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# 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.

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## 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; Pöyry *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, meningitis 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; Pöyry *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,

GenBank accession numbers for the generated full-length CVA nucleotide sequences are: AF465511 (CVA13); AF465512 (CVA15); AF465513 (CVA18); AF465514 (CVA20) and AF465515 (CVA21).

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 four extracellular 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 different 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 IH4 (IgG1) 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 ($5 \times 10^6$–$1 \times 10^7$ cells ml$^{-1}$) were resuspended in electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0·7 mM $Na_2HPO_4$, 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 pcDNA.neo 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 ($1 \times 10^6$) 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-phycoerythrin-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 ($1 \times 10^5$ $TCID_{50}$ 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 purified 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 $1 \times 10^6$ cells in 800 µl DMEM containing 1 % BSA with 300 µl ($\sim 1 \times 10^5$ 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, cell pellets 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/methanol solution (0·1 % crystal violet, 20 % methanol, 4·0 % formaldehyde in PBS) for 24 h, washed in distilled water and the relative absorbance of the stained cell monolayer read on a multiscan ELISA 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% $CO_2$ 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 as previously described (Lindberg *et al.*, 1997). The RNA was hydrolysed by adding NaOH (aq.) to a final concentration of 0·1 M and the solution was incubated at 37 °C for 20 min. The single-stranded viral cDNA was neutralized by adding sodium acetate, ethanol precipitated, dissolved in TE buffer and stored at −20 °C.

**PCR and long-distance PCR.** Several overlapping PCR fragments were generated using a primer-walking strategy. PCR amplifications were performed as described previously using a long-distance PCR protocol, with a mixture of 2·5 U Thermoprime Plus DNA polymerase (Abgene) and 0·06 U Deep Vent DNA polymerase (New England Biolabs) (Lindberg *et al.*, 1997).

**DNA sequencing.** The nucleotide sequences of the CVA13, -15, -18, -20 and -21 genomes were determined from purified PCR products (QIAquick Gel Extraction kit; Qiagen) in a cycle sequencing reaction using the ABI Prism BigDye terminator cycle sequencing ready reaction kit following the manufacturer's instructions (PE Biosystems). Each nucleotide was determined at least twice in each direction. In order to avoid possible inclusion of mutations generated by the amplification process, the final genome sequences were derived from sequences determined for at least two batches of viral RNA, which were extracted and amplified independently. Sequence data were recorded with an ABI Prism 310 Genetic Analyser (PE Biosystems) and assembled/edited using Sequencher 3.0 software (Gene Codes). Sequence alignments were generated using the ClustalW program (Thompson *et al.*, 1994).

## 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

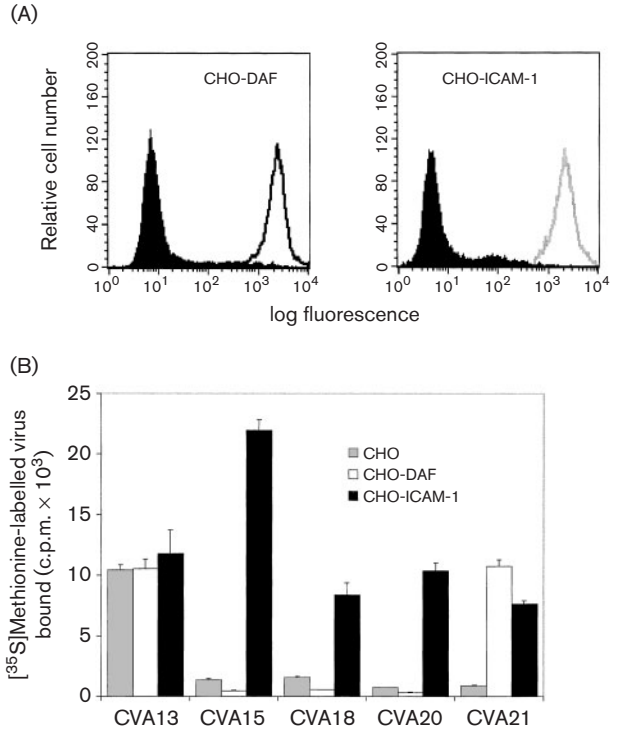


**Fig. 1.** Binding of [$^{35}$S]methionine-labelled CVA13, -15, -18, -20 and -21 to DAF and ICAM-1. (A) Flow cytometric analysis of the level of DAF and ICAM-1 on stably transfected CHO cells (open histograms) compared with conjugate-only controls (filled histograms). (B) Radiolabelled CVA13, -15, -18, -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.

