Structural and Functional Insights into the Interaction of Echoviruses and Decay-accelerating Factor

Many enteroviruses bind to the complement control protein decay-accelerating factor (DAF) to facilitate cell entry. We present here a structure for echovirus (EV) type 12 bound to DAF using cryo-negative stain transmission electron microscopy and three-dimensional image reconstruction to 16-Å resolution, which we interpreted using the atomic structures of EV11 and DAF. DAF binds to a hypervariable region of the capsid close to the 2-fold symmetry axes in an interaction that involves mostly the short consensus repeat 3 domain of DAF and the capsid protein VP2. A bulge in the density for the short consensus repeat 3 domain suggests that a loop at residues 174–180 rearranges to prevent steric collision between closely packed molecules at the 2-fold symmetry axes. Detailed analysis of receptor interactions between a variety of echoviruses and DAF using surface plasmon resonance and comparison of this structure (and our previous work; Bhella, D., Goodfellow, I. G., Roversi, P., Chaudhry, Y., Evans, D. J., and Lea, S. M. (2004) J. Biol. Chem. 279, 8325–8332) with reconstructions published for EV7 bound to DAF support major differences in receptor recognition among these viruses. However, comparison of the electron density for the two virus-receptor complexes (rather than comparisons of the pseudo-atomic models derived from fitting the coordinates into these densities) suggests that the dramatic differences in interaction affinities/specificities may arise from relatively subtle structural differences rather than from large-scale repositioning of the receptor with respect to the virus surface.

Echoviruses are small (~300 Å in diameter) non-enveloped isosahedral viruses classified in the Enterovirus genus of the Picornaviridae family. Echovirus infection is usually mild, although these viruses can cause severe diseases such as aseptic meningitis, encephalitis, and myocarditis. The ~7.5-kb positive sense RNA genome encodes a single polyprotein that is co- and post-translationally cleaved, yielding the structural proteins, enzymes, and additional proteins necessary for virus replication. Picornavirus capsids assemble from four structural proteins (VP1–4) as a pseudo T = 3 icosahedral shell with VP1–3 occupying the three quasi-equivalent positions in the icosahedral lattice and VP4 lying beneath the pentameric apex (1). Echoviruses have a distinctive morphology (common to many picornaviruses) consisting of a star-shaped mesa at the 5-fold symmetry axes surrounded by a deep cleft or “canon” (2, 3).

Substantial variations in receptor usage and cell entry mechanisms are found within the Picornaviridae family. The major receptor group rhinoviruses along with coxsackievirus type A21 use ICAM-1 (intracellular adhesion molecule 1), whereas the polioviruses bind to a protein of unknown function called the poliovirus receptor, and coxsackievirus type B3 binds to the coxsackie-adenovirus receptor. These receptors, which belong to the immunoglobulin-like family of proteins, bind to the capsid surface in the canyon (4–8). Receptor binding in solution induces conformational changes in the capsid akin to those that occur at the cell surface, resulting in loss of VP4, while the normally buried hydrophobic N terminus of VP1 is externalized (9–12). It is hypothesized that this forms a pore in the cell membrane through which the genome passes to enter the cytoplasm. Recent studies of poliovirus virions bound to poliovirus receptor molecules embedded in lipid vesicles indicate that this pore may form at the 5-fold symmetry axis, although it is not clear whether a specific vertex is required to facilitate genome egress (13).

Many enteroviruses bind to decay-accelerating factor (DAF2; CD55), a member of the regulator of complement activation protein family (14–18). DAF is a 70-kDa protein comprising four short consensus repeat (SCR) domains linked by an O-glycosylated serine-, threonine-, and proline-rich linker to a glycosylphosphatidylinositol anchor at the cell membrane. DAF is present on the surface of the majority of serum-exposed cells, where it protects them from complement-mediated lysis by accelerating the decay of the classical and alternative pathway C3 and C5 convertases (19). Unlike the Ig-like receptors, DAF does not induce uncoating in solution, although conformational changes in the virion do occur at the cell surface. This suggests that another molecule is recruited to induce uncoating or, alternatively, that DAF must be presented in the context of a cell membrane (20).

