The Crystal Structure of Coxsackievirus A21 and Its Interaction with ICAM-1

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Summary
CVA21 and polioviruses both belong to the Enterovirus genus in the family of Picornaviridae, whereas rhinoviruses form a distinct picornavirus genus. Nevertheless, CVA21 and the major group of human rhinoviruses recognize intercellular adhesion molecule-1 (ICAM-1) as their cellular receptor, whereas polioviruses use poliovirus receptor. The crystal structure of CVA21 has been determined to 3.2 Å resolution. Its structure has greater similarity to poliovirus structures than to other known picornavirus structures. Cryo-electron microscopy (cryo-EM) was used to determine an 8.0 Å resolution structure of CVA21 complexed with an ICAM-1 variant, ICAM-1K1355. The cryo-EM map was fitted with the crystal structures of ICAM-1 and CVA21. Significant differences in the structure of CVA21 with respect to the poliovirus structures account for the inability of ICAM-1 to bind polioviruses. The interface between CVA21 and ICAM-1 has shape and electrostatic complementarity with many residues being conserved among those CVAs that bind ICAM-1.

Introduction
Coxsackieviruses belong to the family of Picornaviridae, characterized by being small, naked, icosahedral animal viruses with a positive-sense, single-stranded RNA genome (Semler and Wimmer, 2002). The three-dimensional structures of various picornaviruses have been determined to near atomic resolution by X-ray crystallography (Rossmann, 2002). Examples include the major group human rhinovirus (HRV) serotypes 14 (Rossmann et al., 1985) and 16 (Hadfield et al., 1997); the minor group human rhinovirus serotypes 1A (Kim et al., 1989) and 2 (Verdaguer et al., 2000); human poliovirus (PV) serotypes 1 (Hogle et al., 1985), 2 (Lentz et al., 1997), and 3 (Filman et al., 1989); and coxsackievirus serotypes B3 (Muckelbauer et al., 1995) and A9 (Hendry et al., 1999). The external diameter of the viral particles is approximately 310 Å. Packed inside the virion is the approximately 7400 base pair long RNA genome with a small peptide, VPg, covalently linked to the 5′ end. These viruses are assembled from 60 protomers, each composed of one copy of the viral proteins VP1–VP4. The major, external capsid proteins VP1, VP2, and VP3 are arranged in a pseudo T = 3 icosahedral lattice with each protein having a “jelly-roll” structure, consisting of two antiparallel BIDG and CHEF opposing sheets, in which individual β strands are named from A to l along the polypeptide chain. VP4, together with the amino termini of VP1, VP2, and VP3, is situated internally, contributing to the protein/RNA interface.

The Picornaviridae family has been divided into nine genera: Enterovirus, Rhinovirus, Cardioivirus, Aphthovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus, and Teschovirus. Coxsackieviruses and polioviruses belong to the Enterovirus genus, whereas human rhinoviruses belong to the Rhinovirus genus (Stanway et al., 2002). Based on the different pathogenicity and histopathological lesions observed in newborn mice, coxsackieviruses were initially divided into two groups. Group A contains 23 serotypes, and Group B contains 6 serotypes. Group A coxsackieviruses (CVA) induce flaccid paralysis and mainly affect skeletal muscle. Group B coxsackieviruses (CVB) cause spastic paralysis and pathological changes in several tissues (Hyypiä et al., 1993). Later, based on sequence homology, coxsackieviruses were reclassified into three human enterovirus (HEV) clusters, HEV-A, HEV-B, and HEV-C (Stanway et al., 2002), in which CVA21, polioviruses, and another ten CVAs belong to the HEV-C cluster. CVA21 (Figure 1) is closely related to polioviruses, with 56.4%, 72.4%, 74.2%, and 75.4% amino acid identity in VP1, VP2, VP3, and VP4 for PV1, respectively. For nonstructural proteins, such as protease 3C and polymerase 3D, CVA21 has more than 94% amino acid identity. Similar amino acid identity is seen when comparing CVA21 with other polioviruses. The overall genome homology between CVA21 and PVs is between 81.4% and 82.3% (Hughes et al., 1989).