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 ICAM-1 and/or DAF, radiolabelled virus-binding assays were performed using RD cells (which constitutively express DAF) and RD-ICAM-1 cells (RD cells transfected to express ICAM-1). Fluorescence histograms (Fig. 2A) indicated comparable levels of DAF on both RD and RD-ICAM-1 cells with high levels of ICAM-1 expression only on the transfected RD cell line. All strains representing the CVA serotypes tested showed significant levels of binding to ICAM-1 on RD cells as compared with non-transfected cells (Fig. 2B). Furthermore, these data confirmed that CVA13 can bind to ICAM-1 and suggest that the significant levels of attachment to ICAM-1-negative RD cells (Fig. 2B)

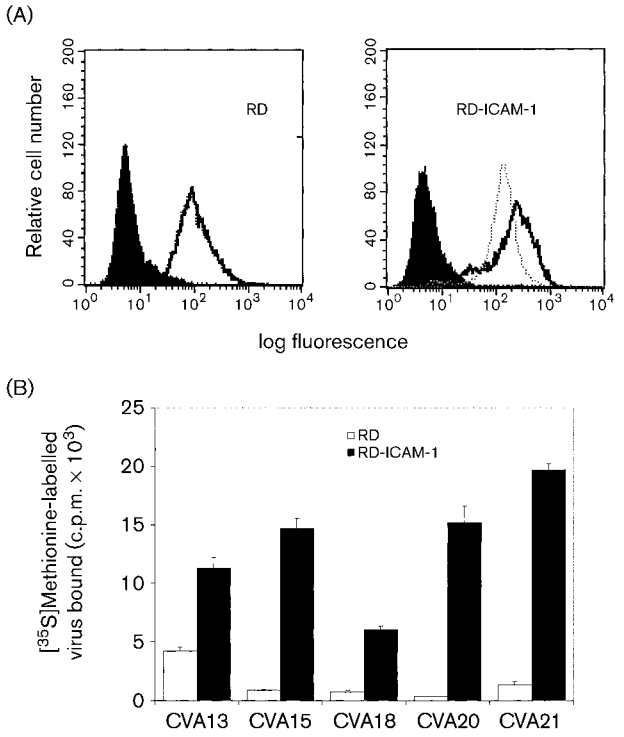


**Fig. 2.** Binding of CVA13, -15, -18, -20 and -21 to RD and RD-ICAM-1 cells. (A) Flow cytometric analysis of the level of DAF (open histogram) and ICAM-1 (dotted histogram) expression on RD and RD-ICAM-1 cells compared with conjugate-only controls (filled histogram). (B) Radiolabelled CVA13, -15, -18, -20 and -21 binding to RD and ICAM-1-expressing RD cells measured by liquid scintillation (Shafren *et al.*, 1995). Results are expressed as the mean of triplicates + SD.

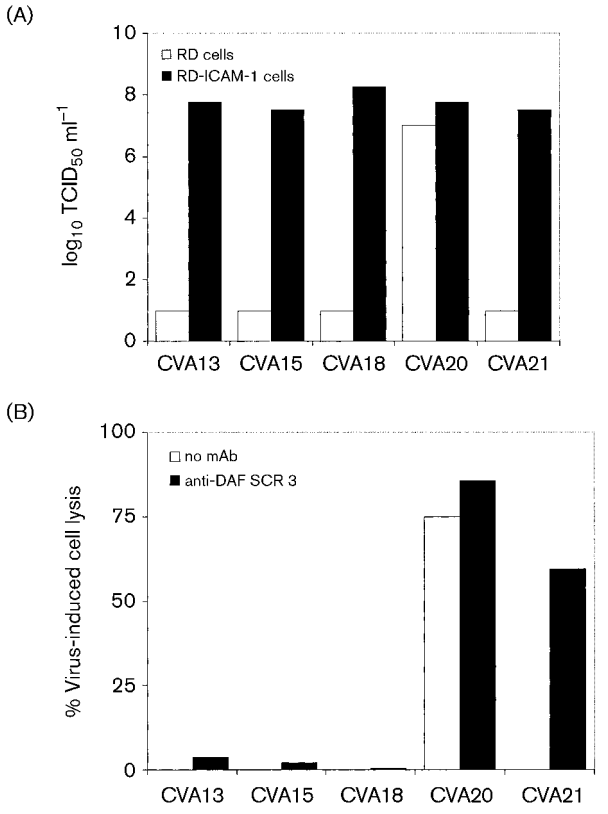


**Fig. 3.** Host-cell lytic infection induced by CVA13, -15, -18, -20 and -21. (A) Virus-induced lytic infection of RD- and ICAM-1-expressing RD cells. Results are expressed as $\log_{10}$ $TCID_{50}$ $ml^{-1}$ of quadruplicate wells. (B) CVA-induced lytic infection of RD cells in the presence and absence of mAb-cross-linked DAF. Results are expressed as mean % virus-induced cell lysis in quadruplicate wells.

