Mapping the binding domains on decay accelerating factor (DAF) for haemagglutinating enteroviruses: implications for the evolution of a DAF-binding phenotype

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Decay accelerating factor (DAF) functions as a cell attachment receptor for a wide range of human enteroviruses, the interaction accounting for the haemagglutination phenotype exhibited by many members of this family. Haemagglutination inhibition assays using purified truncated soluble DAF (sDAF) receptors and short consensus repeat (SCR) domain-specific antibodies have been used to determine the domain(s) of DAF to which the viruses bind. Further sDAF-mediated virus neutralization and biosensor analysis have been used to confirm the virus-binding domains of DAF. Of the four distinct clusters of human enteroviruses, three contain representatives that bind DAF. The majority of DAF-binding enteroviruses occupy the ‘CBV-like’ cluster, and require SCR domains 2–4 for DAF binding. In contrast, the DAF-binding representatives of the ‘ENV70-like’ and ‘PV-like’ clusters require SCR1 for DAF interaction. These studies confirm that DAF binding is a widespread characteristic amongst phylogenetically divergent clusters within the enteroviruses and suggest that the ability to bind DAF may have evolved more than once within this group of viruses.

Introduction

Picornaviruses are small non-enveloped icosahedral, positive-sense, single-stranded RNA viruses that cause a range of diseases in humans and other animals, including paralytic poliomyelitis, encephalitis and myocarditis (Melnick, 1996). Although exhibiting a high level of primary sequence and structural conservation, the enterovirus family of the Picornaviridae, which includes poliovirus (PV), echovirus (EV), the enteroviruses (ENV) and coxsackie A and B viruses (CAV and CBV), cause disease by replication in a divergent range of organ and tissue types. Tissue tropism is at least partly determined by the cellular receptor (Evans & Almond, 1998). Therefore, the identification of receptors and the subsequent characterization of virus binding is a prerequisite to understanding virus pathogenesis.

The human enteroviruses are divided into sub-groups largely on the basis of pathogenicity in experimental animals or, more recently, are numbered in order of identification. Since the virus receptor used influences tropism, and hence pathogenesis, a correlation between the enterovirus sub-grouping and the cellular receptor used for infection would be expected. For example, all serotypes of PV use a member of the immunoglobulin super-family of unknown cellular function [the PV receptor (PVR)] (Mendelsohn et al., 1989). However, the relatively general discrimination made on the basis of pathogenesis conceals significantly wider variation in the range of virus receptors used for cell attachment and infection. Furthermore, molecular techniques enable a distinction to be made between attachment and infection, which can be mediated by the same or different receptors. Since the identification of PVR, a number of additional enterovirus receptors have been identified including: the coxsackie and adenovirus receptor (CAR); the receptor for the coxsackie B viruses (Bergelson et al., 1997a) and, more recently, the αβ integrin (VLA-2), the receptor for EV1 (Bergelson et al., 1992); and decay accelerating factor (DAF; CD55), which was initially identified as the receptor for a subset of the haemagglutinating EV (Bergelson et al., 1994; Ward et al.,...
required for C3 convertase activity (Coyne et al., 1994). Subsequent studies have shown that the haemagglutination (HA) phenotype of enteroviruses, including CAV21 (Shafren et al., 1997a), CBV serotypes 1, 3 and 5 (Shafren et al., 1995) and EV serotypes 3, 6, 6′, 7, 11–13, 19, 21, 24, 25, 29, 30 and 33, is due to interaction with DAF on the erythrocyte (Powell et al., 1998). Although the HA phenotype of CBV3 and CAV21 is a consequence of DAF binding, cell binding and subsequent infection require CAR and ICAM-1, respectively (Shafren et al., 1997a, b). The distinction between the simultaneous ability to bind two attachment molecules and the adaptation of a single isolate to alternative receptors is not always clear (Bergelson et al., 1995; Reagan et al., 1984; Shafren et al., 1995), but it is notable that in the known cases of multireceptor tropism amongst the enteroviruses, DAF is always implicated.

