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Interaction between Echovirus 7 and Its Receptor, Decay-Accelerating Factor (CD55): Evidence for a Secondary Cellular Factor in A-Particle Formation

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Soluble forms of decay-accelerating factor (DAF) (CD55), the receptor for echovirus 7, were synthesized in the yeast *Pichia pastoris*. Purified recombinant protein containing SCR domains 2, 3, and 4, but lacking the serine/threonine rich region, was shown to block infection of susceptible cells by echovirus 7. In contrast to the situation with poliovirus and its receptor, the neutralization of echovirus 7 by soluble DAF was completely reversible and did not lead to the formation of 135S A-particles. Binding of virus to susceptible cells, by contrast, did lead to the formation of A particles, mainly from virus that had been internalized. The data suggest that a secondary factor(s) may contribute to A-particle formation and uncoating of echovirus 7.

Echoviruses (enteric cytopathic human orphan) are members of the *Enterovirus* genus of the family *Picornaviridae*. They possess an icosahedral capsid composed of four viral proteins (VP1 to VP4) surrounding a single-stranded positive-sense RNA genome of approximately 7 kb. The 31 serotypes of echoviruses are associated with a wide spectrum of clinical syndromes, including rashes, diarrhea, aseptic meningitis, respiratory disease, and possibly conditions such as chronic fatigue syndrome (27, 36). This range of clinical manifestations probably reflects virus-tissue tropisms which are mediated, at least in part, by utilization of a range of cellular receptors. Evidence to date suggests that there are at least three specific receptors for echoviruses.

Echoviruses 1 and 8 use the integrin VLA-2 (3), and echoviruses 6, 7, 13, 21, 29, and 33 use the decay-accelerating factor (DAF) (2, 34). Other echoviruses appear to use neither of these, but the identity of their receptor(s) is unknown. DAF is a member of a family of proteins that regulate complement activity (RCA) and functions to protect the host cell from complement-mediated damage by preventing the formation and accelerating the decay of the convertases C3 and C5a (24). Interestingly, two other proteins of the RCA family also serve as virus receptors; membrane cofactor protein (MCP) or CD46 is the receptor for measles virus (25, 28) and CR2 is the receptor for Epstein-Barr virus (15). A feature of RCA proteins is the presence of the short consensus repeat sequences (SCRs) which are approximately 60 amino acids long. DAF has four SCRs which show significant homology to each other and to SCRs from other RCA proteins. Of the four SCRs in DAF, SCRs 2, 3, and 4 are required for decay-accelerating activity, virus binding, and attachment of certain pathogenic *Escherichia coli* strains (4, 6, 7). SCR 1 has recently been shown to bind to CD97 which is expressed on activated leukocytes, suggesting a role in cell adhesion and recruitment during inflammation (19). To date, DAF is the only glycosylphosphatidylinositol-anchored protein identified as a virus receptor, the significance of which is yet to be determined.

It has been suggested that picornavirus receptors function also as mediators of uncoating. This is an attractive hypothesis since it may explain why these viruses, which are normally very stable, readily uncoat after binding. Moreover, entry and uncoating are both early events in virus replication which may proceed simultaneously. For poliovirus and coxsackievirus B3, approximately 50% of virus that binds to cells at 37°C rapidly converts to altered particles (A-particles) which elute from the cell surface (9, 21). A-particles retain RNA but are more labile than native virions. These characteristics support the view that A-particles constitute an intermediate in the uncoating process. Further work has demonstrated that membrane fractions of HeLa cells retained the ability to promote A-particle formation (5, 18, 32). More recent studies, using soluble receptor, have suggested that the virus-receptor interaction per se is sufficient to induce A-particle formation for poliovirus and rhinovirus type 14 (17, 22, 35).

In this report, we describe the characteristics of the interaction between echovirus 7 and soluble DAF produced in the yeast *Pichia pastoris* (8). We find differences from similar work with poliovirus and rhinovirus type 14 that may aid in understanding of the early events in replication for this group of viruses as a whole.

MATERIALS AND METHODS

DAF expression. (i) Cloning. DAF derivatives were produced by overlapping PCR mutagenesis, using the primers described in Table 1. DAF domains were amplified by PCR from pCDM8 containing the complete DAF sequence (34) and cloned into pPic9 (Invitrogen), using the *Xho*I and *Eco*RI sites in the multiple cloning site. The sequence for a C-terminal $(His)_6$ tag was incorporated to permit affinity purification on nickel resin of the expressed protein. Correctly sized clones were identified by restriction digestion and subcloned to include the signal sequence derived from pPic9, into pPic9K using *Eco*RI and *Bam*HI. Standard procedures (26) were used for PCR amplification, restriction digestion, and ligation of DNA fragments.