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

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        ↓VP4
CVA13   MGAQVSSQKVGAHENTNVATGGSTVNYTTINYYKDSASNAASKQDFSQDPSKFTEPVKDV 60
CVA15   MGAQVSSQKVGAHENTNVATGGSTVNYTTINYYKDSASNAASKQDFSQDPSKFTEPVKDI 60
CVA18   MGAQVSSQKVGAHENTNVATGGSTVNYTTINYYKDSASNAASKQDFSQDPSKFTEPVKDV 60
CVA20   MGAQVSSQKVGAHENTNVATGGSTVNYTTINYYKDSASNAASKQDFSQDPSKFTEPVKDI 60
CVA21   MGAQVSTQKTGAHENQNVAANGSTINYTTINYYKDSASNSATRQDLSQDPSKFTEPVKDL 60
        ******:**.***** ***:.***:**************:*::**:**************:
             VP4↓VP2
CVA13   LIKSAPALNSPNIEACGYSDRVMQLTLGNSTITTQEAANSVVAYGVWPSYLSDKDANPVD 120
CVA15   MLKSAPALNSPNIEACGYSDRVMQLTLGNSTITTQEAANSVVAYGEWPSYLSDKEANPVD 120
CVA18   LIKSAPALNSPNIEACGYSDRVMQLTLGNSTITTQEAANSVVAYGVWPSYLSDKDANPVD 120
CVA20   MLKSAPALNSPNVEACGYSDRVLQLTLGNSTITTQEAANSVVGYGQWPTYLNAKDANPVD 120
CVA21   MLKTAPALNSPNVEACGYSDRVRQITLGNSTITTQEAANAIVAYGEWPTYINDSEANPVD 120
        ::*:********:********* *:***************::*.** **:*:. .:*****

CVA13   QPTEPDVSACRFYTLDTVEWDKESKGWWWKLPDALKDMGLFGQNMYYHYLGRSGYTVHVQ 180
CVA15   QPTEPDVSACRFYTLDTVNWNKASRGWWWKLPDALKDMGLFGQNMYYHYLGRSGYTVHVQ 180
CVA18   QPTEPDVSACRFYTLETVEWDRESKGWWWKLPDALKDMGLFGQNMYYHYLGRSGYTVHVQ 180
CVA20   QPTEPDVSACRFYTLQSVEWKTESKGWWWKLPDALKDMGLFGQNMYYHYLGRSGYTVHVQ 180
CVA21   APTEPDVSSNRFYTLESVSWKTTSRGWWWKLPDCLKDMGMFGQNMYYHYLGRSGYTIHVQ 180
         *******: *****::*.*.  *:********.*****:****************:***

CVA13   CNASKFHQGTLGVFAVPEYCLAGDSNSKTNYTSYVNANPGEAGGKFVSTFTPDTG-TSPK 239
CVA15   CNASKFHQGALGVFAIPEYCMACNTEDKTNYVSYVQANPGEAGGVFTDTYNPSNN-ITGA 239
CVA18   CNASKFHQGTLGVFAVPEYCLAGDSNSKTNYTSYINANPGERGGTFVSTFTPDSG-AVPK 239
CVA20   CNASKFHQGALGVFAVPEYCLAGDSDVKNSYTTYKNANPGETGGVFVDSFT--AT-TQPT 237
CVA21   CNASKFHQGALGVFLIPEFVMACNTESKTSYVSYINANPGERGGEFTNTYNPSNTDASEG 240
        *********:**** :**: :* ::: *..*.:* :***** ** *.:::. . .

