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Characterization of the Echovirus 7 Receptor: Domains of CD55 Critical for Virus Binding

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CD55, or decay-accelerating factor (DAF), is a cell surface glycoprotein which regulates complement activity by accelerating the decay of C3/C5 convertases. Recently, we and others have established that this molecule acts as a cellular receptor for echovirus 7 and related viruses. DAF consists of five domains: four short consensus repeats (SCRs) and a serine/threonine-rich region, attached to the cell surface by a glycosylphosphatidyl inositol anchor. Chinese hamster ovary cells stably transfected with deletion mutants of DAF or DAF-membrane cofactor protein recombinants were analyzed for virus binding. The results indicate that the binding of echovirus 7 to DAF specifically requires SCR2, SCR3, and SCR4. There is also a nonspecific requirement for the S/T-rich region which probably functions to project the binding region away from the cell membrane. The three nonpeptide modifications of DAF, N-linked glycosylation, O-linked glycosylation, and the glycosylphosphatidyl inositol anchor, are not required for virus binding. The SCRs of membrane cofactor protein, the closest known relative of DAF, cannot substitute for those of DAF with retention of virus binding activity. The monoclonal antibody used to identify DAF as an echovirus receptor, and which inhibits binding of the virus (monoclonal antibody 854), binds to SCR3.

Echoviruses are nonenveloped single-stranded positive-sense RNA viruses belonging to the *Enterovirus* genus of the *Picornaviridae* family. They are associated with a range of clinical conditions, including diarrhea, rashes, and most notably viral meningitis, being responsible for about 30% of cases. They also cause respiratory illness in young children and occasionally fatal disseminating illness in neonates. Conjunctivitis is also associated with certain strains (19).

The attachment and entry of viruses into host cells via interaction with specific cell surface receptors is a principal determinant of host range and cell tropism and consequently of virus pathogenesis. The cellular receptors for some enteroviruses have been identified. For example, the receptor for poliovirus is a member of the immunoglobulin superfamily, the function of which is not yet known (20), and the receptor for echoviruses 1 and 8 is the integrin VLA-2, which normally binds to collagen and laminin (2, 3). Receptors for other picornaviruses have also been identified. These include the intercellular adhesion molecule 1 (ICAM-1), which is the receptor for the major group of human rhinoviruses (9, 29, 30), and members of the low-density lipoprotein receptor family, which appear to function as receptors for the minor group of human rhinoviruses (10).

Virus-receptor interactions have been characterized for both poliovirus-poliovirus receptor (11, 23, 27), and rhinovirus–ICAM-1 (28). In both cases, the N-terminal region of the receptor has been shown to be important for virus binding. This is also true for some other well-characterized virus-receptor interactions outside the picornavirus family, such as human immunodeficiency virus type 1–CD4 (12) and Epstein-Barr virus–CR2 (5, 13).

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Recently, we and others have identified CD55, or decay-accelerating factor (DAF), as a cellular receptor for echoviruses 7, 13, 21, 29, and 33 (1, 31). DAF is a single extracellular glycoprotein with an M_r of approximately 70,000 attached to the membrane by a glycosylphosphatidyl inositol (GPI) anchor (Fig. 1A) (7, 17, 18). The molecule has five domains, including four short consensus repeats (SCRs), each having approximately 60 amino acid residues (including four cysteines), and a C-terminal serine/threonine-rich region of approximately 70 amino acids which ends in a GPI anchor (4, 17, 18). These SCRs show a low degree of homology to one another, from 18% between SCR2 and SCR4 to 30% between SCR3 and SCR4. Other modifications include a single N-linked oligosaccharide, attached between SCR1 and SCR2, and multiple O-linked oligosaccharides, attached to the S/T-rich region (14).

DAF is a member of the RCA (regulator of complement activation) gene family, which plays a role in down-regulation of complement activity at the cell surface (25). It interacts with the C3/C5 convertase enzymes, which are critical and central in both the classical and alternative complement pathways, with the result that the convertases are disassembled (decay accelerated) and thus inactivated. The interaction between DAF and the C3/C5 convertases has been characterized at the molecular level. All domains other than domain 1 are required for functional interaction with the C3/C5 convertases, although the requirement for the S/T-rich region appears to be steric rather than specific (6).