(ii) Yeast transfection. Linearized pPic9K DAF clones were transfected into spheroplasts of the yeast *P. pastoris* GS115. This was followed by selection on histidine-free medium, since the His4 gene is contained within pPic9K but has been deleted from GS115. The *Pichia* strain was grown to an optical density at 600 nm (OD_{600}) of 0.2 to 0.3, pelleted by centrifugation at 1,500 × *g* for 10 min, and washed sequentially in nano-pure H_2O, SED (1 M sorbitol, 25 mM EDTA, 50 mM dithiothreitol, pH 8), 1 M sorbitol, and SCE (1 M sorbitol, 1 mM EDTA, 10 mM sodium citrate, pH 5.8). Cells were then incubated with Zymolase (Invitrogen) at 30°C for 20 min to give approximately 70% spheroplasts which were harvested at 750 × *g* for 10 min and washed in 10 ml of CaS (1 M sorbitol, 10 mM Tris [pH 7.5], 10 mM $CaCl_2$) and resuspended in 0.6 ml of CaS. For each clone, 10 μg of *Sac*I-linearized pPic9K-DAF DNA was incubated with 0.1 ml of spheroplasts for 10 min and then 1 ml of polyethylene glycol-CaT was added and the mixture was incubated for a further 10 min. Cells were pelleted as above and

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TABLE 1. Primers used in this study

Construct	Amino acids	Primers
1234	35–285	AGCTGCAGGCTCGAGAAAAGAGAGGCTGAAGCTGACTGTGGCCTTCCCCCGGCTACGCTGAATTCCTAGT GATGGTGATGATGGTGTCCTCTGCATTCAGGTGG
123[a]	35–223	AGCTGCAGGCTCGAGAAAAGAGAGGCTGAAGCTGACTGTGGCCTTCCCCCGGCTACGCTGAATTCCTAGT GATGGTGATGATGGTGAATTTCTCTGCACTCTGGC
234	96–286	CCATGCAGGCTCGAGAAAAGAGAGGCTGAAGCTCGTAGCTGCGAGGTGCCAGGCTACGCTGAATTCCTA GTGATGGTGATGATGGTGTCCTCTGCATTCAGGTGG
12[a]	35–161	AGCTGCAGGCTCGAGAAAAGAGAGGCTGAAGCTGACTGTGGCCTTCCCCCGGCTACGCTGAATTCCTAGT GATGGTGATGATGGTGTTTCTTTTTACAAAATTCGACTGC
23	96–223	CCATGCAGGCTCGAGAAAAGAGAGGCTGAAGCTCGTAGCTGCGAGGTGCCAGGCTACGCTGAATTCCTA GTGATGGTGATGATGGTGAATTTCTCTGCACTCTGGC
34	161–286	TCGTGCAGGCTCGAGAAAAGAGAGGCTGAAGCTAAATCATGCCCTAATCCGGGCTACGCTGAATTCCTAG TGATGGTGATGATGGTGTCCTCTGCATTCAGGTGG

[a] Constructs in which overlapping PCR mutagenesis was used to remove the glycosylation site between domains 1 and 2, using the internal primers GGAGAGTT CTGCACTCGTAGCTGCGAGGT and CTCGCAGCTACGAGTGCAGAACTCTTC.

resuspended in 150 μl of SOS (1 M sorbitol, 10 mM $CaCl_2$) in 3× yeast peptone dextrose medium (1%, 2%, and 2%) and incubated for a further 20 min. Then 850 μl of 1 M sorbitol was added, and a 300-μl aliquot was plated onto regeneration dextrose (RD) plates (2% agar, 1% dextrose, 1.34% yeast-nitrogen base [YNB; DIFCO], 40 μg of biotin/ml, and 0.05% [each] amino acids L-glutamic acid, L-lysine, L-leucine, and L-isoleucine) in 10 ml of molten top RD medium (as above with 1% agar). Transfected clones formed visible colonies after 72 h of incubation at 30°C.

(iii) G418 screening. A further round of selection was performed to select for transfected clones which had received multiple copies of the expression cassette. Clones were harvested from the top agar by homogenization in 50 ml of H_2O and allowing the agar to settle out of solution. Cells were counted using a hemocytometer, and approximately 10^4 cells were plated onto RD plates containing G418 at concentrations of between 0 and 20 mg/ml. Colonies growing at the highest concentration of G418 were picked and screened for DAF expression.

(iv) Expression screening. Colonies were grown overnight at 30°C in 25 ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% YNB, 4×10^{-4}% biotin, and 1% glycerol) to an OD_{600} of approximately 1. Cells were harvested by centrifugation at 1,500 × *g* for 5 min and resuspended in BMMY medium (same as BMGY but 0.5% methanol instead of 1% glycerol). Cultures were vigorously aerated by shaking at 200 rpm in baffled flasks for 48 h with methanol added to 0.5% every 24 h. Culture supernatants were harvested by centrifuging out yeast cells at 2,000 × *g* for 20 min. Secreted proteins in the culture supernatant were visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining.