DAF is a member of the regulator of complement activity protein family and prevents the deposition of C3 on the cell surface by enhancing decay of the C3 convertases, so protecting the cell from complement-mediated lysis (Lublin & Atkinson, 1989). The four short consensus repeat (SCR) domains of DAF are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, and separated from the cell membrane by a heavily glycosylated serine- and threonine-rich domain (see Fig. 1). Only SCR domains 2–4 are implicated in virus binding (Evans & Almond, 1998). In the case of EV11, which has similar binding requirements to EV7, we have demonstrated, using surface plasmon resonance analysis, that almost 80% of the binding affinity is located within SCR3 (Lea et al., 1998).

A range of techniques has been used to map the SCR-specificity of DAF-binding viruses. These include the use of SCR-specific MAbs to block binding or infection, virus binding/infection studies using chimeric DAF receptors, and soluble DAF (sDAF) inhibition of virus binding, cell infection or HA. These studies suggest that SCR1 is the principal binding determinant of CAV21 and ENV70 (Karnauchow et al., 1998; Shafren et al., 1997a), SCR2 is the domain bound by rhadomyosarcoma (RD)-adapted CBV3 (Bergelson et al., 1995), and that, where known, SCR3 is implicated in binding the CBV and haemagglutinating EVs (Powell et al., 1998; Shafren et al., 1995).

We have extended these studies using truncated sDAF derivatives, expressed in the yeast *Pichia pastoris*, to determine the SCR domains bound by a range of HA enteroviruses. Our results, together with published data from other laboratories, suggest that, although DAF binding is widespread amongst enteroviruses, there is a clear pattern to the SCR domain(s) required for binding by defined groups of enteroviruses. This suggests that DAF binding may have evolved several times within this group of viruses and that, by inference, there is a selective pressure to bind DAF, indicating that this phenotype is of considerable benefit to the virus.

**Methods**

- **Viruses and antibodies.** EV and coxsackieviruses were a kind gift from Brian Megson, Public Health Laboratory Service, Colindale, London, UK. The isolates had been obtained from the ATCC in 1988 and have been passaged in RD cells. The antibodies used in these studies (BRIC110, BRIC220 and 2D2-1) were a kind gift from G. Daniels at the Bristol blood transfusion service (UK), and have the domain binding specificity (Coyne et al., 1992) shown in Fig. 1. MAb 854 has been described previously (Daniels et al., 1998; Ward et al., 1994).

- **Expression of sDAF deletion mutants in Pichia pastoris.** The expression of sDAF deletion mutants in the *Pichia pastoris* yeast expression system has previously been described in detail (Powell et al., 1997). sDAF containing all the SCRs, but excluding the serine/threonine domain was expressed, as were all contiguous 2 and 3 SCR constructs (sDAF123, sDAF234, sDAF19, sDAF12 and sDAF13) and characterized for correct SCR folding by demonstrating interaction of sDAF fragments with a range of anti-DAF antibodies in an enzyme-linked immunoassay (data not shown). The single N-linked glycosylation site between SCRs 1 and 2 was deleted during the construction of sDAF123 and sDAF12.

- **HA inhibition (HAI) assay using sDAF.** One HA unit is the amount of infected tissue culture (TC) supernatant required to haemagglutinate 50 µl 0.5% human erythrocytes. sDAF deletion mutants were made up to volume in PBS and serially diluted twofold in 50 µl PBS in a 96-well HA plate from a starting concentration of 8 µM. Four HA units

![Fig. 1. Structure of DAF and domain recognition by SCR-specific MAbs. Schematic diagram of the structure of DAF indicating the four SCR domains, the heavily O-glycosylated serine/threonine-rich domain and the GPI anchor. The single N-linked glycosylation site indicated between SCR1 and SCR2 was removed during the construction of sDAF123 and sDAF12. The SCR-specificity of the domain-specific monoclonal antibodies (mAb) is also shown.](image-url)
HAI HAI

preparations (approximately 300
followed by centrifugation through a 30% sucrose cushion. The virus
+
Mapping SCR domain interactions using a biosensor.

staining with crystal violet.

prior to examination for cytopathic effects by light microscopy and
µ
dilutions of sDAF deletion mutants starting from 8


Virus neutralization by sDAF.