We have previously calculated a quasi-atomic resolution model of the echovirus (EV) type 12 receptor complex based on cryo-negative stain transmission electron microscopy and image reconstruction of EV12 bound to a fragment of DAF comprising SCR3 and SCR4 (DAF34) (EM Data Bank code 1057 and Protein Data Bank code 1UPN) (21). This model shows that EV12–DAF binding occurs predominantly between SCR3 and VP2 (residues 142–164). The interaction takes place close to the 2-fold symmetry axes rather than in the canyon, and the receptor

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The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure and a QuickTime movie of the quasi-atomic model.

The three-dimensional reconstructions have been deposited in the Electron Microscopy Data Bank with accession numbers 3182 and 1183.
Echovirus Type 12-Receptor Complex

fragment lies flat against the capsid surface. Comparison of our structure and model with those published for the EV7-DAF complex indicated major differences between these two closely related viruses (Protein Data Bank code 1M11) (22). Previous genetic and biochemical studies of DAF-binding enteroviruses have shown that they can be divided into two major classes: those that bind to SCR1 and those that bind to SCR3 with additional binding to SCR4 and sometimes SCR2 (15–17). Although EV7 and EV12 both bind primarily to SCR3, there is considerable variation in receptor affinity in this class of viruses, and mutagenesis data suggest that individual virus serotypes may interact with different faces of the receptor (23). To test our earlier structural hypotheses and to cast further light on the nature of receptor binding in the echoviruses, we have calculated a three-dimensional reconstruction of EV12 bound to a four-domain receptor fragment (DAF1234) and dissected virus-receptor interactions using a variety of receptor fragments and surface plasmon resonance (SPR).

EXPERIMENTAL PROCEDURES

Production of Virus and Receptor—EV12 was cultivated in rhabdomyosarcoma cells and purified as described previously (21). Soluble DAF comprising SCR1–4 for electron microscopy (EM) analysis was expressed and purified in Pichia pastoris as described previously (20, 24).

For Biacore analysis, DAF3,D A F23, and DAF34 were subcloned from the DAF1234 expression vector into the pQE-30 plasmid (Qiagen Inc.). All constructs were expressed as hexahistidine fusions in M15[pREP4] (Qiagen Inc.). These constructs, along with DAF1234, were purified from inclusion bodies and refolded using established SCR refolding protocols (25).

Electron Microscopy—Preparations of EV12 were labeled with DAF by incubation overnight at 4 °C and prepared for microscopy by cryo-negative staining. 5 µl of virus at a concentration of ~0.2 mg/ml was loaded onto a freshly glow-discharged QUANTIFOIL holey carbon support film (Quantifoil Micro Tools GmbH, Jena, Germany) for ~10 s. The grid was then transferred to a droplet of 20% (w/v) ammonium molybdate solution (pH 7.4) for ~10 s, blotted for 2–3 s, and plunged into a bath of liquid nitrogen-cooled ethane slush. Grids were stored under liquid nitrogen or imaged directly in the transmission electron microscope.

Prepared specimens were imaged in a Jeol 1200 EX II transmission electron microscope equipped with an Oxford Instruments CT3500 cryo-stage (Gatan, Inc., Oxford, UK) at an accelerating voltage of 120 kV. To facilitate correction of the microscope’s contrast transfer function, each field of view was imaged at two levels of defocus. Typically, the first micrograph was recorded between 500 and 1500 nm under focus and the second between 1500 and 2500 nm under focus. Defocus paired images were recorded under low electron dose conditions at a nominal magnification of ×30,000 on Kodak SO163 film.

Image Processing—Micrographs were digitized on a Nikon Super Coolscan 9000 ED CCD scanner at 4000-dpi resolution, corresponding to a raster step size of 2.18 Å in the specimen. Micrographs were converted to PIP format with the BSOFT image processing package (26). Particles were selected and cut out in X3D, and deconvolution of the contrast transfer function was performed using CTFMIX; at this point, defocus paired images of individual particles were merged (27). The orientations and origins of particle images were determined by a modified version of the polar Fourier transform method (PFT2) (28, 29) using an unlabeled low resolution reconstruction of EV12 (21) as the starting model. Successive iterations of polar Fourier transform refinement and three-dimensional reconstruction (EM3DR2) led to the calculation of final reconstructions for EV12-DAF234 and EV12 at 14 Å resolution. Resolution assessment was accomplished by randomly dividing the data set into equal subsets, which were used to generate two independent reconstructions. A number of measures of agreement between these reconstructions were calculated, including the Fourier shell correlation and the spectral signal-to-noise ratio. Reconstructions were visualized in UCSF Chimera using the EMAN radial depth cueing plug-in Isosurface Colorizer (30, 31).

