Cellular receptor interactions of C-cluster human group A coxsackieviruses

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The cellular receptor complex of coxsackievirus A21 (CVA21), a C-cluster human enterovirus, is formed by the subtle interaction of individual cellular receptors, decay accelerating factor (DAF) and intercellular adhesion molecule-1 (ICAM-1). In this receptor complex, DAF functions in the membrane sequestration of the virus, while the role of ICAM-1 is as the functional cellular internalization receptor. However, despite the elucidation of the CVA21–cell receptor interactions, there have been few definite investigations into cellular receptor usage of other coxsackie A viruses (CVAs) belonging to the C-cluster. In the present study, radiolabelled virus-binding assays demonstrated that CVA13, -15, -18 and -20, a subset of the human enterovirus C-cluster, bind directly to surface-expressed ICAM-1, but not to surface-expressed DAF. Furthermore, lytic infection of ICAM-1-expressing rhabdomyosarcoma (RD) cells by this C-cluster subset of viruses was inhibited by specific ICAM-1 monoclonal antibody blockade, except for that of CVA20. Despite possessing ICAM-1-binding capabilities, CVA20 employed an as yet unidentified internalization receptor for cell entry and subsequent productive lytic infection of ICAM-1-negative RD cells. In a further example of C-cluster cellular receptor heterogeneity, CVA13 exhibited significant binding to the surface of CHO cells expressing neither DAF nor ICAM-1. Despite a common receptor usage of ICAM-1 by this subset of C-cluster CVAs, the amino acid residues postulated to represent the ICAM-1-receptor footprint were not conserved.

INTRODUCTION
Phylogenetic comparisons of the amino acid sequences encoding the major structural capsid protein (viral protein 1, VP1) of viruses from the genus Enterovirus, within the family Picornaviridae, have segregated these viruses into four distinct genetic clusters: A, B, C and D (Oberste et al., 1999; Po¨yry et al., 1996). Many of the 23 distinct serotypes of coxsackie A viruses (CVAs), which cause a range of diseases in humans including the common cold, rashes, meningoencephalitis and paralytic illness (Couch et al., 1965; Plummer, 1965), are classified as members of the C-cluster of human enteroviruses (HEV-C). The HEV-C cluster is predicted to include CVA1, -11, -13, -15, -17 to -22 and 24, as well as poliovirus (PV) types 1, 2 and 3 (Oberste et al., 1999; Po¨yry et al., 1996). Similar groupings were obtained by comparing nucleotide and derived amino acid sequences from the 5′ untranslated region (5′UTR), the VP4–VP2 capsid protein and polymerase 3D region, and the 3′UTR (Pulli et al., 1995). At present, however, a significant correlation of these genetically based divisions with specific cellular receptor usage has not been well established.

In preliminary studies of the receptor usage of CVAs, monoclonal antibody (mAb) receptor blockade of intercellular adhesion molecule-1 (ICAM-1) has suggested a shared receptor specificity for CVA13, -15, -18, -20 and -21 (Colonno et al., 1986; Pulli et al., 1995). However, direct virus binding to ICAM-1 was not tested, raising the possibility that the observed inhibition of infection could be as a result of non-specific steric hindrance, as has previously been demonstrated with a mAb directed against CD44 (lymphocyte homing receptor), which protected normally susceptible cells from PV1 infection (Shepley & Racaniello, 1994).

CVA21 utilizes ICAM-1 for cell entry of susceptible cells (Shafren et al., 1997a). In the virus attachment process,
ICAM-1 binds within the deep surface depression, or canyon, surrounding each fivefold vertex of the CVA21 capsids (Xiao et al., 2001) in a similar manner to its interaction with the major group human rhinoviruses (HRVs) (Rossmann et al., 1985). In addition to ICAM-1 usage, CVA21 binds decay accelerating factor (DAF) as a second cellular receptor (Shafren et al., 1997b). DAF is a 70 kDa glycosylphosphatidylinositol-linked complement regulatory protein consisting of fourextracellular short consensus repeats (SCRs) and is expressed almost ubiquitously on cells throughout the mammalian body (Lublin & Atkinson, 1989). DAF is postulated to be utilized by CVA21 in association with ICAM-1 during host-cell entry (Shafren et al., 1997b), functioning primarily as an attachment/sequestration receptor for CVA21, as interactions with DAF alone do not result in lytic infection (Shafren et al., 1997b). However, in the absence of ICAM-1, CVA21 can utilize antibody-cross-linked DAF for cell internalization (Shafren, 1998). Cross-linked DAF on the surface of rhabdomyosarcoma (RD) cells significantly increases the level of CVA21 binding and subsequent lytic infection (Shafren et al., 1998). DAF also serves as a cellular attachment receptor for a number of other enteroviruses, including enterovirus 70 (Karnauchow et al., 1998), several echoviruses (EVs) (Bergelson et al., 1994) and coxsackieviruses B1, B3 and B5 (Shafren et al., 1995).