Virus and
serotype

Interaction with sDAF domain:*

HAI (MAb) HAI (sDAF) Neutralization Biosensor

EV3 + + − − − − + +
EV6 + + − − − − + + +
EV6‡ + + − − − − + + +
EV6‡ + + − − − − + + +
EV7 + + − − − − + + +
EV11 + + − − − − + +
EV12 + + − − − − + + +
EV13 + + − − − − + +
EV19 + + − − − − + +
EV24 + + − − − − + +
EV29 + + − − − − + +
EV33 + + − − − − + +
CBV1 + + − − − − + +
CBV3 + + − − − − + +
CAV21 + + − − − − + +

* SCR domains of DAF that retain the virus binding determinants (+), or those that have no virus binding
activity (−). ±. SCR domains with demonstrable binding activity that is less than 50% of that observable
with sDAF
†† Procedures applied to analysis of any particular virus type.

of TC-infected virus culture were added in 10 µl PBS and the

=−

HEPES-buffered saline (BIAcore) at a rate of 20 µl/min for 100 s. For
dissociation of virus–receptor complexes between injections, 1200

seconds were permitted. Two negative controls were performed. Each
sDAF fragment was also assayed for the ability to interact non-
specifically with a blank sensor chip and gave a negligible shift of 20 ± 10
RU. Subsequently, an irrelevant protein (1%, w/v BSA) was injected over
the immobilized viruses and gave a shift of 150 ± 30 RU (data not shown).
The background binding due to non-specific interactions is
estimated as the sum of these values, i.e. approximately 170 RU. The
actual background values measured were 120 RU for EV6, 130 RU for
EV6‡, 150 RU for EV7 and 200 RU for EV12.

Results

HAI assays

The HA phenotype of EV is explained by the binding of the

of DAF binding amongst human EV to
investigate the receptor domains involved in the virus–
receptor interaction. Two types of HAI assays were performed
on a range of DAF-binding viruses, using antibodies to defined SCR domains of DAF or purified sDAF proteins. Representative results are discussed below and tabulated in Table 1.

**HAI assays with anti-DAF antibodies.** A representative sample of DAF-binding viruses were screened in a HAI assay (Fig. 2, Table 1) following treatment of erythrocytes with each of a panel of anti-DAF MAbs that have previously been mapped to the four SCR domains of the receptor (see Fig. 1). A standardized number of HA units of each virus was tested against doubling dilutions of the antibodies.

MAb BRIC220, which is specific for SCR1, the most membrane-distal domain of DAF, blocked HA by CAV21, but not by any of the other viruses tested. The SCR2-specific MAb BRIC110 inhibited HA by CBV3 and EV13. These two viruses, together with EV7 and EV12, were also blocked from binding DAF on erythrocytes by MAbs 854 and 2D2-1, which are specific for SCR3 and SCR4, respectively. These results suggest that, although there are qualitative differences between EV7, EV12, EV13 and CBV3 receptor binding, in particular the potential involvement of SCR2, all predominantly interact with SCR domains 3 and 4. In contrast, and in line with published results, CAV21 interacted in a distinctly different manner to the other viruses tested, and was only blocked by antibodies to SCR1.

**HAI with sDAF.** The HA reaction mediated by virus binding to DAF can be inhibited by the presence of sDAF or sub-fragments of DAF that authentically mimic the virus binding site (Powell et al., 1997). We have extended our original studies of EV7 to map the binding sites of a range of HA enteroviruses using this approach and show the results obtained from a representative sample of genetically divergent serotypes in Fig. 3 and Table 1. The results obtained generally correlate well with those determined by HAI with anti-DAF MAbs. As previously reported, HA by EV7 is blocked by sDAF and sDAF, a pattern similar to that with EV6 and CBV3. In addition to sDAF and sDAF, EV12 and EV29 are also blocked by sDAF, implying that this two domain construct retains the ability to bind the virus, although the end-point titre is reduced in comparison with the larger receptor derivatives. The HAI pattern obtained with CAV21 is significantly different, and correlates less well with the data obtained with BRIC110 (Fig. 2), in that inhibition is only observed in the presence of sDAF; the truncated sDAF derivatives that retain SCR1 do not block HA by this isolate.