(v) DAF purification. Recombinant DAF was purified by immobilized metal affinity chromatography utilizing the C-terminal histidine tag. Briefly, the culture supernatants were further clarified by filtration through a 2.2-μm-diameter pore. A nickel agarose column was equilibrated with 20 ml of 1× binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris [pH 7.9]), and the induction supernatant was run through the column at a rate of 25 column volumes per hour. The column was then washed with 20 ml of binding buffer and then with binding buffer containing 20 mM imidazole. Proteins were eluted with 300 mM imidazole, and the fractions were examined by SDS-PAGE and silver staining. Fractions were concentrated with a microconcentrator according to the manufacturer's instructions (Centricon), and the purified DAF was resuspended in phosphate-buffered saline (PBS).

Enzyme immunoassay. Purified DAF was characterized by binding to a panel of anti-DAF monoclonal antibodies (MAbs) (854 [34], BRIC110, BRIC220, and BRIC216 [a gift from G. Daniels]) using standard methods.

Virus neutralization assays. One thousand 50% tissue culture infectious units ($TCID_{50}$) of echovirus 7 was incubated with recombinant soluble DAF at various concentrations. After incubation at 37°C for 1 h, the virus-DAF mixtures were applied to monolayers of RD cells on a 96-well plate. The plates were incubated for 24 h and stained with crystal violet.

Virus neutralization dilution assay. Approximately 10,000 $TCID_{50}$ was mock treated or incubated with 8 μM soluble DAF (domains 1, 2, 3, and 4) for 1 h at 37°C in serum-free Dulbecco modified Eagle medium (DMEM). The virus receptor mixture and control virus were then applied to RD cells in a 96-well format, and serial dilutions (1:4) were taken from the first well. The plates were incubated for 48 h and stained with crystal violet.

Standard elution. Approximately 6×10^7 RD or HeLa cells were incubated at 4°C with ^{35}S-radiolabelled poliovirus or echovirus 7 (approximately 250 kcpm at a multiplicity of infection of 3). Following binding on ice for 1 h, cells were centrifuged at 1,000 × *g* for 5 min, washed in ice-cold PBS, and resuspended in 2.4 ml of DMEM–10% fetal calf serum (FCS), and aliquoted into 200-μl fractions. After incubation at 37°C for 2 h, cells were pelleted and the supernatant was harvested and counted in a scintillation counter (see Fig. 5). The cell pellet was washed in PBS and counted. In similar experiments, aliquots of eluted virus were plaqued on RD cells (see Fig. 6) or sedimented in 15 to 45% sucrose gradients for 4 h at 25,000 rpm in a Beckman SW28 rotor. Fractions were quantified by scintillation counting (see Fig. 7).

Elution of internalized virus. Radiolabelled virus was bound to 10^6 cells at 4°C as described above. After binding, virus was allowed to enter by incubation at 37°C for 10 min. The cells were then cooled to 4°C and incubated for 1 h in soluble DAF at a final concentration of 100 μg/ml (sufficient to remove >95% of cell surface virus). The cells were then washed in PBS, resuspended in 200 μl of DMEM, and returned to 37°C. The amount of virus that subsequently eluted over time was measured.

Binding assay 1. Approximately 10^4 cpm of ^{35}S-labelled echovirus 7 (approximately 5×10^5 PFU) was incubated with 5 to 60 μg of DAF/ml in 200 μl of DMEM–10% FCS for 1 h on ice. This mixture was then added to 5×10^6 RD cells and left 1 h on ice. After centrifugation, the supernatant was collected and the cell pellet was washed twice in PBS. Cell-bound virus was then measured by scintillation counting.

Binding assay 2. Approximately 10^4 cpm of ^{35}S-labelled echovirus 7 (approximately 5×10^5 PFU) was incubated with 5×10^6 RD cells for 1 h on ice. The cells were then washed in PBS and resuspended in 200 μl of DMEM–10% FCS containing a range of DAF concentrations. Competition was permitted for 1 h followed by counting of supernatant and pellet as above. In addition, one sample was incubated in PBS containing proteinase K at 0.25 mg/ml for 10 min. Virus released by soluble DAF in this assay was expressed as the percentage of protease K-sensitive virus remaining after incubation on ice for 1 h.

RESULTS

Construction of deletion mutants of DAF. A range of DAF derivatives was PCR amplified from a full-length clone of DAF and cloned into the yeast *P. pastoris* (8) (primers as in Table 1). All possible linear two and three SCR domain constructs were included, some of which had the glycosylation site between domains one and two removed. Expression levels were approximately 5 to 6 mg of DAF/liter of medium for most clones. Construct 12 however yielded approximately three times that amount. As visualized by silver staining after PAGE, the purified products contained no protein bands other than those expected from the clone. Samples subjected to nuclear magnetic resonance analysis produced spectra typical of molecules with SCR domains (1a), suggesting that the recombinant molecules were correctly folded. Moreover, analysis by enzyme immunoassay, using a panel of anti-DAF antibodies, indicated that all the DAF-derived proteins bound, as expected, to a range of MAbs against defined SCR domains (data not shown). The DAF proteins were also tested for their ability to inhibit the binding of a range of domain-specific MAbs (approximately 30 in total) to erythrocytes in a simple hemagglutination assay. All the DAF constructs were able to inhibit hemagglutination as expected, depending on their particular domain structure and the MAb used (10a).