Comparisons of the amino acid sequences constituting the ICAM-1 receptor binding footprint may further the understanding of shared receptor specificity within this subset of C-cluster CVAs. Presently, CVA21 (Hughes et al., 1989) and CVA24 (Supanaranond et al., 1992) are the only serotypes of the C-cluster for whom full-length genomic sequences are freely available, leaving a large void in the amount of information concerning the amino acid composition of the capsid proteins of the remaining viruses.

The aim of this study was to characterize the cellular receptor usage involved in both attachment and infection by CVA13, -15, -18, -20 and -21. In addition, full-length genomic nucleotide sequences of the subset of C-cluster CVAs listed above were generated and used to analyse differences, if any, in the P1 capsid coding region and postulated ICAM-1 footprint.

**METHODS**

**Cells and viruses.** Prototype strains of CVA13 (Flores), CVA15 (G-9), CVA18 (G-13), CVA20 (IH-35) and CVA21 (Kuykendall) were obtained from Margery Kennett, Entero-respiratory Laboratory, Fairfield Hospital, Melbourne, Victoria, Australia. Stock preparations of CVA13, -15, -18, -20 and -21 had previously been passaged between five and eight times in HeLa and/or human lung fibroblasts or HeLa-T cells. In this study CVA13, -15, -18 and -20 were propagated in HeLa-B cells, while CVA21 was propagated in ICAM-1-expressing RD (RD-ICAM-1) cells (Shafren et al., 1997b). HeLa-B and RD cells were obtained from Margery Kennett. Chinese hamster ovary (CHO) cells were obtained from Bruce Loveland, Austin Research Institute, Heidelberg, Victoria, Australia.

**Antibodies.** The anti-DAF mAb HI4 (lgG1) is specific for the third SCR of DAF (Coyne et al., 1992) and was a gift from Bruce Loveland. The anti-ICAM-1 WEHI mAb is directed against the N-terminal domain of ICAM-1 (Berendt et al., 1992) and was supplied by Andrew Boyd, Queensland Institute for Medical Research, Queensland, Australia.

**Cell transfection.** CHO and RD cells expressing DAF and/or ICAM-1 were generated as described previously (Shafren et al., 1997b). Briefly, 500 μl aliquots of cells (5 x 10^6–1 x 10^7 cells ml^-1) were resuspended in electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaHPO_4, 6 mM glucose, pH 7.05) and mixed with 75 μg pEF-BOS ( Mizushima & Nagata, 1990) encoding DAF or ICAM-1 and 5 μg of pCDNAneo in electroporation cuvettes (Bio-Rad). Cells were pulsed at 300 V and 250 μF with a Bio-Rad gene pulser, and then seeded into 24-well tissue culture plates and incubated at 37°C for 48 h until confluent monolayers were formed. Receptor-expressing transfected cells were selected in Dulbecco’s modified essential medium (DMEM) containing G-418 (400 μg ml^-1) and further enriched by fluorescence-activated cell sorting using appropriate anti-receptor mAbs.

**Flow cytometry.** DAF and ICAM-1 surface expression on transfected cells was analysed by flow cytometry. Briefly, dispersed cells (10^5) were incubated on ice with the appropriate anti-DAF or anti-ICAM-1 mAbs (5 μg ml^-1 diluted in PBS) for 20 min. Cells were then washed with PBS, pelleted at 1000 g for 5 min and resuspended in 100 μl R-phycocerythrin-conjugated F(ab')2 fragment of goat anti-mouse immunoglobulin diluted 1:100 in PBS (Dako) and incubated on ice for 20 min. Cells were washed and pelleted as above, resuspended in PBS and analysed for DAF and ICAM-1 expression using a FACStar analyser (Becton Dickinson).

