediated lysis by accelerating the decay of both the classical and alternative pathway C3 and C5 convertases. The regulator of complement activity protein family are defined by the presence of one or more short consensus repeat (SCR) domains, each of about 60 residues. DAF has four such domains, and these are linked to the C-terminal glycosylphosphatidylinositol anchor by a heavily O-glycosylated serine/threonine/proline-rich region (19). Completion control, Enterovirus binding, and other known interactions, including binding of the cellular ligand CD97 and bacterial adhesins, only involve the SCR domains (14, 20–23). Within the DAF binding enteroviruses there is a range of different interactions. Coxsackie A virus type 21 (CV-A21) and Enterovirus type 70 (ENV70) bind the membrane-distal domain SCR 1 (15, 24), whereas the DAF-binding echoviruses all interact with SCR 3, with additional binding to SCR 2 and/or SCR 4 (16). Enterovirus binding to soluble monomeric DAF does not, however, lead to the conformational changes normally associated with uncoupling that these viruses are observed to undergo at the cell surface (25). The status of DAF as a sole cellular receptor has, therefore, been subject to question. Although other molecules have been implicated in cell binding and entry by DAF binding enteroviruses (26–28), candidates that induce irreversible conformational changes in the virion have yet to be identified. Structural analysis of variants of echovirus type 11 that exhibit different cell tropism has, however, provided evidence for an as yet undiscovered canyon binding receptor while implicating a number of residues in the binding interaction with DAF both around the 5-fold symmetry axis and in the EF loop of VP2 (29). The recent analysis of DAF binding in echovirus type 7 by cryo-microscopy and image reconstruction has shown that DAF does not bind in the canyon; rather, it binds to the hyper-variable region of VP2 just outside of the “south” rim of the canyon and also to another hyper-variable region of VP3 (30). In related viruses these regions have been shown to be antigenic and capable of eliciting a neutralizing antibody response, suggesting that the paradigm of segregation of receptor binding regions from those parts of the capsid surface subject to immune surveillance is not strictly adhered to in the enteroviruses. Similarly, receptor binding to hyper-variable epitopes has been demonstrated for the minor group rhinoviruses and foot and mouth disease virus. In these viruses a strategy is employed whereby conserved receptor binding domains are embedded in hyper-variable regions capable of mutation to escape the host immune response (13, 31). In the EV7-receptor complex, bound DAF is located close to and lies across the 2-fold symmetry axes such that symmetry-related molecules are in steric collision (30). This complicates the interpretation of this reconstruction in terms of the known atomic structures for DAF (32–34). There are, however, substantial data regarding specific residues both on the virus capsid and in the SCR 3 and 4 domains of DAF that are involved in receptor binding in the enteroviruses (29, 32).

Echovirus type 12 (EV12) exhibits a distinct interaction with DAF, which predominantly involves SCR domains 3 and 4, a feature shared only with echovirus type 29 (16). A recent mutagenic analysis of DAF structure and function demonstrated that EV12 binding involves a different face of the receptor than that bound by other echoviruses (32). We have, therefore, conducted a structural investigation of the virus-receptor complex and present here a reconstruction of echovirus type 12 bound to SCR domains 3 and 4 of DAF, determined to 16-Å resolution by cryo-negative stain transmission electron microscopy and image reconstruction. Similarly to echovirus type 7, DAF binds to this virus outside the south rim of the canyon close to (but not over) the 2-fold symmetry axes. The distribution of density in our reconstruction is quite different, however, supporting the previous findings that there are at least three distinct virus-DAF interactions within the enteroviruses. By labeling the virus with only two domains of DAF we have been able to determine an unambiguous density for the receptor that is consistent with the crystallographic co-ordinates for DAF (34). Docking of these data into the reconcgneted protein structures shows that DAF binds to EV12 mainly via interactions between SCR 3 and VP2. The orientation determined for the DAF34 fragment allows us to superimpose the crystallographic data from a DAF fragment comprising all four SCR domains (DAF34) onto our model. We find that 60 copies of this protein may be modeled onto the surface of the EV12 capsid without any significant molecular clashes between symmetry-related molecules or the virion itself. This further highlights the differences between the complex seen here and that seen in the earlier investigation of the EV7-DAF interaction.