Docking of Crystallographic Coordinates—The crystallographic coordinates for EV11 (2) and DAF1234 (32) were fitted to the EV12-DAF1234 reconstruction to construct a quasi-atomic resolution model of the virus-receptor complex. The reconstructed density for EV12-DAF1234 was converted to CCP4 format and placed at the origin of a unit cell in space group P21 with a cubic cell edge of 555.9 Å such that theicosahedral 2- and 3-fold symmetry axes coincided with the crystallographic symmetry axes. A pentameric asymmetric unit of the EV11 crystal structure was placed within the unit cell in the same orientation, confirming the hand assignment and scale of the EM density. The main chain atom correlation coefficient for the virus capsid was calculated as 55% between experimental and model densities.

A hybrid DAF structure was calculated to create an optimal fit for each SCR domain in the experimentally derived receptor density as follows. SCR3 and SCR4 were constrained to the previously determined positions. As no further points of contact were found between the virus and receptor, we assumed that the presence of SCR1 and SCR2 would not alter the fundamental interaction. Furthermore, merged densities from SCR2 and SCR3 in symmetry-related molecules across the 2-fold axes precluded further refinement of the previous fit. For SCR3 and SCR4, a real space correlation coefficient of 55.4% was calculated using a 14 Å tagged density map with B-factors set to 100.

To achieve an optimal fit for SCR2, 14 different models of DAF1234 (based on the seven different crystal structures deposited in the Protein Data Bank) were fitted to the existing model as well as 43 models derived from NMR data (32, 33). Isqab was used to superimpose SCR3 of each model on the capsid-docked SCR3 (34, 35). The real space main chain correlation coefficient for SCR2 was used as a scoring function to determine the best interdomain orientation. Fitting of SCR1 was accomplished in the same manner once a satisfactory fit for SCR2 was attained using crystallographic data only, as no NMR data exist for the SCR1-SCR2 linker region. Molecular models were visualized using PyMOL (DeLano Scientific, San Carlos, CA), MolScript (36), and Raster3D (37).

SPR Studies—All SPR measurements were performed on a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden) using HBS-EP (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20) as the running buffer. EV12, EV7, and EV6 were covalently coupled to the surface of a CM-5 sensor chip (Biacore AB) by primary amine coupling. After the activation step, 5–10 µl of virus (diluted to 50 µg/ml in 10 mM sodium formate (pH 3.0)) was injected repeatedly over a single flow channel until the signal was 3000–6000 response units above the (uncoupled) base line. It has been previously shown that low pH does not significantly alter the infectivity of the viruses studied (38). To prevent further coupling reactions, unreacted succinimide esters on the chip surface were displaced by saturating the flow cell with 1 M ethanolamine (pH 8.5). The immobilization procedure was repeated for two different viruses on each of the other two channels. To provide a mock-coupled (no virus) control channel, the fourth channel was inactivated immediately after the activation step.

Experimental runs were based on previous protocols for SPR studies of EV11-207 (38). After coupling the virus to the chip, the system was primed several times to ensure a stable base-line response before the
experiment began. To avoid mass transfer effects, all experiments were performed at a high flow rate (25–30 μl/min) using HBS-EP as the running buffer.

Binding of recombinant DAF<sub>1234</sub> was measured by sequentially injecting 30 μl of a 3/2-fold dilution series in HBS-EP from 8.25 μM (high to low concentration) and also from 30 μM. The injection was performed using the KINJECT command, with a dissociation time of 1000–1400 s, followed by two 20-μl 4 M NaCl wash steps. The experiment was then repeated using the same dilution series except from low to high concentration. This was to confirm that the equilibrium signal for a given ligand concentration did not change with time.

Binding of recombinant DAF<sub>12</sub>, DAF<sub>34</sub>, and DAF<sub>33</sub> was measured by sequentially injecting a 2/3-fold dilution series from 25 μM and from 100 μM (high to low concentration and then low to high concentration). Otherwise, the protocol was identical to the one used for DAF<sub>1234</sub>.