To characterize the binding of echovirus 7 to DAF, we have studied a series of deletion mutants, each lacking a major domain of DAF, and recombinant molecules between DAF and the closely related membrane cofactor protein (MCP). The results of these studies indicate the domains required for echovirus 7 binding and whether these requirements are sequence specific. The conclusions are supported by competition assays between domain-specific monoclonal antibodies (MAbs)

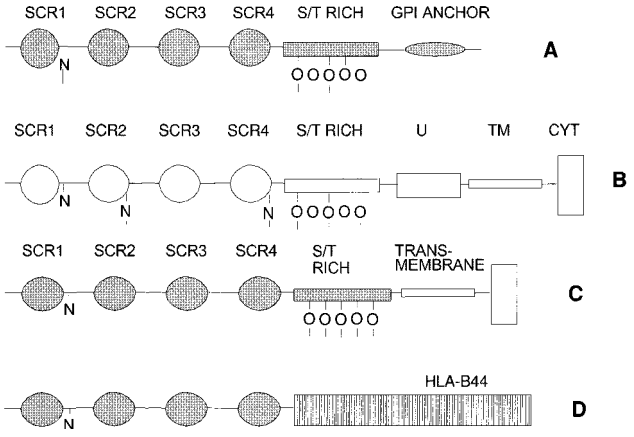

FIG. 1. (A) Schematic representation of the DAF molecule, showing the four SCR domains, the S/T-rich region, the GPI anchor, and sites of glycosylation (N and O linked). The SCR domains consist of approximately 60 amino acids. The relative mass of the complete glycoprotein is 70 kDa, with N-linked glycosylation accounting for about 3 kDa and O-linked glycosylation accounting for about 18 kDa (4, 7, 17, 18). The ΔSCR1 DAF molecule lacks the N-linked oligosaccharide, and the ΔS/T-rich DAF construct lacks the O-linked oligosaccharides. (B) MCP also has four SCR domains and an S/T-rich region but has transmembrane (TM) and cytoplasmic (CYT) domains instead of a GPI anchor. It has a region of unknown function (U) between the S/T-rich and transmembrane domains. MCP has three sites of N-linked glycosylation. (C) The transmembrane form of DAF has the transmembrane and cytoplasmic domains of MCP replacing the GPI anchor. (D) The DAF–HLA-B44 chimera contains SCR1 to SCR4 but lacks the S/T-rich region, and hence the O-linked oligosaccharides and the GPI anchor. These are replaced by the C-terminal region (including the membrane-proximal domains) of HLA-B44.

and echovirus 7. We have also determined the region of DAF bound by the MAb (MAb 854) which blocks binding to and infection of RD cells by echoviruses 7, 13, 21, 29, and 33 and which was used in previous experiments to identify DAF as a virus receptor.

MATERIALS AND METHODS

Materials. Ham's F12 tissue culture medium was obtained from Gibco. Sheep anti-mouse immunoglobulin (^{35}S and biotin labelled) and ^{35}S-labelled streptavidin were obtained from Amersham International Plc. MAb 854 was prepared as described previously (21, 31). Other anti-DAF MAbs (BRIC220, BRIC110, 1H4, and 8D11) have been described previously (6).

CHO cells expressing DAF. The construction of Chinese hamster ovary (CHO) cells expressing DAF and mutants thereof has been described previously (6). Briefly, CHO cells were transfected with the appropriate recombinant molecule within the expression vector SFFV.neo by using Lipofectin and selected for resistance to G418. Clones were selected by several rounds of sorting by using flow cytometry with a polyclonal serum, and limiting dilution was used to produce subclones. Surface expression levels of DAF were then assessed by flow cytometry.