CVA13   REFQPVDYLFGCGVMAGNAFVFPHQIINLRTNNCATLVLPYVNSLAIDCMAKHNNWGIVI 299
CVA15   RKFAAVDYLLGCGVLAGNAFVFPHQIINLRTNNCATLVLPYVNSMAIDCMAKHNNWGIAI 299
CVA18   REFQPVDYLFGCGVMAGNAFVFPHQIINLRTNNCATLVLPYVNSLAIDCMAKHNNWGIVI 299
CVA20   RKFCPIDYLFGCGVLTGNAFVFPHQIINLRTNNSATLVLPYVNSLAIDCMAKHNNWGLAI 297
CVA21   RKFAALDYLLGSGVLAGNAFVYPHQIINLRTNNSATIVVPYVNSLVIDCMAKHNNWGIVI 300
        *:* .:***:*.**::*****:***********.**:*:*****:.***********:.*
                                              VP2↓VP3
CVA13   LPLSKLDYNPDASTKLPITVTIAPMCCEFNGLRNLTIPATQGLPVMSTPGSNQYLTSDNF 359
CVA15   LPLAELDFAEASSTEVPITITIAPMCCEFNGLRNLTTPAKQGLPVMNVPGSNQFLSSDNF 359
CVA18   LPLSKLDYNPDASTKLPITVTIAPMCCEFNGLRNLTIPATQGLPVMNTPGSNQYLTSDNF 359
CVA20   IPLSKLQFPDTSSTEIPITVTIAPMCCEFNGLRNITVPSTQGLPVMNTPGSNQYLTSDNF 357
CVA21   LPLAPLAFAATSSPQVPITVTIAPMCTEFNGLRNITVPVHQGLPTMNTPGSNQFLTSDDF 360
        :**: * :   :*.::***:****** *******:* *  ****.*..*****:*:**:*

CVA13   QSPCALPEFDVTQPIFIPGEVKNLMELAEIDTMIPMDLSEGKRNSMDMYRVKISDAGDRN 419
CVA15   QSPCALPEFDVTPPIHIPGEVKNMMELAEIDTLIPMDLSETKKNTMGMYRVELGTSKSQT 419
CVA18   QSPCALPEFDVTQPIFIPGEVKNLMELAEIDTMIPMDLSEGKKNTMEMYRVKLSDTGNRD 419
CVA20   QSPCALPEFDVTQAINIPGEVKNIMEIAEIDTMIPLNLSDSRKNSMDMYRVPVTTSADLD 417
CVA21   QSPCALPNFDVTPPIHIPGEVKNMMELAEIDTLIPMNAVDGKVNTMEMYQIPLNDNLS-K 419
        *******:**** .* *******:**:*****:**::  : : *:* **:: :    .

CVA13   KPILCLSLSPASDPRLSYTMLGEILNYYTHWAGSLKFSFLFCGSMMATGKLLVAYSPPGA 479
CVA15   APILCLSLSPASEQRLGYTMLGEILNYYTHWSGSLKFTFLFCGSMMATGKILISYAPPGA 479
CVA18   KPILCLSLSPASDPRLSYTMLGEILNYYTHWAGSLKFSFLFCGSMMATGKLLVAYSPPGA 479
CVA20   KPILCLSLSPASDERLSYTMLGEILNYYTHWAGSIKYTFLFCGSMMATGKLLIAYAPPGA 477
CVA21   APIFCLSLSPASDKRLSRTMLGEILNYYTHWTGSIRFTFLFCGSMMATGKLLLSYSPPGA 479
         **:********: **. **************:**::::************:*::*:****

CVA13   QPPQDRKAAMLGTHVIWDIGLQSSCTMVVPWISNTSYRRTVKDDFTEGGYISMFYQTRVV 539
CVA15   KPPKTRKEAMLGTHIIWDIGLQSSATMVVPWISNVMYRRCVKDDFTEGGYISMFYQTKIV 539
CVA18   QPPQDRKAAMLGTHVIWDIGLQSSCTMVVPWISNTSYRRTAKDDFTEGGYISMFYQTRIV 539
CVA20   KPPRTRKEAMLGTHVIWDVGLQSSCTMVVPWISNTAYRRTVEDDFTEGGYISMFYQTKIV 537
CVA21   KPPTNRKDAMLGTHIIWDLGLQSSCSMVAPWISNTVYRRCARDDFTEGGFITCFYQTRIV 539
        :**  ** ******:***:*****.:**.*****. *** ..*******:*: ****::*
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**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).

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 is shown in Table 1. VP2 of CVA13, -15, -18 and -20 exhibited 72·4–80·5 % amino acid identity with VP2 of CVA21, while VP3 of these CVAs displayed 75–77·1 % amino acid identity with VP3 of CVA21. VP1 possessed the most variation in amino acid

**Table 1.** Amino acid sequence identity observed in VP1, -2 and -3 of CVA13, -15, -18 and -20 compared with CVA21

| | Sequence identity (%) | | |
|---|---|---|---|
| | VP1 | VP2 | VP3 |
| CVA13 | 63·0 | 72·4 | 75·0 |
| CVA15 | 69·1 | 80·5 | 76·7 |
| CVA18 | 64·8 | 73·2 | 77·1 |
| CVA20 | 66·1 | 73·9 | 75·0 |