**Experimental Procedures**

**Virus and DAF34 Preparation**—Echovirus type 12 was routinely passaged in rhabdomyosarcoma cells. Ten 1750-cm² roller bottles (Corning Glass) of confluent rhabdomyosarcoma cells were infected with EV12 at high multiplicity (5 or higher), and the infection was allowed to proceed for 24 h at 37°C. Cell-associated virus was released by 2 cycles of freeze-thawing, and the resultant supernatant was clarified by low speed centrifugation (2000 × g for 10 min) and filtered (0.2 µm) to remove particulates. Sodium chloride was added to a final concentration of 2.3% (w/v) followed by 7% polyethylene glycol 8000, and the preparation was stirred at 4°C overnight. The precipitated virus was collected by centrifugation (5000 × g for 30 min) and resuspended in 10 ml of Dulbecco’s modified Eagle’s medium using an 18-gauge needle and syringe. Large particulate material was removed by low speed centrifugation, and the remaining virus was pelleted through a 30% sucrose cushion by centrifugation at 40,000 rpm for 6 h (TH641, Sorval) and resuspended in 1 ml of Dulbecco’s modified Eagle’s medium using an 18-gauge needle and syringe. The partially purified virus was subsequently layered onto the south rim of the canyon close to (but not over) the 2-fold symmetry axes.

**Electron Microscopy—**EV12 preparations were labeled with DAF34 by incubation overnight at 4°C. Unlabeled and labeled virus was then prepared for electron microscopy using the cryo-negative stain technique (36). In brief, a 5-µl droplet of virus or labeled virus was loaded onto a freshly glow-discharged Quantifoil holey carbon support film (Quantifoil Micro Tools GmbH, Jena, Germany) for 30 s. Grids were then transferred to a droplet of ~20% (w/v) ammonium molybdate solution (pH 7.4) for 10 s. Finally, grids were blotted and plunged into liquid nitrogen-cooled ethane slush.

**Vitrified virus preparations** were then imaged in a JEOL 1200 EX II transmission electron microscope equipped with an Oxford instruments cryo-transfer stage. Focal-pair images were recorded at a nominal magnification of 30,000× and defocus of between 300 and 2,000 nm on Kodak SO-163 film under low electron dose conditions.

**Image Reconstruction**—In total 14 focal pairs of unlabeled EV12 and 8 focal pairs of DAF34-labeled EV12 were selected for processing. Micrographs were digitized on a Duvanegan Hi-Scan drum scanner (Duvanegan SA, Lausanne, Switzerland) at a raster step size corresponding to 3.4 Å/pixel in the specimen. Particles were selected from each pair of micrographs using the program X3d, and deconvolution of the contrast transfer function was accomplished using the CTFMIX program, at which point the focal pairs were merged (37). Orientations and origins were determined for the unlabeled EV12 data set using the polar Fourier transform method (PFT) (38) using a starting model that was generated from the crystal structure of echovirus type 1 (39) by the EMAN PDB2MRC program (40). Subsequent iterations of three-dimensional reconstruction (41) and polar Fourier transform refinement led to

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the calculation of a 20-Å resolution reconstruction that was used to determine initial origins and orientations for the DAF34-labeled data set. In both cases origins and orientations were further refined until they ceased changing significantly. Final reconstructions were calculated to 14 Å, and in both cases all inverse eigenvalues were less than 0.01, indicating that the data adequately filled reciprocal space to this resolution. Final resolutions for each reconstruction were estimated by randomly dividing the data sets into two subsets and calculating independent reconstructions, for which a number of measures of agreement were calculated, including the Fourier-shell correlation and the spectral signal to noise ratio. Reconstructions were deposited in the EM data bank (accession numbers are EMD 1057 for the DAF34-labeled virus and EMD 1058 for the unlabeled virus).