Picornavirus infection is initiated by the attachment of the virus to cell surface receptors. Molecular and structural studies have identified diverse classes of receptors (Rieder and Wimmer, 2002). CVA21, as well as the major group HRVs, use intercellular adhesion molecule-1 (ICAM-1, CD54) as their cellular receptor (Olson et al., 1993; Shafren et al., 1997a; Staunton et al., 1990; Xiao et al., 2001) and cause common cold-like respiratory infections. The function of ICAM-1 is as a cell surface glycoprotein that facilitates cell adhesion between leukocytes and endothelium by binding to lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) (Marlin and Springer, 1987) and the plasma protein fibrinogen (Languino et al., 1993). Transgenic mice expressing human ICAM-1 develop classic paralytic poliomyelitis after being infected with CVA21, indicating that CVA21 may share PVs’ poliomyelitis potential (Dufresne and
Figure 1. Structure of CVA21 and Its Differences to PV1

(A) Stereo view of a surface-shaded model of CVA21 calculated to 3.2 Å resolution showing the depressed canyon around each 5-fold axis. The radial distance of the surface is indicated by color, from blue (~120 Å) to red (165 Å).

(B) Stereo view of a ribbon diagram of one CVA21 icosahedral asymmetric unit. VP1, VP2, VP3, and VP4 are colored blue, green, red, and cyan, respectively. One icosahedral asymmetric unit is outlined in both this panel and in (A) by a black triangle with 5-fold, 3-fold, and 2-fold symmetry symbols at the corners and along the base of the triangle.

(C) Plot of Cα atom distance between PV1 and CVA21 versus the residue number. The higher peaks are labeled with their corresponding secondary structure.

(D) Stereo diagrams of the backbone Cα trace of CVA21 viral proteins (red) superimposed with those of PV1 (blue).
Table 1. Data Collection and Structure Refinement of CVA21 Crystals

<table>
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<tr>
<th>pH 5.6</th>
<th>pH 6.4</th>
<th>pH 7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam wavelength (Å)</td>
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<td>1.000</td>
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<td>Detector</td>
<td>ADSC Quantum4</td>
<td>ADSC Quantum4</td>
</tr>
<tr>
<td>Crystal-to-CCD distance (mm)</td>
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<td>250</td>
</tr>
<tr>
<td>Oscillation angle (°)</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Mosaicity (°)</td>
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<td>0.3</td>
</tr>
<tr>
<td>Number of crystals (number of frames)</td>
<td>1 (300)</td>
<td>2 (230)</td>
</tr>
<tr>
<td>Exposure time per frame (s)</td>
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<td>40–45</td>
</tr>
<tr>
<td>Space group (cell dimensions, Å)</td>
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<td>P4₃₂ (a = 348.0)</td>
</tr>
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<td>1,815,084</td>
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<tr>
<td>Number of unique reflections</td>
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</tr>
<tr>
<td>Rejection criterion (I/σ(I))</td>
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<td>3.0</td>
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<tr>
<td>Completeness (%)a</td>
<td>97.4 (87.0)</td>
<td>97.1 (88.9)</td>
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<tr>
<td>Redundancya</td>
<td>11.9 (6.0)</td>
<td>17.0 (11.0)</td>
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<tr>
<td>Rmerge (%)a</td>
<td>9.2 (24.3)</td>
<td>11.9 (23.8)</td>
</tr>
</tbody>
</table>

Average and Phase Extension

Final correlation coefficienta | 0.95 (0.38) | 0.96 (0.68) | 0.96 (0.66) |

Refinement

Final Statistics

| Protein model | Atoms | 6658 |
| Residues | 858 |
| Non-protein atoms | Solvent (waters) | 53 |
| Nucleotide | 23 |
| Myristoyl group | 15 |
| Pocket factor | 16 |
| Ion | 1 |
| RMS deviations from ideality | Bond length (Å) | 0.009 |
| Bond angles (°) | 1.45 |

*Values in parentheses are for the highest-resolution shell (3.31–3.20 Å).

Gromeier, 2004). These data further support the close relationship between PVs and CVA21.

CVA21 can also bind to decay-accelerating factor (DAF, CD55) (Newcombe et al., 2003; Shafren et al., 1997b), a complement regulatory protein. DAF contains four extracellular, short consensus repeats and is anchored by glycosylphosphatidylinositol on the cell surface. DAF is also a cellular receptor for many human enteroviruses (Bergelson et al., 1994, 1995; Bhella et al., 2004; He et al., 2002; Karnauchow et al., 1996). Binding of DAF does not trigger conformational change of the CVA21 particles (Shafren, 1998), nor is it sufficient to produce an infection, requiring ICAM-1 for the cell entry (Shafren et al., 1997b). The major role of DAF may be to capture and concentrate infectious virions on the cell membrane (Newcombe et al., 2004). CVA21 is perhaps the only known virus that can bind to both DAF and ICAM-1. Because both of these cell surface molecules are overexpressed on malignant melanoma cells, CVA21 has been used to selectively lyse tumor cells both in vitro and in vivo (Shafren et al., 2004).