The construction of cells expressing DAF-MCP recombinants is described elsewhere (16). Briefly, this was achieved by PCR amplification of the relevant domain of MCP, and of the DAF clone so as to lack a single domain, followed by ligation. Individual SCRs of MCP were produced by PCR amplification, using Vent DNA polymerase on 10 ng of the CD46 plasmid with primers which matched the individual SCRs. Production of DAF lacking in a single domain was achieved by PCR amplification of the DAF clone in a pBluescript vector, using inverse primers which amplified the DAF clone and the vector but not the SCR inside the primers. The SCR from each MCP clone was then ligated into the DAF clone, and the insertion was verified by DNA sequence analysis. The chimeras replaced DAF SCR2 (amino acids 63 to 126) with MCP SCR2 (amino acids 63 to 125), DAF SCR3 (amino acids 127 to 188) with MCP SCR3 (amino acid 126 to 191), and DAF SCR4 (amino acids 189 to 251) with MCP SCR4 (amino acids 192 to 251) (all numbering starts with the amino terminus of the mature protein).

CHO binding assays. CHO cells were cultured in Ham's F12 with 10% fetal calf serum and penicillin-streptomycin (100 μg/ml of each). Approximately 10^7 cells were harvested in EDTA, washed thoroughly in phosphate-buffered saline (PBS), then split into duplicate fractions, and incubated with 10 μl of ^{35}S-labelled echovirus 7 (8,000 CPM/μl) for 1 h at 4°C in a total volume of 50 μl. Cells were then pelleted (at 4,000 rpm for 5 min) and washed three times in PBS before resuspension in water and scintillation counting. For MAb binding studies, antibody was added for 1 h at 4°C in a total volume of 50 μl. Cells were then pelleted and washed three times with PBS before secondary antibody (sheep anti-mouse, ^{35}S labelled) was added in excess (1:100 dilution). This mixture was incubated for 1 h at 4°C, and the cells pelleted and washed three times in PBS before being resuspended in water and subjected to scintillation counting. Alternatively, a biotin-labelled sheep anti-mouse second antibody was used, and after washing, ^{35}S-labelled streptavidin was added for 1 h at 4°C. This mixture was then washed three times with PBS and scintillation counted.

Competition binding assays. MAbs BRIC220, BRIC110, 1H4, and 8D11 were assessed for the ability to block echovirus 7 from binding to intact DAF. Each MAb, at various dilutions, was bound to CHO cells expressing DAF for 1 h at 4°C in a total assay volume of 50 μl. Echovirus 7 (^{35}S labelled) was then added, and virus binding was measured as described above.

Radiolabelling of echovirus 7. ^{35}S-labelled echovirus 7 was grown and purified as described previously (22) for poliovirus, using RD rhabdomyosarcoma cells. Briefly, RD cells were grown in tubes in roller bottles and infected with virus for 1 h before addition of minimal essential medium deficient in methionine. [^{35}S]methionine was added 6 h later, and infection was allowed to proceed for 24 h. Harvested virus was purified by sucrose gradient centrifugation.

RESULTS

To identify the domains of DAF that are important for binding echovirus 7, ^{35}S-labelled virus was incubated with CHO cells stably expressing deletion mutants of DAF. The panel of DAF mutants included those with deletions of domains SCR1, SCR2, SCR3, and SCR4 and the S/T-rich region. These molecules were first characterized by using MAbs against individual domains of the molecule to ensure that the deletions did not cause a severe disruption of conformation (6). The levels of expression of mutant DAF molecules in the cell lines, relative to a line expressing intact DAF, were measured in radioimmunoassays using MAb 854 (described in reference 31) or MAb 8D11 (Fig. 2A). This assay was performed with an anti-mouse antibody labelled with ^{35}S to measure MAb binding levels. Relative amounts of virus binding to these cells, normalized for DAF expression levels, are shown in Fig. 2B. The results clearly indicate that deletion of domain SCR1 has no effect on virus binding, whereas deletion of any of the other domains abrogates binding completely.

MAb 854 blocks both binding and infection of cells by echovirus 7 in vitro and was used to identify DAF as a receptor for echovirus 7 (31). The cell line expressing the DAF mutant ΔSCR3 was unable to bind MAb 854 but did bind MAb 8D11. A similar result was obtained with a cell line expressing a DAF molecule in which domain SCR3 had been replaced by a foreign domain (see below). By contrast, all cell lines expressing other domain deletion mutants of DAF or DAF molecules with other domain substitutions bound MAb 854. These results suggest that MAb 854 binds to domain SCR3, a result consistent with the fact that this antibody inhibits virus binding. Previous studies have established that MAb 8D11 binds to domain 4 (6).