Isosurfaces calculated from three-dimensional reconstructions were visualized in Iris Explorer (Numerical Algorithms Group, Oxford, UK) using a radial depth cueing module (42). To draw a direct comparison between our reconstructions and the previously determined model for EV7 bound to DAF34, a density map was calculated at 14-Å resolution using the EMAN PDB2MRC program from the deposited co-ordinates of He et al. (30) (PDB code 1M11).

**Fitting of the EV11 and DAF₃₄ Crystal Structures to the Cryo-negative Stain Reconstruction—**Visual inspection of an EV11 virus model (PDB code 1H8T) overlapped with the EV12-DAF₃₄ density map confirmed the correctness of the original hand assignment for the reconstruction. The reconstructed volume was then placed at the origin of a cubic cell with a cell edge a = 599.575 Å, oriented so that its 3- and 2-fold axes coincided with the symmetry axes through the origin of space group P2₃, and was rendered periodic with P2₃ symmetry. The x-ray model for a pentameric symmetric unit of EV11 was oriented and placed around the origin in the same cell. The overall correlation coefficients in real space between the experimental and model density was 0.76.

The x-ray model for DAF domains 3 and 4 (PDB code 1H03) with all B factors set to 95 was manually placed in the EM data bank (accession numbers are EMD 1057 for the DAF34-labeled virus and EMD 1058 for the unlabeled virus). The reconstructed density or incorporation of noise in the final representation of the reconstruction, brought about by the use of the cryo-negative stain technique, prevented the expansion of the virion density or incorporation of noise in the final representation of the volume. The apparent low occupancy in the virus-receptor complex is consistent with biochemical data and previously published reconstructions (16, 30, 48).

**EV12 Decorated with DAF₃₄ Shows a Different Receptor Orientation for EV Compared with EV7—**The reconstruction of labeled EV12 shows a distinct distribution of density compared with the previously published structures for EV7 bound to the three or four SCR domain fragments of DAF, DAF₃₄ and DAF₁₂₃₄ (30). In our reconstruction two symmetry-related DAF molecules are oriented such that they are in a position corresponding to the “ten past eight” setting of hands on a clock when viewed along the 2-fold symmetry axis (Fig. 2B). In the published reconstruction of the EV7-DAF complex the linear density most likely to correspond to the DAF fragment is in a “five to five” position when viewed along the same axes (Fig. 2D). The principle point of contact, however, is similar, located at a puff of density just outside the “southern” lip of the canyon, approximately equidistant between the 5- and 3-fold symmetry axes. Our data reveals only one point of contact between virus and receptor fragment, whereas for EV7 there appears to be three. In our analysis the use of a two-domain fragment and the different mode of receptor binding in EV12 have overcome the problems of interpretation associated with the orientation of the DAF molecule relative to the icosahedral symmetry of the virion experienced by He et al. (30). Inspection of the DAF₃₄-labeled EV12 reconstruction shows a clear region of density that comprises two domains.
fragments approach each other across a 2-fold symmetry axis without coming into steric collision with each other. We are, therefore, able to interpret the reconstructed density unambiguously in the context of the published crystallographic coordinates for SCR domains 3 and 4 of DAF (32).

Determination of a High Resolution Model of Receptor Binding for DAF Binding Echoviruses by Docking Crystallographic Coordinates into the Low Resolution Reconstruction—Because the structure of EV12 has not been determined at atomic resolution, we have fitted the known crystallographic coordinates for EV11 (PDB codes 1H8T (29)) into our reconstruction. EV11 should provide a good model for EV12 because these two viruses are 79% sequence identical in the capsid proteins. In fact we see that positioning of the EV11 coordinates in the EV12 density gives a close fit to the surface structure of the reconstruction, reflected in the fact that a map calculated at 16 Å from the x-ray structure contains density that correlates well with the experimental density observed in the reconstruction (real space correlation of 0.76 in the correct hand).