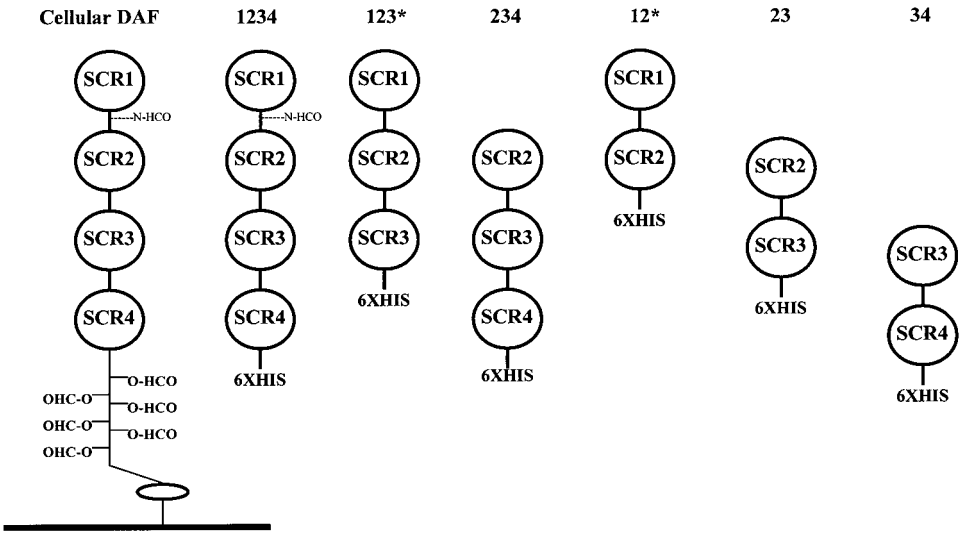

FIG. 1. Structure of recombinant DAF molecules expressed in the yeast *P. pastoris*. * indicates constructs in which overlapping PCR mutagenesis was used to remove the glycosylation site between domains 1 and 2, using the internal primers GGAGAGTTCTGCACTCGTAGCTGCGAGGT and CTCGCAGCTACGAGTGCAGA ACTCTTC.

Virus neutralization. Soluble DAF deletion mutants were tested for their ability to protect susceptible cells from infection in a virus neutralization assay. Consistent with previously published results (6), only clones that expressed domains 2, 3, and 4 together retained the ability to block infection (i.e., DAF constructs 1234 and 234 [Fig. 1]). Concentrations of approximately 0.5 μM protein were sufficient to protect a susceptible cell monolayer from infection in a $TCID_{50}$ assay as described in Materials and Methods (Fig. 2). The results also indicated that neither the presence of domain 1 nor the glycosylation site between domains 1 and 2 were required for neutralization activity.

Previous work with other enteroviruses and rhinoviruses has indicated that incubation with soluble receptor results in an irreversible loss of infectivity (17, 22). We therefore investigated whether incubation of echovirus 7 with DAF would produce a similar result to imply an analogous mechanism of neutralization. However, when the reaction mixture from a neutralization assay was serially diluted, infectious virus could be detected, at the same titer as in untreated controls, in wells containing the lowest concentrations of DAF (Fig. 3). This indicates that DAF neutralization is a reversible process. Indeed after dilution, DAF-treated virus retained the same infectivity as untreated virus even after prolonged, overnight incubations with soluble DAF (data not shown).

Receptor-mediated conformational change. Since, unlike the situation with poliovirus and the poliovirus receptor, the mechanism of neutralization of echovirus 7 by DAF appeared