Notwithstanding the high sequence similarity between polioviruses and CVA21, polioviruses use poliovirus receptor (PVR, CD155) instead of ICAM-1 or DAF as their cellular receptor (Belnap et al., 2000b; He et al., 2000; Xing et al., 2000). Although both ICAM-1 and PVR bind into the “canyons” surrounding each icosahedral 5-fold vertex, the orientation of these long molecules is very different (Xiao et al., 2001). Binding of ICAM-1 or PVR to the corresponding virus surface triggers conformational changes (Arita et al., 1998; Shafren et al., 1997a) that release a “pocket factor,” causing destabilization of the virus and release of the genome (Rossmann, 1994). The pocket factor is an unidentified fatty acid-like molecule situated in a hydrophobic pocket underneath the floor of the canyon within VP1 (Filman et al., 1989; Hendry et al., 1999; Rossmann, 1994). Hydrophobic compounds can replace the pocket factor and inhibit the disassembly of virions (Smith et al., 1986).

A natural variant of ICAM-1, ICAM-1Kan, was first found within certain African populations in which malaria is endemic (Fernandez-Reyes et al., 1997). ICAM-1 is used by the malarial parasite Plasmodium falciparum-infected erythrocytes as a receptor to attach to other cells. The only amino acid sequence dif-
Figure 2. Stereo Diagrams of Densities in Selected Regions of CVA21

(A) One putative guanine nucleotide stacks against Trp2038.
(B) Pocket factor (yellow).
(C) Myristylation (yellow) of VP4 N termini.
(D) A possible calcium atom (yellow) at the N-terminal region of VP1.

Difference between ICAM-1 and ICAM-1\textsuperscript{Kilifi} is Lys29, which is a Met in ICAM-1\textsuperscript{Kilifi}. Although individuals that are homozygous for the Met29 allele have increased susceptibility to cerebral malaria, they also have a reduced immune responsiveness because ICAM-1\textsuperscript{Kilifi} reduces its avidity to LFA-1 and loses its binding ability to fibrinogen. These observations imply a possible balance in selection between pathogen infection and inflammatory responses (Craig et al., 2000). In vitro ELISA experiments and cryo-electron microscopy (cryo-EM) reconstructions have shown that ICAM-1\textsuperscript{Kilifi}, unlike normal ICAM-1, is unable to bind to HRV16. However, both variants of ICAM-1 can bind to HRV14 with different affinity (Xiao et al., 2004).

ICAM-1 has five extracellular immunoglobulin-like superfamily (IgSF) domains, a transmembrane domain, and a short carboxyl-terminal cytoplasmic tail. The five extracellular IgSF domains form a long rod-shaped molecule with a length of approximately 190 Å (Staunton et al., 1990). The structures of N-terminal domains D1 and D2 (Bella et al., 1998; Casasnovas et al., 1998; Shimaoka et al., 2003) and of domains D3, D4, and D5...
Figure 3. Cryo-EM Map of CVA21/ICAM-1 Complexes

Densities corresponding to CVA21, ICAM-1\textsuperscript{KilifiFc}, and ICAM-1 are colored purple, blue, and green, respectively.

(A) Stereo view of CVA21 complexed with ICAM-1\textsuperscript{KilifiFc} calculated to 26 Å.

(B) Stereo view of CVA21 complexed with ICAM-1.

(C) Stereo diagram of ICAM-1\textsuperscript{KilifiFc} density (blue) around one 2-fold axis superimposed with ICAM-1 density (green). Two ICAM-1\textsuperscript{Kilifi} molecules are bent closer to the 2-fold axis than are those in ICAM-1. C\textalpha trace of fitted ICAM-1\textsuperscript{Kilifi} (red) was superimposed with the original X-ray structures (yellow) without changing the elbow angles.

(D) One-eighth of the complex cryo-EM map calculated to 26 Å resolution at 0σ (standard deviation of the map) above the average value showing a layer of low height densities corresponding to the domain D4, D5, and Fc parts of ICAM-1\textsuperscript{KilifiFc}.