To analyze the equilibrium constant for the dissociation (K<sub>D</sub>) of the ligand virus, the corresponding FC4 mock response signal was aligned and subtracted from each measurement using BIAevaluation (Biacore AB). The average equilibrium response (R<sub>eq</sub>) was determined for each ligand concentration (<i>C</i>) and plotted on a graph of R<sub>eq</sub> (response units) versus <i>C</i> (molar). A nonlinear fit of the 1:1 Langmuir binding model to the data yielded an estimate for the equilibrium dissociation constant (K<sub>D</sub>).

RESULTS

Three-dimensional Reconstruction of EV12 Bound to DAF<sub>1234</sub>—42 defocus paired micrographs of unlabeled EV12 and 96 paired micrographs of DAF<sub>1234</sub>-labeled EV12 were selected for image processing on the basis of ice thickness, virion numbers, defocus, and astigmatism. 1796 unlabeled and 1742 labeled particle image pairs were selected and corrected for the effects of the microscope’s contrast transfer function. Several rounds of iterative orientation refinement and three-dimensional reconstruction were performed until stable orientations and origins were achieved for each data set. In total, 1501 images of unlabeled and 1339 images of labeled virions were used to calculate final reconstructions (Fig. 1). The resolution assessment for these reconstructions gave values of 14 and 16 Å, respectively (Electron Microscopy Data Bank accession numbers 1182 and 1183).

We have previously calculated a structure for EV12 bound to a two-domain fragment of DAF (DAF<sub>34</sub>) (21). This structure has clear regions of contiguous density, and two domains could be readily defined (Fig. 1B). The rod-shaped fragment was found to bind to the capsid surface close to the 2-fold symmetry axes, oriented such that it lay approximately equidistant between the 3- and 5-fold axes, pointing to two neighboring 2-fold symmetry axes. When viewed along the 2-fold axes, two DAF<sub>34</sub> molecules appear as hands on a clock in a “ten past eight” orientation. The crystallographic structure was readily docked into the reconstruction, unambiguously identifying the domain lying closest to the 2-fold axes and in contact with the capsid surface as SCR3. Our structure for EV12-DAF<sub>1234</sub> (Fig. 1C) is consistent with our previous reconstruction and model, containing density that overlaps with that previously found in the EV12-DAF<sub>34</sub> structure. Further density is present lying across the 2-fold symmetry axes, extending out of SCR3, in a “six o’clock” orientation. This density bends sharply away from the capsid surface, close to the 3-fold axis. As this globular region (which appears to consist of two distinct lobes) is farthest from SCR3 and SCR4 and is consistent with our previous quasi-atomic resolution model for EV12-DAF<sub>1234</sub>, we attribute it to SCR1. We consider that the larger region of density lying across the 2-fold axis most likely comprises SCR2 and the SCR2-proximal region of SCR3 from two symmetry-related molecules. SCR1 and SCR2 do not make additional contacts with the capsid surface; therefore, our previous description of contact residues involved in this interaction remains valid and unchanged.

Construction of a Quasi-atomic Model for EV12-DAF<sub>1234</sub>—Fitting SCR2—Starting from our previous model for EV12-DAF<sub>34</sub>, a hybrid EV12-DAF<sub>1234</sub> structure was constructed by successively fitting SCR2 and SCR1 into the reconstructed density for the virus-receptor complex (Fig. 2) (Protein Data Bank code 2C8I). SCR3 and SCR4 were constrained to their positions in our previous model, as we consider this fit to be robust, and there is no evidence at this resolution for a change in the orientation of the molecule. SCR3 from each of 14 different crystallographic (Fig. 2A) and 43 different NMR (Fig. 2B) models was overlaid on the capsid-docked SCR3, and the SCR2 fit was scored according to correlation with the EM density. Of the crystallographic models, chain B from Protein Data Bank deposition 1OK3 was found to have the SCR2-SCR3 interdomain angle giving the highest correlation with the EM density (55%) (Fig. 2C) (32). The 43 NMR-derived models for SCR2 and SCR3 (33) exhibit a large spread of interdomain angles that fan out parallel to the capsid surface. Only three chains showed any significant correlation with the EM density, and none of these models were com-

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**FIGURE 1.** Stereo views of surface-rendered three-dimensional reconstructions of unlabeled and DAF-labeled EV12. A, 14-Å resolution reconstruction of unlabeled EV12. B, reconstruction of EV12 bound to DAF<sub>12</sub> at 16-Å resolution showing clear density that we attribute to the two-domain receptor fragment; C, additional density seen in the 16-Å resolution reconstruction of EV12 bound to all four SCR domains of DAF.
combinable with the overall packing seen for all four domains in our reconstruction.