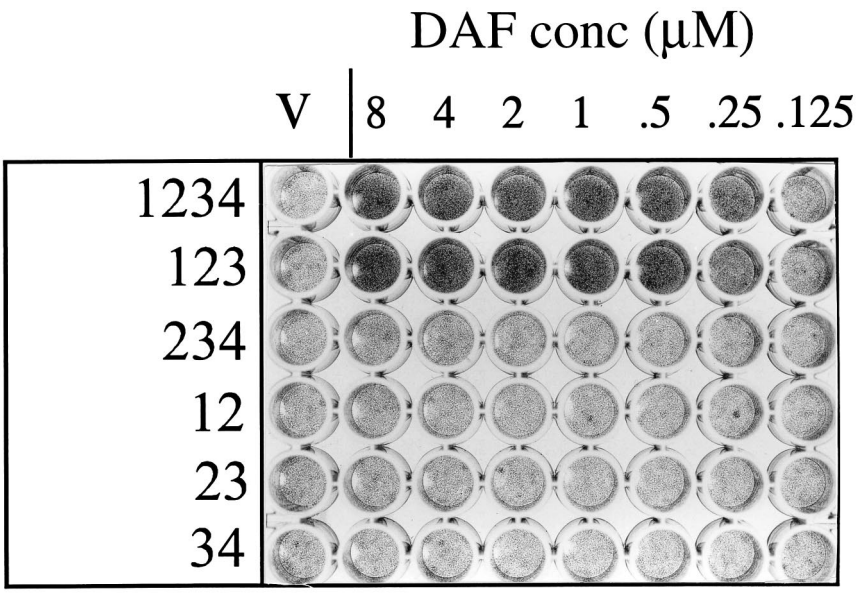

FIG. 2. Neutralization of echovirus 7 by recombinant soluble DAF. Various dilutions of recombinant DAF proteins (domains indicated) were preincubated with 1,000 $TCID_{50}$ of echovirus 7. The mixture was then added to RD cell monolayers in a 16-well plate and incubated at 37°C for 24 h.

FIG. 3. Infectivity of virus after incubation with soluble DAF (sDAF) construct 1234. Approximately 20,000 $TCID_{50}$ was mock treated or incubated with soluble 8 μM DAF at 37°C for 1 h. This mixture was applied to RD cells and serially diluted 1 to 4 across the plate. The cells were incubated at 37°C for 48 h.

reversible, we compared these viruses for receptor-induced conformational changes. First, radiolabelled echovirus 7 and poliovirus were pelleted and recentrifuged in sucrose density gradients. For both viruses the characteristic profile of 160S native virions and 80S empty capsids was observed (Fig. 4A). We then treated 10^6 PFU of pelleted radiolabelled echovirus 7 with a 10-fold excess of the amount of DAF required to block virus infection and looked for the formation of a 135S particle analogous to poliovirus A-particles. No alteration in sedimentation profile was found, either by the formation of 135S particles or by a change in the ratio of 160S native virus particles to 80S particles (Fig. 4B). Moreover, the DAF-treated 160S peak retained VP4 as revealed by SDS-PAGE (data not shown).

Elution of echovirus 7 and Poliovirus. The above experiments suggested that soluble DAF does not, on its own, induce echovirus 7 to form A-particles. We next investigated whether echovirus 7 formed 135S particles upon interaction with permissive cells. Other enteroviruses are known to elute from cells as A-particles after incubation at 37°C. We therefore monitored elution of highly purified, radiolabelled echovirus 7 and poliovirus from HeLa or RD cells. Virus was bound to cells at 4°C, followed by washing, and then permitted to elute for 2 h at 37°C (Fig. 5). The amount of eluted radioactive virus was measured, and its infectivity was assayed by plaque formation on RD cells (Fig. 6). About 55% of bound poliovirus eluted from both cell lines. In contrast, a much smaller fraction of echovirus 7 eluted; approximately 12% from RD cells and only 3% from HeLa cells. However, the infectivity of eluted material (as measured by PFU/count per minute [cpm] ratios before and after binding and elution) was significantly reduced (>99%) for both viruses. This suggested that A-particles of echovirus 7 were being produced upon interaction with the cell. To confirm this conclusion, eluted material was subjected to sucrose gradient centrifugation. In common with the eluted poliovirus control, eluted echovirus 7 from RD and HeLa cells sediments at approximately 135S (Fig. 7). These results indicate that interaction with permissive cells does cause the formation of A-particles, even though interaction of virus with soluble DAF alone does not. This is consistent with the notion that there may be secondary binding steps at the cell surface, as has been observed for other picornaviruses (14, 29).

Elution of virus from cells. There are two potential routes of virus elution from the cell. Virus may be eluted directly from the cell surface after binding DAF, or it may be eluted at a later stage after complex formation, possibly from an internal cellular vesicle. We therefore investigated these alternatives by measuring elution of echovirus 7. In pilot experiments, greater than 95% of virus bound to the cell at 4°C was eluted by treatment with 100 μg of soluble DAF/ml. If incubation was shifted to 37°C for 10 min prior to DAF treatment, less than 30% of virus was removed by this treatment. This suggests that the virus either enters the cell at 37°C or becomes associated with a high-affinity complex at the cell surface. Unlike virus bound only at 4°C, this tightly associated virus was also insensitive to removal by proteinase K treatment of the cells. To measure whether the tightly associated virus was qualitatively different from that eluted by DAF treatment, this fraction was analyzed as follows. Virus was bound to the cell at 4°C and then further incubated at 37°C for 10 min. Any remaining cell