The conclusions presented above on virus binding to DAF deletion mutants were broadly corroborated by the results of competition binding experiments between echovirus 7 and a series of MAbs (Fig. 3). In this case, CHO cells expressing the complete DAF molecule were preincubated with antibodies against known individual domains of DAF at a range of dilutions (Fig. 3A), after which radiolabelled virus was added (Fig. 3B). MAb 1H4, which is known to be directed against SCR3, inhibits virus binding by over 90%, indicating the importance of this domain. MAbs 8D11 and BRIC110, which bind to SCR4 and SCR2, respectively, inhibit virus binding by 77 and 49%, confirming the importance of these domains also. Surprisingly, MAb BRIC220, directed against domain SCR1, inhibited virus binding by 35%, suggesting some steric effect or possibly induced conformational change of one of the other domains.

To assess which regions of the DAF molecule are specifically required for echovirus 7 binding, we analyzed a series of domain replacements. These replacements were constructed by

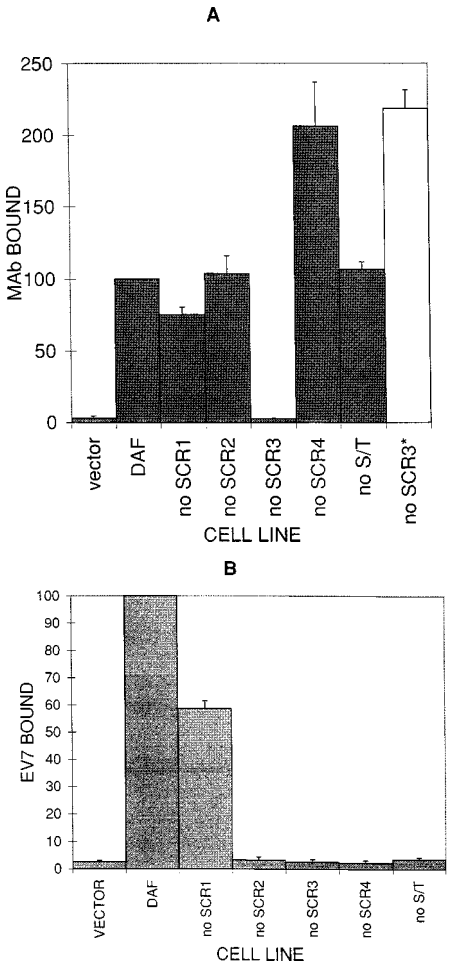

FIG. 2. Binding of virus and antibodies to cells expressing partially deleted DAF molecules. "no SCR1" refers to the molecule with SCR1 deleted (referred to as ΔSCR1 in the text), "no SCR2" refers to the molecule with SCR2 deleted, etc.; "no S/T" refers to the molecule with the S/T-rich region deleted. Error bars indicate standard errors calculated from at least three separate experiments. (A) Levels of expression of DAF and DAF deletion mutants in CHO cell lines. MAb 8D11 was used to measure expression of the construct lacking SCR3 (8D11 binding to this cell line is marked "no SCR3*" and is represented by the hollow bar), whereas the levels of expression of the remaining molecules were measured by using MAb 854 (represented by filled bars). Antibody binding to each cell line is expressed as a percentage of the binding of antibody to cells expressing the intact DAF molecule. (B) Binding of radiolabelled echovirus 7 (EV7) to CHO cell lines expressing DAF. Each value represents the percentage of virus bound by cells expressing intact DAF, after adjustment for levels of expression of recombinant molecules determined as described for panel A.