We have previously commented on the transitory nature of the HA phenotype displayed by certain EV serotypes which have a weak affinity for DAF (Powell et al., 1998), and consequently display weak HA reactions. This can be seen in Fig. 3 for EV29 (and to a lesser extent EV6') in the presence of
sDAF123, which display pinprick ‘buttons’ that are unrelated to sDAF concentration, and which can clearly be distinguished from HAI on the same plate.

**Virus neutralization by sDAF deletion mutants**

We have previously demonstrated that sDAF123, or derivatives that retain the ability to bind virus, are able to reversibly neutralize virus infection of susceptible cell lines by sterically preventing the virus from interacting with cellular DAF (Powell et al., 1997). We have investigated the ability of a range of enteroviruses to be neutralized by sDAF123, or the truncated forms of the soluble receptor (see Table 1). In the case of EV6’, 6’, 7, 12, 13, 29 and 33, the pattern of neutralization observed correlated precisely with the HAI receptors.

Direct measurement of SCR binding using a biosensor

Real time biological interactions can readily be detected by measuring changes in surface plasmon resonance; the use of this technique for studying picornavirus–receptor interactions has recently been demonstrated for human rhinovirus and ICAM-I (Casasnovas & Springer, 1995) and EV11 binding to sDAF (Lea et al., 1998). We have used this approach at a qualitative level to investigate simple positive binding interactions for sDAF truncated receptors and EV6, 6’, 7 and 12. The approach used was essentially as described previously (Lea et al., 1998), with similar levels of virus immobilized on the sensor chip and the interaction with sDAF, or a non-specific protein (BSA), present in the soluble phase, determined. In each case, sample injection resulted in an increase in the signal (measured in RU) over the background level, reflecting bulk flow effects. Signals higher than those observed with the control BSA were considered specific. For example, EV7 generated a signal of 150 RU with 1% BSA (data not shown), similar to that seen with sDAF123 or any of the two domain sDAF products (Fig. 4). In contrast, sDAF123 and sDAF234 both generated significantly higher signals, indicative of a specific association between the virus and the receptor derivative, in line with the studies we have previously published (Lea et al., 1998).

Using this approach with EV6, 6’, 7 and 12, results were obtained that are consistent with those demonstrated by HAI and/or soluble receptor-mediated virus neutralization. sDAF123, sDAF15 and sDAF23 displayed no reactivity with any of the viruses tested. As expected, all reacted with sDAF123 and, in line with other assays, sDAF234. Of the viruses tested, only EV12 reacted with sDAF234.

**Discussion**

Having initially been identified as an EV receptor (Bergelson et al., 1994; Ward et al., 1994), further studies have shown that DAF binding is widespread amongst the human enteroviruses, including at least 11 serotypes of EV, CBV1, 3 and 5, CAV21 and ENV70 (Karnauchow et al., 1996; Powell et al., 1998; Shafren et al., 1995, 1997a). Proteins other than DAF may also be involved in virus binding or infection; at least in the case of CBV3 and CAV21, there is compelling evidence that cell surface proteins including CAR or ICAM-1, αβ microglobulin (Ward et al., 1998) or the complement regulatory protein CD59 (I. Goodfellow & D. J. Evans, unpublished results), although probably not directly binding to the virus, are part of the virus–receptor complex that forms during cell infection. This situation is further complicated by the natural variation seen in the receptor specificity of primary isolates (Bergelson et al., 1997b) and the apparent ease with which variant viruses can be selected that gain or lose the ability to interact with certain receptors. The HeLa cell receptor for ENV70 has been reported as DAF (Karnauchow et al., 1996), but the isolate available in our laboratory had been repeatedly passaged in RD cells and had lost the ability to haemagglutinate erythrocytes (data not shown), presumably due to the loss of the ability to bind DAF. Notwithstanding

![Fig. 4. Qualitative biosensor analysis of enterovirus–DAF binding. Approximately 10000 RU of each of the four indicated serotypes was independently immobilized on the biosensor chip; the interaction with truncated sDAF protein was monitored for 1200 s.](image-url)
these points, the incidence of DAF binding amongst the human enteroviruses is striking.