(E–H) Cryo-EM densities around one 2-fold axis showing one pair of ICAM-1\textsuperscript{KilifiFc} and part of the virus surface at 26, 16, 11, and 9 Å respectively. The left and right panels show the densities at 0.25 and 1.0σ, respectively, above the average density height.
Table 2. Fitting X-Ray Structures into Cryo-EM Density

<table>
<thead>
<tr>
<th>Structure</th>
<th>Residue Range</th>
<th>Sumf b</th>
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<tbody>
<tr>
<td>ICAM-1Kilifi domain D1</td>
<td>1–83</td>
<td>1.31 (1.36) c</td>
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<tr>
<td>ICAM-1 domain D2</td>
<td>84–185</td>
<td>0.88</td>
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<td>ICAM-1 domain D3</td>
<td>186–281</td>
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<td>ICAM-1 domain D4</td>
<td>282–366</td>
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</tr>
<tr>
<td>ICAM-1 domain D5</td>
<td>367–450</td>
<td>0.15</td>
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<tr>
<td>CVA21 VP1 total</td>
<td>1016–1298</td>
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<tr>
<td>CVA21 VP1 N terminus</td>
<td>1016–1078</td>
<td>0.93</td>
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<td>CVA21 VP1 C terminus</td>
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Elbow Angles among ICAM-1 Domains

<table>
<thead>
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<th>Elbow Angle with Next Domain</th>
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<td></td>
<td>Cryo-EM</td>
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<tr>
<td>D1</td>
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</tr>
<tr>
<td>D2</td>
<td>Pro115, Phe185</td>
</tr>
<tr>
<td>D3</td>
<td>Pro217, Tyr281</td>
</tr>
<tr>
<td>D4</td>
<td>Phe283, Tyr367</td>
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<tr>
<td>D5</td>
<td>Pro398, Pro450</td>
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Overall Contact Statistics

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<th>Number of Ion Pairs</th>
<th>Number of Other Interactions</th>
<th>Total Number of Interactions</th>
<th>H Bond</th>
<th>Electrostatic Interaction</th>
<th>VDW Interaction</th>
<th>Total Interaction</th>
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<td>19</td>
<td>6</td>
<td>22</td>
<td>41</td>
<td>-30.3</td>
<td>-394.3</td>
<td>62.9</td>
<td>-487.5</td>
</tr>
<tr>
<td>CVA21/ICAM-1</td>
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<td>22</td>
<td>8</td>
<td>25</td>
<td>47</td>
<td>-32.9</td>
<td>-516.0</td>
<td>75.9</td>
<td>-624.8</td>
</tr>
</tbody>
</table>

a) Amino acids in the virus are numbered nxxx, where n is the VP number and xxx is the amino acid sequence number.
b) Sumf is the normalized mean density over all atoms calculated by the program EMfit (see Experimental Procedures).
c) The value in parentheses is sumf of ICAM-1 with Lys29 instead of Met29 as in ICAM-1Kilifi.
d) VDW = van der Waals.

(Staunton et al., 1988). Enzymatic digestion (Tomassini et al., 1989) and X-ray crystallography (Bella et al., 1998; Casasnovas et al., 1998; Yang et al., 2004) have shown that at least seven of these can be glycosylated. Domain D1 does not have any glycosylation sites, whereas domain D2 is the most heavily glycosylated domain, containing four carbohydrate sites associated with Asn103, Asn118, Asn156, and Asn175. Domain D3 has two glycosylation sites located at Asn240 and Asn269. Glycosylation of domain D3 may play a role in ICAM-1 dimerization (Reilly et al., 1995). The glycosylation sites have been used as anchors to fit the X-ray structure of ICAM-1 domains D1 and D2 into the low-resolution cryo-EM maps of various virus/receptor complexes (Kolatkar et al., 1999; Xiao et al., 2001).

X-ray crystallography and cryo-EM image reconstructions have been combined to study a number of unstable picornavirus/receptor complexes (Rossmann et al., 2002). One of these was a 26 Å resolution cryo-EM map of a complex of CVA21 with ICAM-1, which had been interpreted by using the homologous PV1 structure to represent the virus and the D1-D2 ICAM-1 X-ray crystallographic structure of the receptor (Xiao et al., 2004).
Here, we report the crystal structure of CVA21 determined by X-ray crystallography to a resolution of 3.2 Å, as well as a cryo-EM reconstruction of CVA21 complexed with ICAM-1Kilifi to a resolution of 8.0 Å. These results provide more accurate structural information of the virus/receptor interface.

Results and Discussion

Structure of CVA21

The crystals of CVA21 belong to space group P4_2_2_1, with 346.3 ≤ a ≤ 348.0 Å (Table 1). The Matthews coefficient (2.5 Å^3/Da) shows that there are two virus particles in the unit cell. Thus, each particle must be situated at a special position with 23 symmetry. Given the icosahedral symmetry of the virus, this implies that the noncrystallographic symmetry redundancy is 5. The best R factor, obtained for crystals at pH 6.4, was 0.225 (Table 1). No significant difference was found for structures determined from crystals grown at pH values ranging from 5.5 to 7.2.