Initial manual positioning of the DAF34 fragment (PDB code 1H03 (32)) was found to give an optimal fit, as verified by calculation of the real space correlation coefficients between the density for the receptor and differently oriented copies of the atomic model (Fig. 3). Further rigid-body refinement of the SCR 4 component led to the final calculation of the fully fitted virus-receptor complex. A robust fit of crystallographic data has been calculated, allowing us to unambiguously determine that the SCR 3 domain lies closest to the icosahedral 2-fold symmetry axes and contains the largest proportion of the area of contact with the virion surface. We were also able to determine which face of the receptor provides the primary site of interaction (Fig. 4). The highest correlation for the optimally positioned receptor fragment was 0.79, with the occupancy of the receptor set to 0.15, confirming that only a small percentage of the potential receptor binding sites are occupied under these conditions. This is perhaps not surprising given that the affinity of DAF for EV11 (the only virus-DAF interaction which has been measured quantitatively) is known to be in the μM range (48). This is a relatively weak interaction by comparison to those virus-receptor interactions previously investigated by cryomicroscopy and image reconstruction, such as those of the
polioviruses and rhinoviruses, which have receptor affinities in the nanomolar range (49, 50). The high quality of fit for the DAF34 x-ray model in our reconstruction provides evidence of the relative rigidity of these two domains of the DAF molecule and contrasts strikingly with the flexibility observed in studies of a DAF34 domain pair by NMR (33).

Analysis of the atomic resolution virus-receptor complex, produced by fitting the x-ray models to the reconstruction, shows that residues in the capsid protein VP2 are mainly involved in DAF interactions (Fig. 5). In fact, despite the different receptor orientation, just under half of the capsid residues buried in the EV12-DAF complex were found to also contribute to binding of DAF in the EV7-DAF complex, although the part of the receptor contacted by any of these residues varies due to the different mode of DAF association (Refs. 16 and 32 and Fig. 5C). A single virus residue, VP2$_{161}$, contributes just more than 10% of the total surface area buried in the interaction and is clearly critical to this binding event. In terms of the key DAF residues buried in the complex it is noteworthy that Glu$_{334}$ in SCR 3 is among the three residues found to contribute more than 10% to the buried surface area, since our earlier mutational studies implied that this residue was critical for EV12 binding but not involved in binding EV11 (32). The other two residues contributing more than 10% of the buried surface area are Gln$_{139}$ (also in SCR 3) and Arg$_{212}$ (in SCR 4). All three of these residues lie in close proximity on the surface of DAF$_{34}$ close to the SCR 3/4 interface, and their participation in the binding site suggests that recognition of DAF by EV12 is likely to be highly dependent on the relative orientation of these two SCR modules. Further evidence supporting our model of this virus-receptor interaction includes the observation that EV12 binds a range of primate DAF proteins; of the 19 DAF residues buried in this EV12-DAF complex, 17 are conserved across all primate and human DAFs that are known to bind EV12. In fact the two variable residues within the virus footprint on the receptor contribute less than 5% to the total binding area. Other DAF binding echoviruses interact with less conserved domains of the receptor and generally do not bind a wide range of primate DAF proteins (51).