**Radiolabelled virus-binding assays.** The procedure for obtaining purified viruses of high ^35^S activity was as follows. Confluent monolayers of HeLa or RD-ICAM-1 cells in six-well tissue culture plates were inoculated with 500 μl of the appropriate virus (1 x 10^6 TCID_50 ml^-1) for 1 h at 37°C. Unbound virus was removed by washing three times with methionine/cysteine-free DMEM (ICN Biochemicals) and, following the addition of methionine/cysteine-free DMEM, cell monolayers were incubated for a further 2 h before addition of 300 μCi [^35^S]methionine Trans-Label (ICN Radiochemicals). Infected monolayers were then incubated at 37°C in a 5% CO_2 environment for 12 h. Following three freeze/thaw cycles, viral lysates were collected in 5–30% sucrose (Shafren et al., 1995). Fractions were collected from the bottom of each tube and monitored by liquid scintillation counting on a 1450 Microbeta TRILUX (Wallac).

Radiolabelled virus-binding assays were performed by incubating 1 x 10^6 cells in 800 μl DMEM containing 1% BSA with 300 μl (~1 x 10^6 c.p.m.) of [^35^S]methionine-labelled virus for 2 h at room temperature. Cells were then washed four times with serum-free DMEM, pelleted dissolved in 200 μl 0.2 M NaOH/1% SDS and the level of [^35^S]methionine-labelled virus bound determined by liquid scintillation counting.

**Virus infectivity assay.** Confluent monolayers of RD and RD-ICAM-1 cells in 96-well plates were inoculated with tenfold serial dilutions (100 μl per well in quadruplicate) of CVA13, -15, -18, -20 and -21 and incubated at 37°C in a 5% CO_2 environment for 48 h. To quantify cell survival, plates were incubated with a crystal violet/ formaldehyde in PBS) for 24 h, washed in distilled water and the relative absorbance of the stained cell monolayer read on a multi-scans ELLISA plate reader (Flow Laboratories) at 540 nm. Fifty per cent end-point titres were calculated using the method of Reed & Muench (1938), where a well was scored as positive if the absorbance
was less than the no virus control minus three standard deviations (SD). Where cell monolayer pretreatment with anti-receptor mAbs was required, cells were incubated with 100 μl per well of anti-DAF IH4 mAb or anti-ICAM-1 WEHI mAb (1 μg ml⁻¹) for 1 h at 37 °C. Cell monolayers were then inoculated with quadruplicate samples of the tenfold serial dilutions and incubated at 37 °C in a 5 % CO₂ environment for 48 h before staining as described above.

**Viral RNA extraction and cDNA synthesis.** Confluent cell cultures of HeLa cells were infected with CVA13, -15, -18, -20 and -21 and incubated at 37 °C until complete lytic infection of the cell monolayer (24–48 h post-inoculation). Virions were pelleted from the medium by high-speed centrifugation for 16 h at 80 000 g and resuspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7-5). Viral RNA was extracted from the pellet with proteinase K/SDS followed by phenol/chloroform/isoamyl alcohol treatment as described previously (Lindberg et al., 1992).

For cDNA synthesis, viral RNA was reverse transcribed using Superscript II (Life Technologies) and the primer Notdt25 for 2 h at 45 °C.

**RESULTS**

**Cellular attachment receptor usage of C-cluster CVAs**

CVA21 uses DAF as an attachment receptor and ICAM-1 as a functional internalization receptor (Shafren et al., 1997b). However, the precise cellular receptor usage of other C-cluster CVAs is not known. Preliminary studies using mAb blockade of ICAM-1 have suggested indirectly that ICAM-1 is involved in cell entry of CVA13, -15, -18 and -20 (Colonno et al., 1986; Pulli et al., 1995), but direct binding was not investigated. To determine whether CVA13, -15, -18 and -20 bind directly to ICAM-1 and/or DAF, radiolabelled virus-binding assays were performed using stably transfected CHO-DAF or CHO-ICAM-1 cells. Fluorescence histograms (Fig. 1A) revealed a high level of ICAM-1 and DAF surface expression on the appropriate transfected cells. The CVA serotypes 15, 18, 20 and 21 bound to surface-expressed ICAM-1 (Fig. 1B), whereas only CVA21 exhibited significant levels of binding to DAF. Somewhat surprisingly, CVA13 bound equally to all three CHO cell types suggesting that this serotype can attach to cell surfaces independently of ICAM-1 and DAF via interactions with an as yet unidentified cell-surface molecule(s).