As expected, among the known structures of picornaviruses, CVA21 has the greatest similarity to polioviruses (Figure 1). The biggest differences occur at the chain termini and in some loop regions, such as the carboxyl termini of VP1 and VP3 (forming part of the canyon rim), parts of the internal VP4 protein, the B-C and D-E loops of VP1 (forming the 5-fold vertex of the virus), and the protruded E-F loop region of VP2 (the “puff” region) (Figure 1). The puff region, which is especially variable among picornaviruses, is one of the large contact regions between CVA21 and ICAM-1 and between PV and its receptor PVR.

Non-Protein Components that Bind to the CVA21 Capsid

As observed in other picornaviruses, including PV (Filman et al., 1989), HRV14 (Arnold and Rossmann, 1988), HRV16 (Hadfield et al., 1997), CVB3 (Muckelbauer et al., 1995), and CVA9 (Hendry et al., 1999), there is a disc-like electron density in the CVA21 map stacked against the highly conserved Trp2038 (amino acids are numbered nxxx, where n is the VP number and xxx is the amino acid sequence number). A guanine nucleotide was built into this density because tryptophans usually stack with guanines (Jones et al., 2001) (Figure 2A).

The drug binding pocket in VP1 was found to be filled with a pocket factor represented by a thin, but long, density. The 2.2 Å resolution map of PV1 (Protein Data Bank [PDB] accession number 1HXS) had been interpreted as having a palmitate molecule in the drug binding pocket. However, the length of the pocket factor density in CVA21 better resembles a two-carbon-shorter myristate molecule (Figure 2B).

It has been shown that the N terminus of VP4 of at

Figure 4. Cryo-EM Densities of CVA21/ICAM-1

(A) Stereo diagram of cryo-EM densities within one asymmetric unit, in which densities belonging to ICAM-1Kilifi, VP1, VP2, and VP3 are colored yellow, blue, green, and red, respectively.

(B) Same densities as in (A), but in transparent cyan and fitted with backbone Cα trace of ICAM-1Kilifi (yellow), VP1 (blue), VP2 (green), and VP3 (red).

(C) Cryo-EM difference densities corresponding to the ICAM-1Kilifi (yellow), VP1 (blue), VP2 (green), and VP3 backbone (red) fitted with backbone structures of ICAM-1Kilifi (yellow), VP1 (blue), VP2 (green), and VP3 (red).

(D) One-quarter of the central slab (−20 to 20 Å on the z axis) of cryo-EM density (cyan) fitted with backbone structures of ICAM-1Kilifi (yellow), VP1 (blue), VP2 (green), and VP3 (red), and VP4 (black).
least four genera of picornaviruses is myristylated (Chow et al., 1987). A myristate-like density can be identified as being associated with the amino end of VP4 in CVA21 (Figure 2C), although the last two carbon atoms cannot be seen in the electron density. Similar observations have been made for the amino ends of VP4 in PV1 (Chow et al., 1987), PV2 (Lentz et al., 1997), PV3 (Filman et al., 1989), HRV16 (Hadfield et al., 1997), CVA9 (Hendry et al., 1999), and CVB3 (Muckelbauer et al., 1995), although no myristate could be seen in maps of HRV1A (Kim et al., 1989), HRV14 (Arnold and Rossmann, 1990), or HRV2 (Verdaguer et al., 2000). The myristylation has been postulated to mediate attachment of viral capsid proteins to the cell membrane during virus assembly inside the cell and during entry from outside the cell (Belnap et al., 2000a; Chow et al., 1987).

In the structure of HRVs, divalent cations were found along the 5-fold and 3-fold axial cavities, thereby possibly increasing the stability of the virions (Zhao et al., 1997). No equivalent densities were found in the CVA21...
map. However, a high-density peak was found surrounded by polar groups belonging to Ser1021, Thr1022, Ser1024, and Asn1063 (Figure 2D). Thus, based on the coordination geometry (Zhoa et al., 1997), a putative metal ion (probably Ca\(^{2+}\), Zn\(^{2+}\), or Mg\(^{2+}\)) was built into this density. Although EDTA was used in the virus purification and was also used at low concentration in the final crystallization conditions, the putative metal atom was buried inside the virus at the N terminus of VP1, preventing it from being chelated by EDTA.