By superimposing SCRs 3 and 4 from the recently determined structure of all four DAF SCR domains (DAF$_{1234}$) (34) onto our docked DAF$_{34}$ model, we are able to produce a model for EV12 in complex with DAF$_{1234}$. In contrast to the EV7-DAF reconstruction this procedure produces a model in which the only atomic clashes between symmetry-related DAF molecules occur between the flexible loop that links strands 4b and 5a of SCR 3 (as defined in Williams et al. (32)) and the bulge that interrupts strand 2 in SCR 2 (Fig. 6). Because the SCR 3 loop is the only portion of the DAF structure seen to vary between different crystal structures and since a minor rearrangement of this loop would prevent any steric clashes between the two copies of DAF, we believe this superposition is likely to represent a reliable model for the interaction between DAF$_{1234}$ and EV12. The interaction seen between the 2-fold symmetry-related copies of DAF is intimate and is reminiscent of the head-to-tail interactions seen in crystals of DAF$_{1234}$ (34); the linkers between SCRs 2 and 3 appear to form a rough sheet across the 2-fold axis. In agreement with earlier studies (16, 32, 51) this model predicts that SCR 2 will be minimally (and SCR 1 not at all) involved in virus binding (Fig. 6B).

Implications for the Evolution of a DAF Binding Phenotype in the Enteroviruses—We have presented the first data that unambiguously delineates the nature of the interaction between an echovirus (type 12) and its receptor DAF. Our finding that this interaction is substantially different from that suggested by previous studies (both structural and mutational) has implications for the significance of the DAF binding phenotype. It has been shown that both EV7 and EV11 bind to DAF in a substantially different manner to EV12 and possibly to each other despite their conserved characteristic of interacting with the membrane-proximal SCR domains (16, 30). This is surprising given the high level of sequence identity between these viruses, particularly EV11 and -12. It is striking, however, that the 21 residues that contribute significantly to the binding site suggest that recognition of DAF by EV12 is likely to be highly dependent on the relative orientation of these two SCR modules. Further evidence supporting our model of this virus-receptor interaction includes the observation that EV12 binds a range of primate DAF proteins; of the 19 DAF residues buried in this EV12-DAF complex, 17 are conserved across all primate and human DAFs that are known to bind EV12. In fact the two variable residues within the virus footprint on the receptor contribute less than 5% to the total binding area. Other DAF binding echoviruses interact with less conserved domains of the receptor and generally do not bind a wide range of primate DAF proteins (51).

Several possible explanations might be postulated to explain the diversity of interactions that exist between the enteroviruses and DAF. It has been suggested that DAF binding could have evolved independently in these viruses rather than being present as an ancestral trait inherited through many genera-
FIG. 5. A comparison of the low resolution three-dimensional reconstruction of EV12-DAF₃₄ (A) and a space-filling representation of the EV12-DAF₃₄ complex (B), generated using the crystallographic co-ordinates for EV11 and DAF₃₄. Radial depth-cueing emphasizes the distance between atoms or regions of density and the center of the virion such that dark colors are close to the center and light colors are farther away. EV12 (and EV11) is colored in shades of blue, whereas DAF₃₄ is colored in green. A space-filling representation of the EV7-DAF₁₂₃₄ complex (30) (C) highlights the different orientation of DAF bound to these two viruses. The model deposited under PDB code 1M11 contains α-carbon atoms only; this view is therefore rendered with the atomic radii for each atom set to 3.5Å. EV7 is colored in shades of purple, and the receptor is in red. A close-up view of DAF₃₄ shown as in panel B but rotated 180° about a vertical axis exposes the residues buried in the virus-
rhinoviruses capable of infecting cells in an ICAM-1-independent manner, and the reversion of non-DAF binding EV11 mutants to a DAF binding phenotype (29, 52, 53). Although the evolution of a novel receptor binding site might seem intuitively improbable for a virus that has an already functional interaction with this receptor, consideration of the high mutation and recombination rates in these viruses as well as the numbers of virions produced in the course of an infection makes such events plausible. It, therefore, seems conceivable that selective pressure to retain the DAF-utilizing phenotype but alter the receptor binding site to evade immune surveillance might give rise to such alterations in this interaction. Clearly, regardless of the evolutionary route these viruses took to arrive at the situation we find today, where several viruses bind the receptor by very different mechanisms, the fact that this situation exists indicates that this phenotype is of biological significance and must confer considerable evolutionary advantages.

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