Resolving Steric Collisions across the 2-fold Symmetry Axes—Our previous model for EV12-DAF1234 predicted a minor steric collision between the SCR2-proximal region of SCR3 and SCR2 of symmetry-related receptor molecules (21). This collision is still found in the revised model presented here. The program “contact” (CCP4 (35)) was used to determine the extent of steric interference between SCR2 and SCR3 of the 2-fold symmetry-related partner (Fig. 2D). This revealed two significant clashes. The first (between Arg102 and Arg103 of the symmetry-related molecule) is easily resolved by rearrangement of the arginine side chains. The second is less straightforward to resolve. An overlap between a loop on SCR3 (residues 174–180) and a face of the symmetry partner SCR2 (composed of residues 95–98 and 75–77) was found. A steric clash at this position could lead to a rearrangement of the SCR3 loop as previously predicted (21). Simple manual torsion of this loop away from this contact places it within a bulge of density observed in the EM reconstruction that cannot be explained by other portions of the DAF structure as seen in any of the crystallographic or NMR models for FIGURE 2. Calculation of a quasi-atomic model for EV12-DAF.

A, variation in SCR2 orientation for the 14 crystal forms of DAF1234, with each model superimposed onto the capsid-docked SCR3. Only SCR2 is shown for each model. B, variation in SCR2 orientation for the 43 different NMR models. C, the optimal SCR2 position is from chain B of the x-ray structure of Protein Data Bank code 1OK3. D, points of contact on DAF1234 with the symmetry partner across the 2-fold axis. The green surface represents a steric clash between Arg102 and Arg103 and identical residues of the symmetry partner. This is resolved by side chain rearrangement. The blue surface is a van der Waals contact between Pro137 and Pro109 of the symmetry partner. The red and orange surfaces are an overlap between the main chain atoms of residues 174–180 of SCR3 (red) and a surface composed of residues 95–98 and 75–77 of the symmetry partner SCR2 (orange). This clash can be resolved only by a remodeling of loop 174–180. E, electron density of SCR1. The strong density at the center of each lobe is shown as a red mesh, whereas the lower contours are shown as a blue mesh. The major and minor lobes, as well as the position of SCR2, are highlighted. F, superposition of all 14 possible SCR1 orientations from the crystal structures. These orientations are consistent only with the minor lobe density. G, optimal “minor lobe” SCR1 model from the side. Also highlighted is the remodeled loop 174–180 on SCR3. H, complete DAF model based on a hybrid of the original DAF1234 fit (with the remodeled loop 174–180 on SCR3) and the two crystal structures that gave optimal SCR1 and SCR2 positions (green). Also shown in magenta is the alternative position for SCR1 proposed to explain the major lobe density. The symmetry partner DAF molecule is shown in red. I, radially depth-cued atomic model of the virus capsid (blue) decorated with 60 copies of the DAF1234 hybrid model (green).
FIGURE 3. DAF1234 interaction with EV12, EV7, and EV6. A–C, typical surface plasmon resonance data and $K_D$ fits for DAF1234 flowing over EV12, EV7, and EV6, respectively. In each case, the interaction sensorgrams for five different concentrations of DAF1234 are shown on the left, whereas the nonlinear fits of the equilibrium data to the 1:1 Langmuir binding model are shown on the right. The final values for the fitted parameters ($K_D$ and $R_{\text{max}}$) are also given. Residual plots and $\chi^2$ values are included to demonstrate the quality of the fit in each case. R.U., response units.
**Echovirus Type 12-Receptor Complex**