**Cryo-EM Reconstruction**

The cryo-EM reconstruction of CVA21/ICAM-1\(^{\text{Kilifi}}\) had a resolution of 8.0 Å, a considerable improvement on the earlier 26 Å resolution results (Xiao et al., 2001). All five extracellular domains (D1–D5) of ICAM-1 were visible in the previous study (Figure 3B). Although in the current CVA21/ICAM-1\(^{\text{Kilifi}}\) work only about three domains (D1–D3) are visible, nevertheless, if the resolution of the data is reduced to 26 Å resolution, all five domains, including the Fc fusion component, become visible (Figures 3A and 3D). It would appear that the increased flexibility of the molecule at each successive elbow between sequential domains is the cause of the loss of visibility of the outer ICAM-1 domains. At low resolution, the molecule looks much the same no matter which of the many possible structures are viewed. However, at high resolution, each of the different structures will be better resolved but present only as a small fraction of the total number of molecular conformations, resulting in low and unrecognizable density (Figures 3D–3H). This is shown by the decrease in density height along the ICAM-1 molecule in which the ratio of the relative heights of the densities for domains D1, D2, D3, D4, and D5 are approximately 1.0, 0.7, 0.3, 0.2, and 0.1, respectively. As the average density height of domain D1 is approximately 0.8 of the viral capsid protein shell (Table 2), presumably only about 80% of the ICAM-1 binding sites are occupied.

**ICAM-1 Dimerization**

Dimerization enhances ICAM-1’s ability to bind to LFA-1 (Reilly et al., 1995), as well as to HRV (Casasnovas and Springer, 1995). Furthermore, dimeric ICAM-1 more effectively triggers the HRV conformational changes that initiate viral infection (Martin et al., 1993). Although soluble ICAM-1 is mainly monomeric in vitro, full-length ICAM-1 molecules form homodimers on cell surfaces in vivo (Miller et al., 1995; Reilly et al., 1995) with intermolecular contacts between opposing domains D3, D5, and the transmembrane regions (Jun et al., 2001; Miller et al., 1995). The ICAM-1\(^{\text{Kilifi}}\)/Fc protein dimerizes in solution (Craig et al., 2000), as is also the case with ICAM-1 fragments fused with various immunoglobulin molecules (Martin et al., 1993). Similarly, there are no direct contacts between ICAM-1 molecules bound to the surface of CVA21, whereas when ICAM-1\(^{\text{Kilifi}}\)/Fc was bound to CVA21, domains D3, D5, and Fc in adjacent 2-fold-related molecules are in contact (Figures 3A, 3C, 3E, and 3F). Thus, the Fc component appears to act in a manner equivalent to the transmembrane domains or immunoglobulin fusion fragments at the carboxyl end of ICAM-1. This similarity suggests that, on the cell surface, the virus might bind to a dimer of the full-length ICAM-1. The contacts between the 2-fold-related D3 domains appear to be via the carbohydrate associated with Asn240, as has been previously suggested (Reilly et al., 1995).

**The Interface between CVA21 and ICAM-1\(^{\text{Kilifi}}\)**

In the previously published 26 Å resolution description of the CVA21/ICAM-1 complex, the density representing ICAM-1 has a smooth surface (Figure 4C, left panel). The current 8.0 Å resolution cryo-EM map provides more surface features than before (Figure 4C, right panel), and these features can be used for an accurate fit of the ICAM-1 structure into the cryo-EM density.

The tip of the ICAM-1 D1 domain (D-E loop, B-C loop, G-F loop) contacts the VP1 βG and βH strands as well as the VP1 G-H loop. The edge of the ICAM-1 D1 domain, formed by the βG strand, contacts the VP1 E-F loop and the VP3 G-H loop. The opposite edge of the same ICAM-1 domain, formed by the βD and βE strands, contacts the puff region of VP2. However, in PVs, the structure and sequence of the highly variable puff and the G-H loop are completely different, probably accounting for the inability of ICAM-1 to bind to PVs (Figures 1, 5C, and 7B). Belnap et al. (2000b) had found that the pocket factor in poliovirus was missing when PVR was bound in the canyon. This would verify the hypothesis that receptor attachment into the canyon dislodges the pocket factor, thereby destabilizing the virus. However, the ability to determine the absence or presence of pocket factor at 8 Å resolution has been questioned (Xiao et al., 2004), and that remains true in the current study.

ICAM-1 binds into the same site within the canyon of both CVA21 and HRVs, although the orientation of ICAM-1 relative to the respective viral surfaces is quite different (Figure 5B). The interface between ICAM-1 and CVA21 has 30% less buried surface area and 40% smaller calculated interaction energy than the interface between ICAM-1 and HRV14 (Xiao et al., 2004) (Table 2). The shape correlation (S\(_{sh}\), see Experimental Procedures) for ICAM-1 binding to HRV14, CVA21, or HRV16 is approximately 0.69, 0.65, and 0.54, respectively, consistent with the observed inability of ICAM-1\(^{\text{Kilifi}}\) binding to HRV16 (Xiao et al., 2004). The S\(_{sh}\) for antibody-antigen complexes is comparable at about 0.65 (Epa and Colman, 2001; Lawrence and Colman, 1993).