We are interested in the origin and evolution of DAF binding by the human enteroviruses. To this end, we have investigated the domains of DAF with which the DAF-binding haemagglutinating enteroviruses interact by HAI assay, virus neutralization and, for a limited number of viruses, by measuring the direct interaction of virus and receptor using a biosensor. We have previously demonstrated, using EV7, that HAI and virus neutralization using sDAF enables the identification of the receptor domains with which the virus interacts (Powell et al., 1997), and that these results correlate well with the observed binding to receptor variants expressed at the surface of transfected cells (Clarkson et al., 1995).

The majority of viruses used in this study have been tested using at least two assays and the results obtained, summarized in Table 1, are generally in good agreement with alternative assays reported here and previous published studies. As expected, and irrespective of the type of assay used, all viruses interacted with sDAF<sub>1234</sub>, and the EV and CBV isolates also bound sDAF<sub>234</sub>. These results are in agreement with our previous studies of EV7, using sDAF and CHO cells expressing chimeric DAF receptors (Clarkson et al., 1995; Powell et al., 1997), in which we suggested that the integrity of the virus binding site on the receptor required the presence of the three SCR domains, even though blocking studies with MAb 854 (anti-SCR3), and subsequent biosensor analysis of EV11–DAF binding (Lea et al., 1998), suggested that SCR3 was the principal domain involved in the interaction. The HAI results using CBV3 are also in agreement with antibody inhibition of DAF binding in which SCR2 and SCR3 were implicated (Martino et al., 1998; Shafren et al., 1997 b). In contrast, deletion of SCR2 alone abrogated binding to cell-surface expressed DAF by an RD-adapted CBV3 (CBV3-RD) (Bergelson et al., 1995), suggesting that there are differences in the specificity of binding within this group of viruses. This conclusion could also be drawn from the differential reactivity of sDAF<sub>234</sub> to the viruses tested. EV12 and 29 alone exhibited binding to the two membrane-proximal SCR domains of sDAF in HAI assays (Fig. 3), a result in agreement with the antibody-mediated HAI and biosensor studies on EV12 (Figs 2 and 4). CAV21 was efficiently blocked in an HAI assay by BRIC220 (Fig. 2), an SCR1-specific MAb, in agreement with previous domain mapping studies for this virus (Shafren et al., 1997 a). HAI assays using sDAF<sub>12</sub> exhibited no inhibition of CAV21, sDAF<sub>123</sub>, showed only very weak inhibition and sDAF<sub>1234</sub> blocked HA significantly less well than for any of the other viruses tested (Fig. 3). The P. pastoris-expressed sDAF proteins used in these studies fold in an authentic manner, as confirmed by MAb binding analysis (Daniels et al., 1997), inhibition of complement fixation assays (I. Goodfellow & B. Sim, unpublished data) and preliminary NMR structural analysis (data not shown). However, the yeast-expressed sDAF<sub>1234</sub> differs from the cellular protein in that it is only partially glycosylated (approx. 70% by SDS–PAGE; data not shown) at the single N-linked site between SCR1 and SCR2 (Asn<sub>64</sub>), which was also removed during the construction of plasmids encoding sDAF<sub>12</sub> and sDAF<sub>123</sub>. We therefore interpret the CAV21 HAI results as indicating that the nature or completeness of the glycosylation of sDAF<sub>1234</sub>, or the lack of carbohydrate on sDAF<sub>123</sub> or sDAF<sub>12</sub> may affect CAV21–DAF interactions. Although there is a precedent for this interpretation – ENV70, a virus that binds SCR1 (Karnauchow et al., 1998), exhibits a neuraminidase-sensitive HA phenotype.