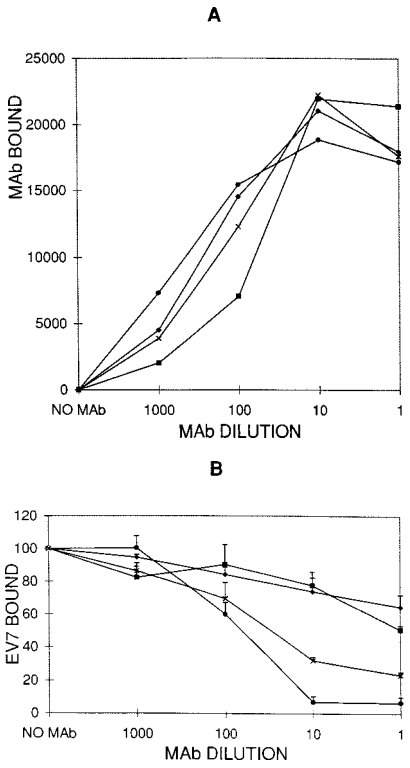

FIG. 3. Antibody saturation and antibody-virus competition studies with cells expressing intact DAF. ◆, BRIC220 (SCR1); ■, BRIC 110 (SCR2); ●, 1H4 (SCR3); ×, 8D11 (SCR4). (A) Binding of anti-DAF MAbs to CHO cells expressing intact DAF. Results are expressed as counts per minute of bound secondary antibody. (B) Competition studies between echovirus 7 (EV7) and anti-DAF MAbs for binding to intact DAF. MAbs, at the range of dilutions shown, were bound to CHO cells expressing intact DAF, and radiolabelled echovirus 7 was then added. Results are expressed as percentages of virus bound to DAF in the absence of antibody and include error bars indicating standard errors as calculated from at least three separate experiments. All dilutions are of hybridoma culture media and are expressed as dilution factors.

using MCP for the SCRs and HLA-B44 for the S/T-rich region. MCP, recently identified as the receptor for measles virus (8, 24), is also a member of the RCA gene family and is the closest known relative of DAF. It has a predicted domain structure which is highly similar, except that it has an additional region of unknown function following the S/T-rich region and a transmembrane and cytoplasmic domain instead of a GPI anchor (Fig. 1B) (15). We were therefore able to construct straightforward replacements of DAF domains SCR2, SCR3, and SCR4 (SCR2DM to SCR4DM) by using this molecule. We also replaced the GPI anchor of DAF with the membrane-spanning and cytoplasmic domains of MCP (Fig. 1C). To replace the S/T-rich region, we used the membrane-proximal and C-terminal regions of HLA-B44 (Fig. 1D). Expression levels of these molecules were assessed by measuring the binding of MAb 854 as shown in Fig. 4A. As MAb854 was known to bind to SCR3, the expression of mutant SCR3DM, which has this domain replaced by the equivalent domain of MCP, was determined by using MAb 8D11. The level of MCP expression was determined using anti-MCP MAb E4.3 (data not shown). The conformational integrity of the remaining DAF domains within these molecules was checked by determining binding to the panel of MAbs as described previously (16). As can be seen in Fig. 4, neither MAb 854 nor echovirus 7 binds to MCP. The results of studies of virus binding on these recombinant molecules (Fig. 4B) indicate that domains SCR2, SCR3, and SCR4 of DAF cannot be replaced by the equivalent MCP domains. This finding agrees with the data presented above for deletion mutants and confirms that domains SCR2, SCR3, and SCR4 of DAF are essential for virus binding.

DAF has three nonpeptide posttranslational modifications: (i) an N-linked oligosaccharide (attached between SCR1 and SCR2), (ii) multiple O-linked oligosaccharides (in the S/T-rich region), and (iii) a GPI anchor. A possible role for the N-linked oligosaccharide in virus binding is ruled out by the data presented in Fig. 2B, which show that the DAF mutant lacking SCR1, which also lacks this modification, binds virus. Data presented in Fig. 4B indicate that the O-linked oligosaccharides are also not required, since the DAF–HLA-B44 recombinant, which consists of the four SCRs of the DAF molecule attached directly to the HLA-B44 molecule, also binds virus. The DAF–HLA-B44 recombinant also lacks the S/T-rich re-