Theoretical energy calculations showed that electrostatic interactions contribute 80% of the contact energy (Table 2). There are four regions of ionic networks in the ICAM-1/CVA21 interface (a, b, c, and d in Figures 6D and 7), as has also been observed for the interface between HRVs and ICAM-1 (Xiao et al., 2004). However, for ICAM-1\(^{\text{Kilifi}}\), which has Lys instead of Met at residue 29 in the interface, there is one less ionic interaction, which suggests that ICAM-1\(^{\text{Kilifi}}\) may have a weaker interaction with CVA21 than ICAM-1, as is also the case for HRV16 (Xiao et al., 2004).

There are five available sequences of HEV-C class CVAs that bind ICAM-1 (Newcombe et al., 2003). Many of the CVA21 residues that are involved in forming the
interface with ICAM-1 are conserved (Figure 7B). However, some charged residues (Arg1161, Asp1221, and Asp2167) are not conserved, indicating that they may not be contributing significantly to the virus/receptor affinity. Four adjacent Pro residues (Pro1156, Pro1157, Pro1160, and Pro1162) associated with the ionic site...
“a” (Figure 7A) are entirely conserved, indicating an invariant conformation of this component of the ICAM-1 binding surface. Apart from the charge interactions, there are large areas of hydrophobic complementarity in the CVA21/ICAM-1 interface. However, these regions, apart from the interaction of Phe/Tyr1223 with Leu30 of ICAM-1 in the B region, are not well conserved among other ICAM-1 binding CVAs, further indicating the importance of the charge interactions.

Conclusions
The availability of atomic resolution structures of both virus (CVA21) and virus receptor (ICAM-1), together with an improved cryo-EM map of their complex, has provided accurate information regarding the interface between virus and receptor, indicating which residues are likely to be most important in forming a stable complex. These results can now be used for functional investigations with site-directed mutagenesis.

Experimental Procedures

Samples
CVA21 (strain Kury kendall) was prepared by an established lab protocol (Xiao et al., 2001). The fusion protein, combining ICAM-1D3–D5 at the amino end with an Fc fragment (consisting of the two human IgG1 domains linked by four amino acid residues) at the carboxyl end (ICAM-1D3–D5Fc), was produced as described previously (Craig et al., 2000).

Crystallization of CVA21
After CVA21 was purified as described by Xiao et al. (2001), the virus was at a concentration of 7 mg/ml in a buffer of 200 mM NaCl, 10 mM Tris (pH 7.2), 0.1 mM EDTA. The mother liquor consisted of 300 mM NaCl, 200 mM citrate buffer (pH 5.6, 6.4, 7.2), 0.5% glycerol. Crystallization trials were set up by mixing 8 µl virus sample with 8 µl mother liquor in sitting drops. Sealed crystallization trays were incubated at 4°C for 2–3 days until the crystals grew to a maximum size of 0.2 × 0.2 × 0.1 mm.

In order to provide cryoprotection, glycerol and PEG400 were added to the mother liquor to a concentration of 20% and 10%, respectively, prior to crystal freezing. Several data sets (Table 1) were collected on frozen (100 K) crystals at the Advanced Photon Source of Argonne National Laboratory.

The DENZ0 program (Otwinowski and Minor, 1997) was used for indexing and integrating the diffraction data, and the SNP program in the DPS suite (Rossman and van Beek, 1999) was used for scaling and merging the data from different frames. Starting phases were calculated to 4 Å resolution by using the known PV1 coordinates (PDB accession number 2PLV). Crystallographic and noncrystallographic symmetry (NCS) averaging were carried out in real space by using the program ENVELOPE (Rossman et al., 1999). All of the cryo-EM micrographs were recorded on a Philips FEG300 microscope at a magnification of 300 kV. Micrographs were then scanned on a Zeiss Phodi s SCSI scanner with a step size of 7 µm. Subsequently, four pixels were averaged together in order to minimize space and computing time. The final pixel size corresponded to 2.98 Å on the sample. The program RobEM was used to box and preprocess the box images. There were 33 micrographs, including 7 focal pairs (14 micrographs), used for the reconstruction. The defocus distance ranged from 0.7 to 3.0 µm. The EM reconstruction procedures, including the contrast transfer function corrections and orientation and translation determinations, were performed as described previously (Xiao et al., 2004). A total of 4704 particles, selected from 7979 boxed particles based on the correlation coefficient between the raw image and the corresponding calculated projection, were included in the reconstruction. The final resolution was estimated by finding where the Fourier shell correlation fell below 0.5 by using Fourier coefficients based only on the viral protein shell and D1 of the ICAM-1 density situated between 104 Å and 177 Å radius.