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**Table 2. Genetic clusters of human enteroviruses**

The human enteroviruses can be grouped into four clusters, of which the ‘CAV16-like’ A cluster contains no known DAF-binding viruses. The remaining three clusters are indicated, listing the viruses for which sequence data are available on which the groupings were made [based on the data of Pöyry et al. (1996) and Pulli et al. (1995)]. Viruses known to bind DAF are underlined. Other receptor usage identified within each cluster is indicated. Viruses for which a receptor has yet to be identified are given in italics.

<table>
<thead>
<tr>
<th>CBV-like (B-cluster)</th>
<th>PV-like (C-cluster)</th>
<th>ENV70-like (D-cluster)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAV type</td>
<td>9</td>
<td>1, 11, 13, 15, 17, 18, 19, 20, 20a, 20b, 21, 22, 24</td>
</tr>
<tr>
<td>CBV type</td>
<td>4, 5</td>
<td>–</td>
</tr>
<tr>
<td>ENV type</td>
<td>69</td>
<td>–</td>
</tr>
<tr>
<td>EV type</td>
<td>1, 4, 6, 7, 11, 12, 27, 30</td>
<td>–</td>
</tr>
<tr>
<td>PV type</td>
<td>–</td>
<td>68, 70</td>
</tr>
<tr>
<td>Additional receptor usage</td>
<td>CAR (CBV1, 3, 5)</td>
<td>PVR (PV1–3)</td>
</tr>
<tr>
<td></td>
<td>VLA-2 (EV1)</td>
<td>ICAM-1 (CAV13, 15, 18, 20, 21)</td>
</tr>
</tbody>
</table>
(Utagawa et al., 1982) suggesting that sialic acid and N-linked carbohydrate may be involved in the binding – further studies are required to determine whether the differences noted in CAV21 binding reflect a direct interaction with the carbohydrate moiety, or if glycosylation may be required for the correct presentation of a binding site occupying the N-terminus of DAF.

How has DAF binding evolved to be such a widespread characteristic of the human enteroviruses? One possibility, which these studies do not rule out, is that the ancestral enterovirus possessed this characteristic which has subsequently been retained by many representatives due to selective pressure. Alternatively, the ability to bind DAF may have evolved multiple times within the human enteroviruses. The latter conclusion is supported by distribution of DAF-binding isolates amongst three of the four clusters of human enteroviruses identified in phylogenetic studies (see Table 2; Pöyry et al., 1996; Pulli et al., 1995): CAV21 in the ‘PV-like’ C-cluster, ENV70 in the ‘ENV70-like’ D-cluster, and the remainder (CBV1, 3 and 5, EV6, 7, 11, 12 and 30) in the ‘CBV-like’ B-cluster. In the absence of sequence data from the haemagglutinating EV omitted from Table 2, for which the DAF-binding specificity is known (EV6, 6”, 13, 19, 24, 29 and 33; see Table 1), it seems highly likely that these serotypes would also occupy the ‘CBV-like’ B-cluster. It is striking therefore, that the two broad classes of DAF interaction, with SCR1 (CAV21, ENV70) or SCR2–4 (the rest), are located in distinct clusters. Furthermore, the separate clustering of CAV21 and ENV70 (Table 2), and the presumed rhinovirus/poliovirus recombinant origin of CAV21 (Hughes et al., 1989), suggest that the ability to bind SCR1 may have evolved independently in these two viruses. Further studies are under way to determine whether the differences in binding catalogued between representatives of the ‘CBV-like’ cluster, for example the ability to bind sDAF14 by EV12 and 29, represent subtle variation within the group, or the independent evolution of a distinct binding footprint.

These interpretations make the assumption that DAF binding is a physiologically relevant phenotype that has evolved because it offers a selective advantage to the virus within the host, which seems reasonable in light of the DAF binding observed in cardiovirulent coxsackieviruses and clinical isolates (Bergelson et al., 1997b; Martino et al., 1998). The nature of this advantage is the subject of further study.

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References


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