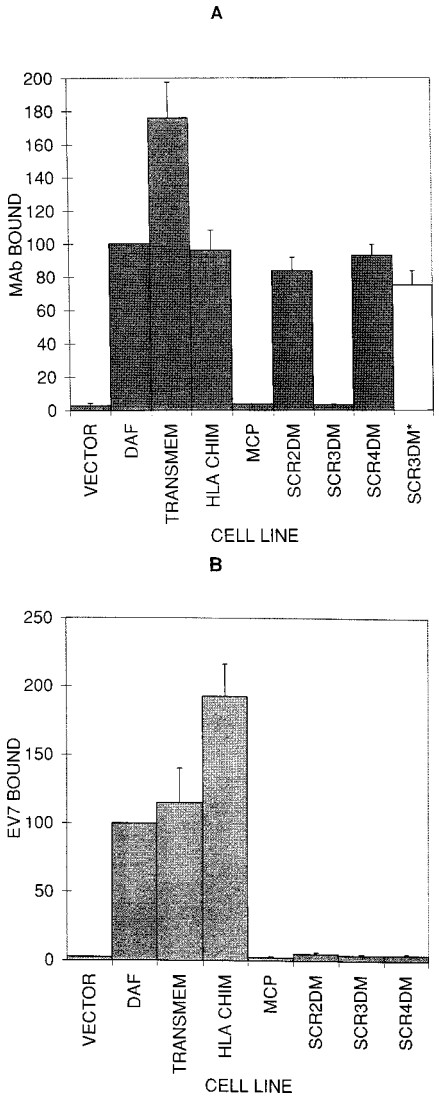

FIG. 4. Binding of virus and antibodies to cells expressing recombinant DAF molecules. TRANSMEM, DAF with the transmembrane and cytoplasmic domains of MCP; HLA CHIM, DAF with the C-terminal region of HLA-B44. Values include error bars representing the standard errors calculated from at least three separate experiments. (A) Binding of anti-DAF MAbs 854 and 8D11 to CHO cell lines. MAb 8D11 was used to measure expression of SCR3DM (marked "SCR3DM*" and represented by a hollow bar), whereas the levels of expression of the remaining molecules were measured by using MAb 854 (represented by filled bars). Antibody binding to the various cell lines is expressed as a percentage of the binding of antibody to cells expressing the intact DAF molecule. (B) Binding of echovirus 7 (EV7) to CHO cell lines expressing DAF and DAF recombinants. Each value represents the percentage of virus bound by cells expressing intact DAF, after adjustment for levels of expression of recombinant molecules determined as described for panel A.

gion, showing that this region is also not essential for virus binding. This last result contrasts with that presented above indicating that deletion of the S/T-rich region abrogates virus binding (Fig. 2B). The logical conclusion from these two observations is that the S/T-rich region is not involved directly in virus binding but plays a role nonspecifically, possibly by projecting the SCR domains away from the cell surface. The data in Fig. 4B also show that the GPI anchor is not required for virus binding, since the DAF–HLA-B44 and DAF-MCP-transmembrane molecules, both of which lack the GPI anchor, are able to bind virus.

DISCUSSION

By exploiting MAb 854, which blocks the binding to and infection of cells by echovirus 7, we have previously established that DAF (CD55) is the cellular receptor for echovirus 7 and a number of related echoviruses. A similar conclusion was reached independently by Bergelson and colleagues, who characterized the protein recognized by another MAb which blocked infection of HeLa cells by echovirus 7 (1). DAF is a previously characterized member of the RCA gene family, whose function is to cause the breakdown (decay acceleration) of the C3/C5 convertases central to both the classical and alternative complement pathways. DAF has also been shown to act as a receptor for the surface lectin-like molecule Dr and related adhesins (afimbrial adhesin I, afimbrial adhesin III, and diarrhea-associated F1845) of *Escherichia coli* (26).