**TABLE I**

|                | EV12 | EV7 | EV11* | EV6 |
|----------------|------|-----|-------|-----|
| **DAF1234**    |      |     |       |     |
| $K_i \pm \Delta K_i$ ($\mu M$) | 0.7 ± 0.2 | 0.9 ± 0.2 | 3.4 ± 0.4 | 4 ± 2 |
| $\Delta G^\circ$ assoc (kJ/mol) | −34.8 ± 0.7 | −34.4 ± 0.6 | −31 | −30.8 ± 0.7 |
| No. of fits | 8 | 10 | 17 | 17 |
| Mean $\chi^2$ (S.D. in mean $\chi^2$) | 2.0 (2.0) | 3.3 (2.0) | 1.4 (1.1) | 1.4 (1.1) |

| **DAF23**      |      |     |       |     |
| $K_i \pm \Delta K_i$ ($\mu M$) | 8.1 ± 0.4 | 27 ± 1 | 9.3 ± 0.8 | ND |
| $\Delta G^\circ$ assoc (kJ/mol) | −29.0 ± 0.1 | −26.1 ± 0.1 | −29 | ND |
| No. of fits | 5 | 5 | ND | ND |
| Mean $\chi^2$ (S.D. in mean $\chi^2$) | 3.4 (1.1) | 2.1 (0.7) | ND | ND |

| **DAF3**       |      |     |       |     |
| $K_i \pm \Delta K_i$ ($\mu M$) | 16 ± 2 | ND | ND | ND |
| $\Delta G^\circ$ assoc (kJ/mol) | −27.3 ± 0.3 | ND | ND | ND |
| No. of fits | 6 | ND | ND | ND |
| Mean $\chi^2$ (S.D. in mean $\chi^2$) | 4.0 (0.9) | ND | ND | ND |

| **DAF34**      |      |     |       |     |
| $K_i \pm \Delta K_i$ ($\mu M$) | 1.2 ± 0.9 | 5.1 ± 0.3 | 19.2 ± 0.4 | ND |
| $\Delta G^\circ$ assoc (kJ/mol) | −33 ± 1 | −30.2 ± 0.2 | −27 | ND |
| No. of fits | 8 | 6 | ND | ND |
| Mean $\chi^2$ (S.D. in mean $\chi^2$) | 4.3 (0.8) | 2.4 (0.6) | ND | ND |

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* Values previously reported by Lea et al. (38) are shown for the sake of comparison with binding energies converted from kcal/mol to kJ/mol.

* Values are given as error-weighted means from independent fits of a 1:1 Langmuir binding model.

* Values are the standard molar Gibbs free energy change for association: $\Delta G^\circ_{\text{assoc}} = RT\ln(K_i/C^0)$, where $R = 8.314$ J/K/mol, $C^0 = 1 \mu M$ (standard state concentration), and $T = 298$ K.

* The means ± S.D. in $\chi^2$ are included to show that the fits are accurate and are consistently so:

$$
\chi^2 = \frac{\sum_{i=1}^{n} (r_i - \bar{r})^2}{n - \rho}
$$

where $r_i$ and $\bar{r}$ are the fitted and experimental values, respectively, at a given point; $n$ is the number of data points; and $\rho = 2$.

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DAF (Fig. 2G). The density observed in the EM reconstruction is therefore consistent with dual occupancy of symmetry-related receptor-binding sites, bringing DAF molecules into close proximity across the 2-fold axes and causing a subtle rearrangement of this flexible surface loop.

**Construction of a Quasi-atomic Resolution Model for EV12-DAF**

**Fitting SCR1**—The density for SCR1 is less easy to reconcile to the crystal structures for DAF, as it is significantly larger and comprises two clearly defined lobes that we termed the major and minor lobes (Fig. 2E). Overlaying the SCR2 component of each of the 14 crystallographic models for DAF onto the docked SCR2 in our developing model places SCR1 in close proximity to the minor lobe. The optimal fit for SCR1 was given by DAF chain B from Protein Data Bank deposition code 1O/7Y, with a correlation coefficient of 58%. This model places the hydrophobic core of SCR1 at the center of the minor lobe density (Fig. 2F).

We interpret this density as implying flexibility of the SCR1-SCR2 interface, leading to two possible orientations for SCR1. The two possible orientations may be due to an inherent flexibility in this region of the molecule or, alternatively, to conformational changes induced by the close packing of the receptor onto the virus surface. Applying a small tilt to the SCR1-SCR2 linker brings SCR1 into register with the major lobe density and allows construction of a model (with two orientations for SCR1) that fully explains the density seen (Fig. 2, G–I).