Fitting the X-Ray Structure into the Cryo-EM Densities
Coordinates of CVA21 determined from X-ray crystallography were used to calculate an 8.0 Å resolution map. This map was used to determine the pixel size of the cryo-EM map of CVA21/ICAM-1D3–D5 by using the program EMFit (Rossmann, 2000; Xiao et al., 2004). Only the density corresponding to atoms in the virus capsid was used to establish the accurate magnification of the cryo-EM map. The accurate pixel size for the EM map was found to be 2.65 Å. A difference map was then computed between the cryo-EM complex map and the map based on the atomic structure of the virus to obtain the density corresponding to the 60 bound ICAM-1 molecules. The crystal structure of ICAM-1 D1 and D2 (PDB accession number 1IAM), with Lys29 in domain D1 modeled as a Met to represent the structure of ICAM-1D3–D5, was then fitted into the difference map by using the program EMFit (Rossmann et al., 2001) as described previously (Xiao et al., 2004). The sumf² values (Table 2) were normalized with respect to the root mean square (rms) deviation from the mean density within the protein shell of the virus. The crystal structure of ICAM-1 D3–D5 (PDB accession number 1P53) was used to fit into a map of the complex calculated to only 16 Å resolution instead of the final 8 Å, because the density for the ICAM-1 D3–D5 is poor when higher resolution data are used (see Results and Discussion). Nevertheless, the values of sumf² were calculated for D3–D5 based on the higher 8 Å resolution cryo-EM map (Table 2) in order to make the values comparable to those of domains D1 and D2.

Elbow Angles
The elbow angles between neighboring domains were calculated as described previously (Xiao et al., 2004). Two Cα atoms were selected for each domain (Table 2) to define their long axes. The elbow angle between two domains was defined as being the angle between their long axes (Table 2). Although the elbow angle between domains D1 and D2, between D3 and D4, and between D4 and D5, have been characterized crystallographically (Kolatkar et al., 1999; Yang et al., 2004), this is, to our knowledge, the first direct determination of the elbow angle between D2 and D3.

Energy Minimization
Although in the fitting procedure the number of atoms of ICAM-1 in negative or zero density of the difference map was minimized, the rigid-body fitting still generated a few ICAM-1 D1 side chains that would be in steric conflict with the virus side chains. Therefore, the CVA21/ICAM-1 complex was subjected to limited energy minimization by using the program CHARMM (Brooks et al., 1983). Only atoms within the virus/receptor interface were allowed to move in order to eliminate steric collisions. After the minimization, the potential energies of the interactions between ICAM-1 and CVA21 were also calculated by CHARMM (Table 2).

Interface Analyses
Residues in the virus/receptor interface were identified as those in CVA21 that had atoms less than 4 Å from any atom in ICAM-1D3–D5. The buried surface area and the number of contacts were calculated.
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lated with the program CNS (Brünger et al., 1998) (Table 2). The surface electrostatic potential of CVA21 and ICAM-1 was calculated with the program DelPhi (Gilson and Honig, 1988) and was plotted onto the CVA21 surface. The program SC (Lawrence and Colman, 1993) was used to calculate the shape correlation ($S_x$) among different virus/receptor complexes. $S_x$ measures the geometric surface complementarity of protein/protein interfaces.

Acknowledgments

We thank Suchetana Mukhopadhyay and Ricardo A. Bernal for help with crystallization and structure determination. We also thank Wei Zhang, Xing Zhang, Wen Jiang, and Wah Chiu for help in improving the resolution of the cryo-EM map. We are grateful to Jia-huai Wang for providing us with the coordinates of the complete ICAM-1 structure, to William I. Whitson for parallel computer support, as well as to Sharon S. Wilder, Cheryl A. Towell, and Sheryl L. Kelly for help in the preparation of the manuscript. The work was supported by National Institutes of Health grants AI 11219 to M.G.R. and AI 15122 and GM 65030 to E.W., a Keck Foundation grant to M.G.R, D.C., Hogg, N., and Newbold, C. (2000). A functional analysis of a cellular adhesion molecule-1 transgenic mice. Proc. Natl. Acad. Sci. USA 95, 13636–13641.


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Accession Numbers

The refined CVA21 atomic coordinates have been deposited with the PDB as accession number 1Z7S. The atomic coordinates of the CVA21/ICAM-1Kilifi complex, which were fitted into the cryo-EM map and then energy minimized, have also been deposited with PDB accession number 1Z7Z. The cryo-EM map has been deposited with the Macromolecular Structure Database at the European Bioinformatic Institute as accession number EMD-1114.