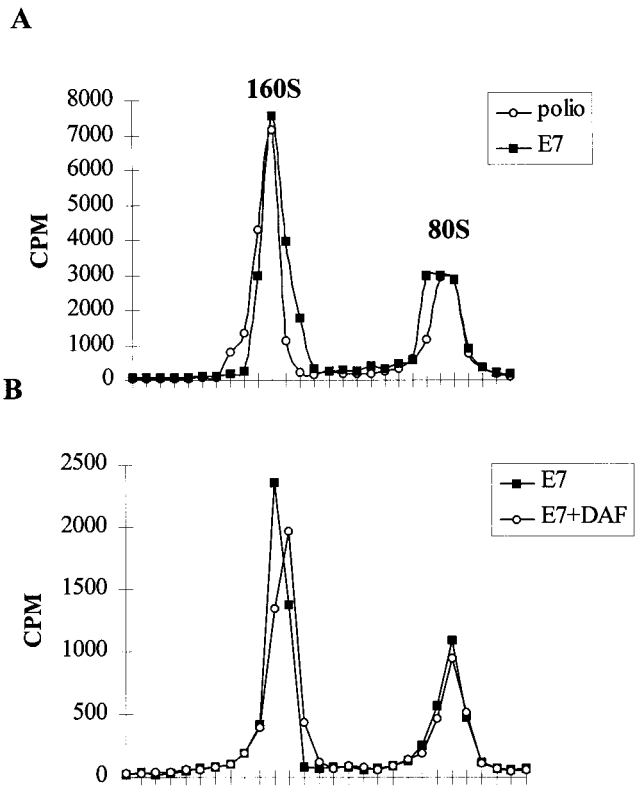

FIG. 4. Sedimentation profile of echovirus 7 in the presence and absence of DAF. Partially purified radiolabelled echovirus 7 and poliovirus were sedimented in 15 to 45% sucrose gradients. The 160S peak corresponds to infectious virus, and the 80S peak corresponds to empty capsids. (A) Sedimentation of partially purified poliovirus and echovirus 7 (E7). (B) Sedimentation of E7 with and without DAF treatment at 200 μg/ml.

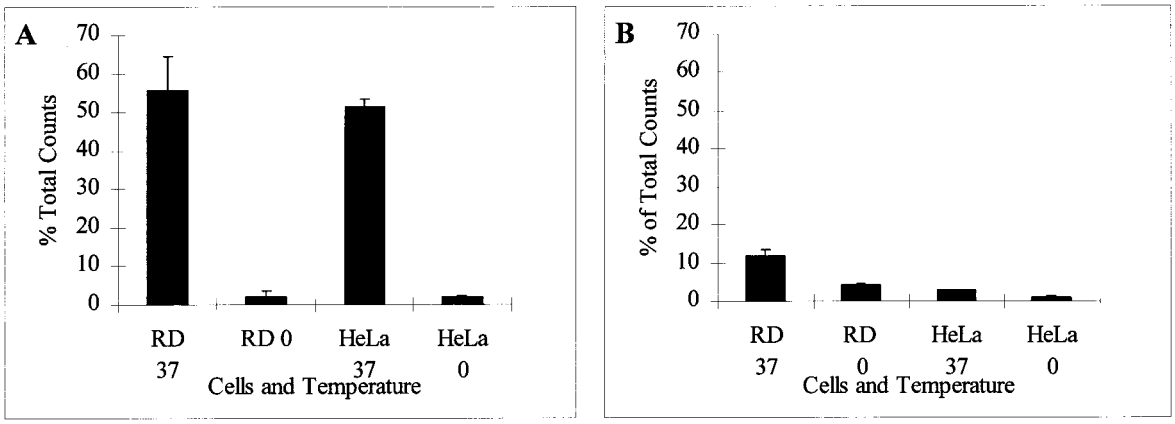

FIG. 5. Elution of poliovirus and echovirus 7 from cells. Radiolabelled poliovirus and echovirus 7 were bound to cells at 4°C and allowed to elute by incubation for a further 2 h at 37°C. (A) Poliovirus eluted from RD and HeLa cells at 4 and 37°C. (B) Echovirus 7 eluted from RD and HeLa cells at 4 and 37°C. Points represent the average of three assays, and the error bars indicate the range of values obtained.

surface virus was then competed off with soluble DAF. Elution of the soluble DAF-resistant fraction was then measured over a 2-h period. The proportion of virus eluted from RD cells over this time period was identical to the proportion that was observed to be recoverable as A-particles in the experiments described in Fig. 5. This indicates that A-particles are formed from the tightly associated fraction that is proteinase K insensitive and resistant to removal by excess soluble DAF.

DISCUSSION

We, and others, have previously identified DAF as the cellular receptor for echovirus 7 and related enteroviruses (2, 34). Here we describe the use of the *P. pastoris* yeast expression system to produce large amounts of soluble DAF, including a range of deletion mutants lacking various domains, in order to characterize its interaction with virus.