**Comparison of DAF Binding by Echoviruses Using SPR**—To compare in detail the interactions between DAF and several closely related echoviruses, we used SPR as previously used to dissect the interaction between EV11-207 and DAF (38). Inspection of the control-corrected sensograms (Fig. 3) reveals that, as was the case for EV11-207, the DAF interaction with both EV7 and EV12 is characterized by very rapid on- and off-rates. Binding reached equilibrium within 10 s of the start of the injection, and dissociation was complete within 30 s of the end. Identical injection sensograms were obtained for equivalent measurements in the high-to-low and low-to-high concentration series, indicating that the chip did not deteriorate significantly during the experiment and that DAF1234 was successfully washed from the surface between injections. Dissociation constants for the interaction with DAF1234 have been calculated for EV12, EV7, and EV6, and these values are summarized in Table 1 together with the previously measured values for EV11 (38). For both EV7 and EV12, the DAF1234 affinities are the same within the experimental errors. However, EV6 has a lower DAF1234 affinity. In terms of $\Delta G^\circ$, this corresponds to an 11% difference in binding energy. The measured $K_D$ for EV6 is also identical (within experimental error) to the published value of 3.4 ± 0.4 μM for EV11 (38).

To further dissect the interactions, we sought to use receptor fragments (DAF23, DAF3, and DAF34) to aid in the understanding of which portions of the receptor are recognized by the different viruses. For DAF3, significantly higher receptor concentrations were required to observe a clear binding signal, consistent with a lower overall affinity for DAF3 compared with intact DAF1234. The on- and off-rates were very rapid; equilibrium was reached within 5 s of injection, and dissociation was complete within 10 s of the end of the injection. Sequential injections of 8.25 μM DAF1234 and 67 μM DAF3 over the same chip showed a surprising difference between EV7 and EV12 receptor recognition (Fig. 4, A and B). For the DAF1234 injection, the EV7 equilibrium response was much higher than the response seen on the EV12 channel, whereas for the DAF3 injection, the converse was true. Although the absolute magnitude of the signal cannot be trivially interpreted, this switching (in which the virus channel gives the largest response) demonstrates that EV2 interacts much more tightly with DAF3 compared with EV7. This is despite the very similar affinities of both viruses for the full construct. Both virus channels showed a dose-dependent signal, but for the range of DAF3 concentrations studied (67 to 7 μM), only EV12 yielded good
fits for a 1:1 binding model (Table 1). As Fig. 4C and Table 1 show, despite the nearly 6-fold difference in dissociation constants observed in the interaction of the viruses and DAF1234, DAF3 contributes >50% of the binding energy in all cases (range of 63–81%). The EV7-DAF interaction is somewhat different in character from the EV11 and EV12-DAF interactions, being significantly more dependent on DAF2 and DAF4. (37% of the binding energy derives from these two domains, cf. 21 and 19% for EV12 and EV11, respectively.)

FIGURE 4. DAF3 interactions with EV12 and EV7. A, control channel-subtracted SPR sensorgrams for DAF3 flowing simultaneously over EV12 (blue trace) and EV7 (red trace). B, control channel-subtracted SPR sensorgrams for DAF1234 flowing simultaneously over the same flow channels as in A. This apparent “switching” (in which the virus gives the largest response) indicates that the two viruses interacted with DAF3 very differently. R.U., response units. C, comparison of the affinity (K_D) of each virus (EV12, EV7, EV11, and EV6) for DAF1234 (left) and comparison of the affinity (Gibbs free energy) of each virus for individual SCR domains (right). Despite the significantly different K_D values for the various viruses, EV12, EV7, and EV11 all interacted predominately with SCR3.

DISCUSSION

Flexibility in the DAF Molecule—The role of flexibility in the function of multidomain molecules such as DAF has been much debated and is a difficult issue to resolve because the major structural techniques tend to yield differing results, with NMR emphasizing flexibility and x-ray crystallography giving a more static view. Although at lower resolution than either of these techniques, the reconstruction presented here provides a functionally important view of DAF in complex with a natural ligand and it is therefore worth noting that the domain arrangement seen here is trivially interpreted using the crystallographic structure for DAF in isolation. This seems to support the earlier indirect data (32) suggesting that the domain arrangement seen in the isolated structure is a good representation of a functional form of this molecule. It also supports the idea that, despite the small interaction surfaces between SCR domains, these domains may be arranged to generate a relatively rigid molecule. However, the density clearly provides evidence for limited flexibility of the SCR1-SCR2 linker, leading to the two orientations for SCR1.