The domains of importance in the binding interaction between DAF and echovirus 7 have now been identified. Unlike previously characterized virus receptors, such as the poliovirus receptor and ICAM-1, the N-terminal domain of DAF (SCR1) appears not to be required for binding virus, since a DAF mutant lacking this domain appears to bind virus normally. Surprisingly, our data indicate that all of the remaining domains (SCR2, SCR3, and SCR4, and the S/T-rich region) are necessary, since removal of any of them abolishes virus binding. The possibility that the overall conformational structures of the deletion mutants of DAF were disrupted seems unlikely because of the fact that each mutant was shown in both immunoprecipitation and FACScan analyses to be recognized by the appropriate MAbs (6). Moreover, the ΔSCR2 and ΔSCR4 molecules were recognized by some of the Dr adhesins as assayed by Nowicki et al. (26). We have also exploited the DAF deletion mutants to map the location of the binding epitope for MAb 854, the antibody used to identify DAF as an echovirus receptor, to SCR3. MAb 854 does not bind to the deletion mutant lacking this domain (ΔSCR3) or to the recombinant DAF (SCR3DM) in which SCR3 has been replaced by the SCR3 of MCP.

The interactions of DAF with the *E. coli* Dr and related adhesins, which are believed to be important in the colonization of host tissues by pathogenic *E. coli*, have also been characterized at the molecular level (26). All show a requirement for the SCR3 domain and the S/T-rich region, although as for the C3/C5 convertase binding, the requirement for the S/T-rich region appears to be steric. The requirements for the other domains differ between the adhesins, although as in the binding between DAF and the C3/C5 convertases, the first domain, SCR1, appears not to be involved.

Echovirus 7 shows no discernible interaction with MCP (CD46), the closest known relative of DAF and another member of the RCA gene family. MCP also possesses four SCRs, with SCR3 and SCR4 showing the closest homology to DAF. By replacing SCR2, SCR3, and SCR4 of DAF with the equivalent domains of MCP, we have established that the requirement for these domains for virus binding is specific, since DAF molecules with each of these regions substituted fail to bind virus. By contrast, the requirement for the S/T-rich region appears to be nonspecific, since a DAF-HLA recombinant molecule possessing all four SCRs but lacking the S/T-rich region can bind virus. This finding suggests a steric role for the S/T-rich region, such as projection of the four SCRs away from the cell surface. The O-linked and N-linked glycosylations of DAF also appear to be unnecessary for echovirus 7 binding, since each can be removed (in the SCR1 deletion and in the DAF-HLA recombinant, respectively) without significant effect. Similarly, the GPI anchor is unimportant, since the two

transmembrane forms of DAF (Fig. 1C and D), both of which lack a GPI anchor, show full binding activity. The specific requirement for domains SCR2, SCR3, and SCR4 in virus binding is similar to the requirements of the interaction between DAF and the C3/C5 convertases (6). SCR3 is also clearly required for the interactions between DAF and the adhesins of *E. coli* (26). Interestingly, none of these ligands show a requirement for SCR1. All ligands tested so far seem to require the S/T-rich region, but in each case this requirement appears to be steric since membrane-proximal domains from other proteins can effectively substitute for this region.

Competition binding to DAF between echovirus 7 and a panel of anti-DAF MAbs confirmed the importance of SCR3 and SCR4 for echovirus 7 binding. The effect of antibodies against SCR2 was less pronounced given that the earlier binding studies had shown a clear requirement for this domain. A likely explanation is that the antibody used recognizes a site on SCR2 distinct from that recognized by the virus. The slight inhibiting effect of an antibody against domain SCR1 (BRIC220) was surprising given the apparent lack of requirement for this domain in binding. It is possible that binding of this antibody to domain SCR1 sterically hinders the binding of virus to one or more of the other domains.

Although DAF clearly plays a role in the binding of echovirus 7 and as such is essential for entry into host cells, the addition of human DAF to hamster CHO cells is insufficient to render them fully susceptible to virus infection. This observation is comparable to findings of studies identifying ICAM-1 as the receptor for major group rhinoviruses. Work is under way to further define the function of DAF in echovirus 7 entry and to identify other cellular factors that may influence full permissiveness for echovirus 7 in murine cells.

The observation that echovirus 7 specifically requires three SCRs of DAF for binding invites speculation about whether the interaction centres on a canyon region on the icosahedral virus particle. Future work will involve the identification of specific contact amino acids on both DAF and echovirus 7, using mutagenesis and the selection of virus variants which may be resistant to neutralization by soluble receptor. Ultimately it is desirable to analyze the interactions by crystallographic studies.

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