To characterize further the viral attachment of the C-cluster subset CVA13, -15, -18, -20 and -21 to DAF and ICAM-1, (A) Flow cytometric analysis of the level of DAF and ICAM-1 on stably transfected CHO cells (open histograms) compared with conjugate-only controls (filled histograms). (B) Radiolabelled CVA13, -15, -18, -20 and -21 binding to DAF- and ICAM-1-expressing CHO cells measured by liquid scintillation. Results are expressed as the mean of triplicates + SD.

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may possibly occur via interactions with a similar unidentified attachment receptor that is also endogenously expressed by CHO cells.

**ICAM-1 is not the only cellular receptor required for cell infection by C-cluster CVAs**

Studies of the receptor usage of CVA21 have indicated that, whilst this virus can bind to either DAF or ICAM-1, the presence of ICAM-1 is required for CVA21 to achieve host-cell lytic infection (Shafren et al., 1997a, b). When ICAM-1 is not present, CVA21 is capable of initiating host-cell entry via antibody-cross-linked DAF (Shafren, 1998). To determine whether ICAM-1 is the cell internalization receptor employed by CVA13, -15, -18 and -20, virus infectivity assays using RD and RD-ICAM-1 cells inoculated with tenfold serial dilutions of each CVA serotype were performed (Fig. 3). All CVA serotypes lytically infected cells expressing ICAM-1; however, CVA20 was capable of infecting RD cells in the absence of ICAM-1, at a level similar to that observed in ICAM-1-expressing RD cells, which suggests the use of an alternative internalization receptor (Fig. 3A).

To investigate whether this subset of C-cluster CVAs is capable of utilizing mAb-cross-linked DAF in the absence of ICAM-1, RD cells were pretreated with anti-DAF SCR 3 mAbs prior to viral infection. Only CVA20 and CVA21 mediated lytic infection of RD via interactions with antibody-cross-linked DAF (Fig. 3B). However, as CVA20 also lytically infected RD cells in the absence of cross-linking anti-DAF mAbs, it is most likely, as above, that this virus is utilizing an unknown internalization receptor that is not DAF.

**Comparison of the putative receptor-binding footprint of C-cluster CVAs**

The nucleotide sequences coding the entire P1 region of the viral genomes of CVA13, -15, -18 and -20 have not been previously determined. Comparison of the amino acid sequences of capsid structural proteins encoded therein would allow a possible characterization of the specific amino acids utilized during receptor binding. Full-length nucleotide sequences of each of the CVA serotypes were generated by sequencing multiple PCR-generated viral
amplicons. The deduced amino acid sequences encoding the entire P1 region of the CVA13, -15, -18, -20 and -21 genomes were aligned using the ClustalW program (Thompson et al., 1994) to allow identification of any significant conserved regions with the known picornavirus receptor-binding footprints (Fig. 4). The amino acid sequence identity within VP1, -2 and -3 of CVA13, -15, -18 and -20 compared with CVA21 of these CVAs displayed 75–77% amino acid identity with VP3 of CVA21. VP1 possessed the most variation in amino acid

**Fig. 4.** Multiple amino acid alignments of the four viral capsid proteins (VP1–4) of CVA13, -15, -18, -20 and -21. Arrows indicate protein cleavage sites. Identical amino acids are represented by an asterisk, and strongly and weakly conserved amino acid residues by a colon and a dot, respectively. Sequence alignments were generated using the ClustalW program (Thompson et al., 1994).

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sequence, with CVA13, -15, -18 and -20 sharing 63–69.1% identity with VP1 of CVA21.