For both poliovirus and rhinovirus type 14, interaction with receptor molecules (poliovirus receptor and ICAM-1, respectively) on the cell leads to the formation of structurally altered viruses (A-particles) characterized by a release of VP4, loss of receptor binding potential, sedimentation at 135S, and loss of infectivity (12, 16, 18, 20). Moreover, soluble forms of the receptors also both induce A-particle formation *in vitro* and neutralize infectivity (17, 22, 35). Our results show that echovirus 7 forms A-particles upon interaction with susceptible cells, but in contrast to rhinovirus type 14 and poliovirus, does not do so after interaction with soluble receptor. We do not believe that this is due to a deficiency in the integrity of our recombinant DAF since the protein is able to neutralize virus in a way that is entirely consistent with our previous observations that domains 2, 3, and 4 are both necessary and sufficient for virus binding at the cell surface (6). Moreover, our soluble DAF reacts as expected with all available antibody preparations (>30 have been tested [10a]), and the levels required to block 1,000 $TCID_{50}$ of virus in our assay (approximately 0.5 μM [Fig. 2]) compare favorably with the 10 μM concentration of ICAM-1 required to block infection by rhinovirus type 14 (17).

Our data suggest that echovirus 7 A-particles produced upon interaction with the cell are derived slowly and principally from virus that has become tightly associated, possibly through internalization (Fig. 8). This is in contrast to the majority of eluted poliovirus A-particles which are produced rapidly at the cell surface (21). This leaves open the possibility that

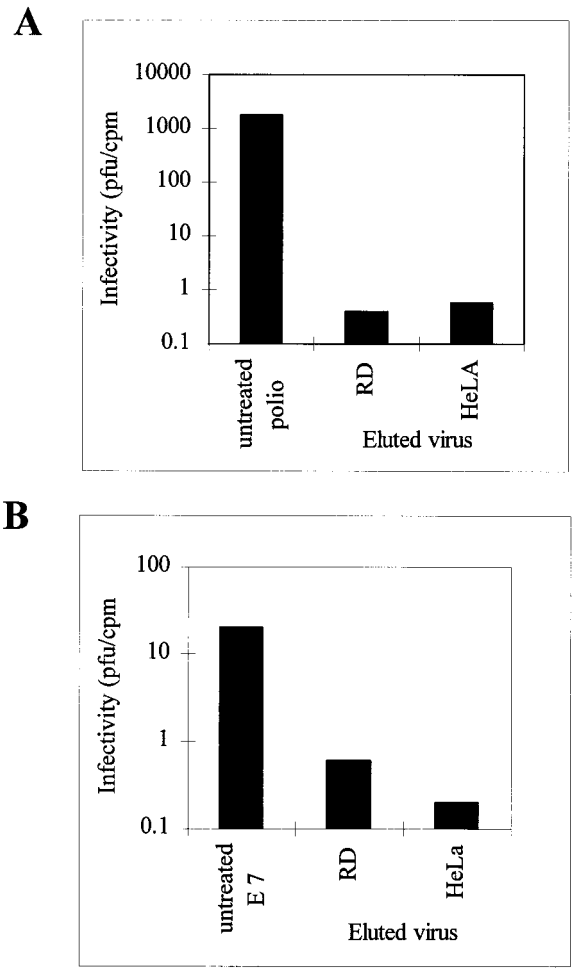

FIG. 6. Infectivity of poliovirus and echovirus eluted from RD and HeLa cells. Radiolabelled poliovirus and echovirus 7 (E7) was bound to cells at 4°C. Virus eluted after 2 h at 37°C was assayed for infectivity relative to untreated virus. (A) Infectivity of poliovirus eluted from RD and HeLa cells. Column 1 represents the infectivity in PFU/cpm of the unadsorbed virus. (B) Infectivity of E7 eluted from RD and HeLa cells. Column 1 represents the infectivity in PFU/cpm of the unadsorbed virus.