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 $\beta$C 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

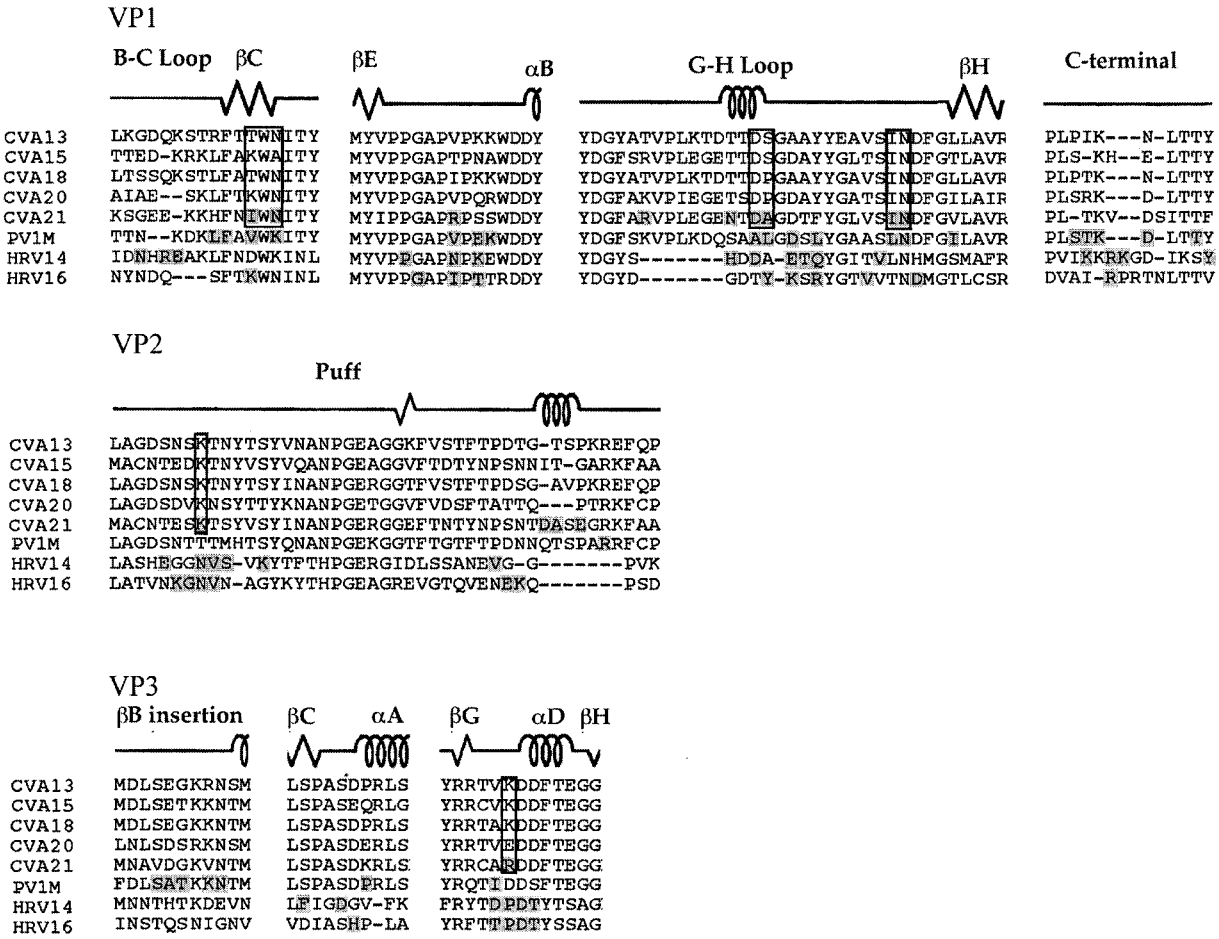


**Fig. 5.** Amino acid sequence alignments for CVA13, -15, -18, -20, -21, PV1, HRV14 and HRV16 in the regions of the viral capsid proteins involved in putative receptor binding. Amino acids residues located in the receptor-binding footprints of ICAM-1 on HRV14, HRV16 (Kolatkar *et al.*, 1999) and CVA21 (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 $\beta$ 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 twofold surface depression consisting of residues from VP2 and VP3 (Shafren *et al.*, 1997b). Recently, the DAF-binding footprint on the echovirus 7 (EV7) capsid was shown to be located close to the icosahedral 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, $\alpha_v$ integrins and $\beta_2$-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 1 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 (Belnap *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.

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