A common core of somewhat conserved residues exists between the ICAM-1-binding footprint on the capsids of CVA21, HRV14 and HRV16 and those forming the poliovirus receptor (PVR) footprint on PV1 (Xiao et al., 2001). To investigate the degree of conservation of the ICAM-1 footprint on the capsids of CVA13, -15, -18 and -20, the deduced capsid protein sequences (Fig. 4) were aligned with those from CVA21, HRV14, HRV16 and PV1 (Fig. 5). The greatest degree of similarity in residues predicted to form an ICAM-1 footprint was observed between that of CVA21 and CVA13, -15, -18, -20, especially in the βC strand and the G–H loop of VP1 (Fig. 5). There was little or no conservation of the ICAM footprint residues located in the ‘puff’ region of VP2 among the CVAs, most probably due to its exposed nature on the capsid surface and high level of immune recognition. The presence of core residues constituting the ICAM-1 footprint does not guarantee similarity in the nature of the biophysical interaction of ICAM-1 with capsids of different viruses. Despite an apparent similarity between the CVA21 and HRV ICAM-1-binding footprint, it is predicted that in CVA21 the ICAM-1 molecule leans slightly to the east-southeast of the capsid canyon, while in HRVs the ICAM-1 receptor molecule leans to the southwest (Xiao et al., 2001).

**DISCUSSION**

The experimental findings presented in this study demonstrate that, similar to CVA21, the C-cluster subset of CVA including CVA13, -15, -18 and -20 employs ICAM-1 as a cellular receptor for binding and cell entry. However, the

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**Fig. 5.** Amino acid sequence alignments for CVA13, -15, -18, -20, -21, PV1, HRV14 and HRV16 in the regions of the viral capsid proteins involved in putative receptor binding. Amino acids residues located in the receptor-binding footprints of ICAM-1 on HRV14, HRV16 (Kolatkar et al., 1999) and CVA21 (Xiao et al., 2001) and PVR on PV1 (Belnap et al., 2000) are shaded. Specific residues involved in ICAM-1 binding to CVA13, -15, -18 and -20 have not been identified; however, ‘loosely’ conserved amino acids in these serotypes compared with those involved in ICAM-1 binding to CVA21 are shown (open boxes). This figure is adapted from a previous report that examined the ICAM-1-binding footprint of the CVA21 (Kuykendall) capsid (Xiao et al., 2001).
members of the above C-cluster subset differ from CVA21 in that they are unable to bind to DAF. In a similar vein, CVA13 binds to CHO cells lacking both DAF and ICAM-1, while CVA20 is able to undergo a productive infection of RD cells in the absence of surface-expressed ICAM-1.

ICAM-1 is employed by CVA21 as the functional internalization component of the cell-receptor complex (Shafren et al., 1997b) and in the same role by the major group HRVs (Colonno et al., 1986; Greve et al., 1989). Despite the common use of ICAM-1 among the major group HRVs, the specific residues lining the ‘footprint’ directly involved in receptor binding differ significantly between various serotypes (Kolatkar et al., 1999). Radiolabelled virus-binding assays as well as virus infectivity assays have demonstrated that there is a common use of ICAM-1 as an attachment and internalization receptor by CVA13, -15, -18, -20 and -21 (Figs 1, 2 and 3). This common receptor usage suggests that these serotypes have evolved from a common ancestor specific for ICAM-1 and may be as a result of the ICAM-1-binding residues being located in a deep capsid canyon (Rossmann et al., 1985).

Cryo-electron microscopy of CVA21 complexed with its ICAM-1 receptor has identified a receptor footprint located within the capsid canyon, which is a region residing within the VP1 β barrel of the canyon adjacent to a hydrophobic pocket (Xiao et al., 2001). Site-directed mutagenic studies of VP1 amino acid residues lining the base of the canyon have identified regions specifically involved in receptor binding (Colonno et al., 1988). This demonstrates the importance of genetic conservation between viruses of the same receptor specificity. While amino acid comparisons of CVA13, -15, -18, -20 and -21 indicate some sequence divergence in this region of the structural proteins responsible for receptor binding, the capacity to utilize ICAM-1 for attachment and infection indicates that critical residues must have been retained (Figs 4 and 5).

While the additional capacity of CVA21 over CVA13, -15, -18 and -20 to utilize DAF as a cellular attachment receptor may be the result of capsid structural changes, this cannot be concluded from the results presented here until the DAF binding footprint of CVA21 is determined. The DAF-binding region of CVA21 is postulated to reside in a two-fold surface depression consisting of residues from VP2 and VP3 (Shafrén et al., 1997b). Recently, the DAF-binding footprint on the echovirus 7 (EV7) capsid was shown to be located close to theicosahedral twofold axes consisting of residues from VP2 and VP3 (He et al., 2002). The structural similarities between the EV7 and CVB3 capsids (Filman et al., 1998; Muckelbauer et al., 1995) predict that CVB3 may also bind DAF within the twofold depression (He et al., 2002). Interaction between DAF and the EV11 capsid is postulated to occur at the fivefold axes (Stuart et al., 2002), also outside the capsid canyon.