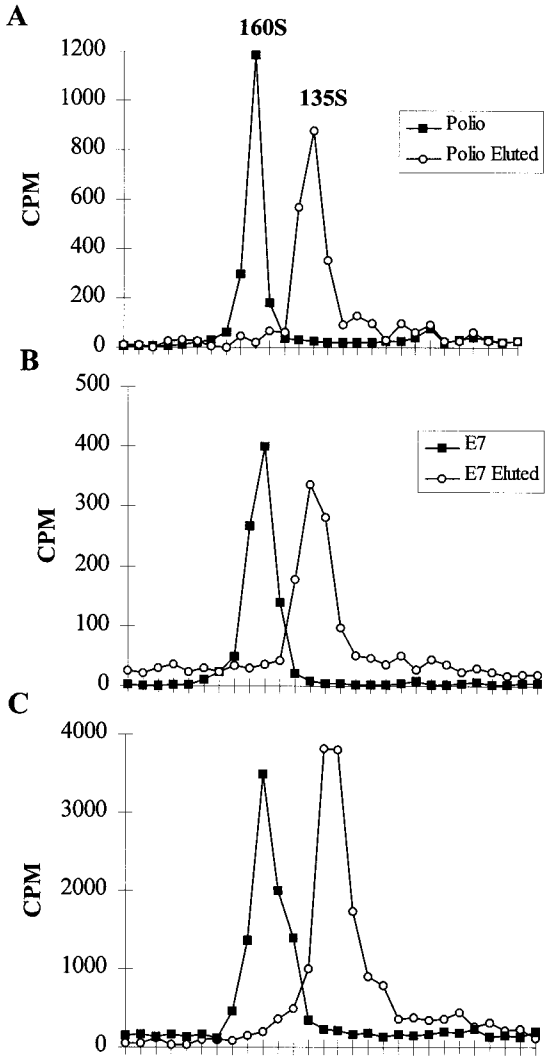

FIG. 7. Sedimentation profiles of poliovirus and echovirus 7 (E7) eluted from HeLa and RD cells. Radiolabelled virus was bound to cells at 4°C and then allowed to elute at 37°C for 2 h. The eluted fraction was then centrifuged on linear 15 to 45% sucrose gradients. The 160S peak represents purified whole virus, and the 135S peak corresponds to A-particles released from the cell. (A) Poliovirus eluted from HeLa cells. (B) E7 eluted from HeLa cells. (C) E7 eluted from RD cells.

poliovirus and echovirus 7 135S particles infect cells via different mechanisms after binding to their receptors. The reason why echovirus 7 requires an event in addition to receptor binding, which may be internalization and/or interaction with a secondary factor(s), is unknown. However, DAF, being glycosylphosphatidylinositol anchored and readily released from the cell surface by endogenous phospholipases, is present as a free molecule in serum and tissues and is expressed abundantly on nonsusceptible cells such as erythrocytes (11). If interaction with DAF alone were sufficient to trigger A-particle formation, free echovirus might be inactivated by such DAF and this would be disadvantageous to the virus. A better strategy therefore might be for the virus to be triggered by a secondary interaction before committing itself to the irreversible events of A-particle formation and uncoating.

Consistent with the failure to form A-particles *in vitro*, the neutralization of echovirus 7 infectivity by soluble DAF is completely reversible. This contrasts with the observations of Philipson et al. (30), who purified a complex from erythrocytes which irreversibly neutralized echovirus 7. However, the high level of impurities (including 31% lipid) in those experiments makes it impossible to conclude that the solubilized receptor alone caused the neutralization. Indeed, it is possible that the complex (which was of M_r >200,000) contained additional factors necessary for A-particle formation. Specific accessory factors or coreceptors, have been identified for other viruses (1, 13, 33). Moreover, two-stage binding of enterovirus has been described previously (23), and after initial binding at 4°C further interactions occur at 37°C which make dissociation of virus and receptor more difficult to achieve by treatment with chymotrypsin (5). An additional role for the cell may be the provision of a low-pH intracellular environment. However, our own preliminary experiments *in vitro*, plus the work of Philipson et al. discussed above, do not support the simple model that A-particle formation is triggered by DAF binding plus a lowering of pH alone.

The currently favored model for entero- and rhinovirus infection is that the 135S A-particle is an intermediate in the infection process (10) and that loss of VP4 results in the extension of a helix motif from VP1 which may bind a cellular membrane and produce a pore through which viral RNA is ejected into the cytoplasm (16). Alternatively, it has been suggested that A-particles may be a dead end product and that productive virus infection occurs through an alternative, receptor-mediated process (31). Echovirus 7 represents a further example of a picornavirus that forms A-particles. The fact that these are produced, even though the soluble receptor interaction alone does not induce their formation, supports the argument that they do indeed represent a stage in virus disassembly. Further work is required however, to determine the precise route of entry and uncoating, including the role of the 135S particle.

We are currently pursuing three-dimensional structural studies on DAF which should provide further insight into the echovirus 7-DAF interaction and, in particular, the requirement for three domains. Further details of the essential steps

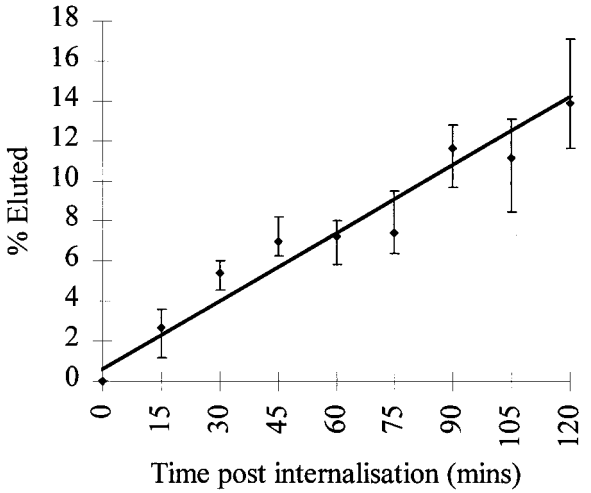

FIG. 8. Elution of echovirus 7 from RD cells. Radiolabelled virus was bound to RD cells at 4°C and then allowed to initiate entry into the cells by incubation for 10 min at 37°C. Excess adsorbed virus was removed by treatment on ice with soluble DAF at 100 μg/ml. Elution of internalized virus (resistant to removal by soluble DAF) was then measured over time. Points represent the average of three assays, and the error bars indicate the range of values obtained.

in virus attachment and uncoating may allow the identification of targets for anti-picornavirus agents.

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