Structural Views of Receptor Recognition by the Echoviruses—We have previously compared our work with that of He et al. (22), in which three-dimensional reconstructions were calculated for EV7 bound to two fragments of DAF: DAF34 and DAF1234 (21). Clear differences were observed between EV12-DAF34 and EV7-DAF; the majority of the receptor density in the EV7-DAF234 structure appeared rod-shaped and lay across the 2-fold symmetry axes in a “five-to-five” orientation. He et al. have proposed an atomic resolution model for EV7-DAF based on fitting

FIGURE 5. Comparison of the preliminary reconstruction of EV12 DAF1234 and the proposed atomic model for EV7-DAF1234. A and B, model for EV7-DAF1234 proposed by He et al. (22), in which DAF occupies one of two mutually exclusive orientations across the icosahedral 2-fold symmetry axes. C, low resolution preliminary reconstruction of EV12-DAF1234 showing attenuated density for SCR3 (asterisk) and fusion of SCR4 from 5-fold symmetry-related DAF molecules with SCR2 and SCR3, close to the 2-fold symmetry axes (arrow).
Echovirus Type 12-Receptor Complex

experiments, and it has been deposited in the Protein Data Bank (code 1M11). In this model, sterically collision between symmetry-related molecules at the icosahedral 2-fold axes prevents full occupancy such that DAF can occupy one of two alternative orientations, occluding the symmetry-related receptor-binding site (Fig. 5). The striking differences between our structure for EV12-DAF34 and the model of He et al. led us to propose major differences in the receptor binding properties of EV12 and EV7, a hypothesis supported by mutagenesis data and evidence for variation in virus binding to primate DAF molecules (23, 39).

We have presented a structure for EV12 in complex with all four SCR domains of DAF and a revised quasi-atomic resolution model, confirming the validity of our previous model and suggesting that structural rearrangements allow two receptor molecules to pack tightly together at the 2-fold symmetry axes.

Preliminary reconstructions calculated in the course of this work highlighted unexpected similarities between the densities we have calculated and those published for EV7-DAF1234 (22). The resolution of our preliminary reconstruction is worse than 30 Å and under these circumstances, density from SCR4 of a 5-fold symmetry-related DAF molecule merges with SCR2 close to the 2-fold symmetry axes. Interpretation of this reconstruction is further complicated by the apparent attenuation of SCR3. These similarities led us to additional close inspection of the published reconstructions for EV7-DAF1234 and EV7-DAF34 (22). We now suggest that the observed receptor density seen in the EV7-DAF34 reconstruction may be better accounted for by a DAF orientation similar to that proposed in our model for EV12-DAF1234.

Plasticity in Virus Receptor-binding Sites—Although we now propose a gross similarity between EV7 and EV12 in their receptor binding modes, major differences remain to be explained. Our SPR studies suggest that EV11 and EV12 share very similar interactions with DAF, primarily contacting DAF3 (with a minor contribution from DAF2), whereas EV7 must have significant contacts with all three C-terminal SCR domains. The hypothesis that these viruses contact DAF in different ways is supported by our earlier mutagenesis data demonstrating that mutation of Glu134 of DAF abolishes binding to EV12 while leaving the EV7-DAF interaction unaffected (23).

In addition to the subtle but clear differences between echovirus-DAF interactions described here, there are also clear differences in DAF binding properties between different enteroviruses. The recently determined structure for coxsackievirus type B3 bound to DAF reveals that, in this virus, the DAF-binding site is grossly located in a region of the protein that can tolerate extensive mutation, a phenomenon previously observed in foot and mouth disease virus (40). The mutable nature of enterovirus DAF-binding sites may also account for the observed variation in DAF binding properties. Plasticity of receptor binding interactions has been documented in influenza virus. Receptor binding is accomplished by the hemagglutinin glycoprotein in influenza virus, which binds to sialic acid moieties on host cell-surface glycoproteins. Hemagglutinin molecules from different influenza virus subtypes have been crystallized with small molecule human receptor analogs, revealing substantial differences in the mode by which these proteins bind (41). These studies illustrate how the selective pressure of the host immune response combined with an advantageous receptor tropism results in surprising differences in receptor binding for closely related RNA viruses. Further studies should reveal any common motifs conserved between different binding sites, leading to the description of a potential target for antiviral therapies.

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