While this study has focused on the use of DAF and ICAM-1 as cellular receptors by C-cluster CVAs, there are a large number of different cell surface molecules utilized as receptors for picornaviruses. Apart from DAF, α, integrins and β2-microglobulin utilized by various EVs, heparan sulfate (HS) is also commonly used as a picornaviral attachment receptor (Goodfellow et al., 2001). The results presented here have suggested the use of additional receptors other than DAF or ICAM-1 in the cell attachment/internalization of the C-cluster CVA serotypes. CVA13 was capable of attaching to CHO cells not expressing DAF or ICAM-1, suggesting the use of another discrete attachment receptor. Enzymatic removal of HS from the surface of CHO and RD cells by heparinase I was used to investigate whether HS was the unidentified attachment receptor for CVA13; however, results were inconclusive (data not shown). Furthermore, CVA20 lytically infected ICAM-1-negative RD cells despite an inability to bind to DAF, suggesting the use of another receptor(s) in facilitating host attachment and cell entry. Given that the previously identified picornaviral receptors responsible for mediating host cell lytic infection, e.g., ICAM-1, the coxsackievirus-adenovirus receptor and PVR, all bind within the capsid canyon and all belong to the immunoglobulin supergene family (Beinap et al., 2000; He et al., 2001; Kolatkar et al., 1999), it is tantalizing to postulate that the receptor utilized by CVA20 may also belong to this class of molecule. The high level of lytic infection of RD cells by CVA20, despite minimal levels of cell attachment, highlights the efficiency of this unidentified receptor in mediating virus cell entry. The use of an additional attachment receptor by CVA13 is particularly interesting in light of previous findings reporting that CVA13 and -18 should be regarded as the same serotype as a result of their high VP1 nucleotide (77.2%) and amino acid (95.1%) sequence identities (Oberste et al., 1999). The observed differences in receptor usage by these CVA serotypes may be the result of evolution and adaptation to an ever-changing environment, in much the same way as CVA21 may have adopted the use of DAF as a co-receptor to extend its tissue tropism and host range.

Differences exist in receptor usage of a group of C-cluster CVAs that all share the capacity to infect cells via ICAM-1 interactions. The genetic relationship between CVA13, -15, -18, -20 and -21 at the amino acid level for the entire P1 region of the viral genome was determined in this study (Fig. 4), a significant extension on previous investigations involving solely VP1 (Oberste et al., 1999). Amino acid alignments of potential residues, located in the capsid canyon, constituting receptor-binding footprints of a number of CVAs have revealed that, despite variations in these regions, ICAM-1 usage is still permitted.

It is apparent from the data presented here that not all CVAs assigned to the C-cluster of human enteroviruses share the same cellular receptor usage patterns. Although CVA13, -15, -18, -20 and -21 bind to ICAM-1 to facilitate cell attachment and subsequent entry, major receptor usage differences are highlighted by: (i) CVA13 binding to an
unidentified attachment receptor, present on both CHO and RD cells, which does not facilitate cell internalization (data not shown); (ii) CVA20 binding to an unidentified cellular receptor that permits cell entry and lytic infection; and (iii) CVA21 binding to and utilizing cross-linked DAF for cell entry. Elucidation of these differences will require further analysis and modelling of capsid structure, particularly in the region responsible for DAF binding on CVA21. The use of an antiviral compound such as pleconaril, which binds within the hydrophobic pocket of the canyon floor (McKinlay et al., 1992), may provide insights into the location of the additional, presently unknown receptor-binding sites utilized by CVA13 and -20.

**NOTE ADDED IN PROOF**

Since the submission of our manuscript the sequences of CVA13, CVA15, CVA18, CVA20 and CVA21 have been confirmed by Brown et al., J Virol 77, 8973–8984.

**ACKNOWLEDGEMENTS**

This research was supported by generous grants from the Greater Building Society, Hunter Medical Research Foundation, National Health and Medical Research Council of Australia and The Knowledge Foundation of Sweden. We gratefully acknowledge those investigators mentioned in the text for the provision of mAbs and viruses that enabled this study to be undertaken.

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