

Interaction with Coxsackievirus and Adenovirus Receptor, but Not with Decay-Accelerating Factor (DAF), Induces A-Particle Formation in a DAF-Binding Coxsackievirus B3 Isolate

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Although many coxsackie B viruses interact with decay accelerating factor (DAF), attachment to DAF by itself is not sufficient to initiate infection. We examined the early events in infection that follow virus interaction with DAF, and with the coxsackievirus and adenovirus receptor (CAR). Interaction with soluble CAR in a cell-free system, or with CAR on the surfaces of transfected cells, induced the formation of A particles; interaction with soluble or cell surface DAF did not. The results suggest that CAR, but not DAF, is capable of initiating the conformational changes in the viral capsid that lead to release of viral nucleic acid.

All tested coxsackie B virus (CB) isolates interact with the coxsackievirus and adenovirus receptor (CAR) and can attach to and infect nonpermissive rodent cells transfected with CAR (2, 28, 41). Some isolates of CB1, CB3, and CB5 also bind decay accelerating factor (DAF); however, although these viruses attach to DAF on the cell surface, no infection occurs in the absence of CAR (3, 37, 38). A number of other enteroviruses—including many echoviruses (1, 42, 43), coxsackievirus A21 (CA21) (38), and enterovirus 70 (25)—also attach to DAF but fail to infect DAF-transfected cells. It thus appears that virus interaction with CAR, but not with DAF, leads to a postattachment event that is essential for infection to proceed. This event has not been defined.

CB3, when bound to the cell surface in the cold, can be recovered after exposure to low pH or after treatment with detergents (8). However, at physiologic temperatures, cell-bound virus becomes resistant to elution (47) and infectious virus cannot be recovered until new virions are produced. Although the events that occur during this “eclipse” period remain incompletely understood, resistance to acid elution is thought to reflect a multivalent “tight” interaction between virions and receptors or entry of virions into the cell (9). During eclipse, enterovirus capsids undergo conformational changes that lead to release of viral RNA into the cytoplasm (16). After attachment, most cell-associated virus is converted to an irreversibly altered form, the A particle, which shows reduced sedimentation velocity in sucrose gradients, has lost the internal capsid protein VP4, and no longer interacts with cellular receptors or infects receptor-bearing cells (11, 12, 27). Despite some questions about their role in infection (13), a variety of evidence suggests that A particles are essential intermediates in the uncoating process (21, 23).

The first DAF-binding CB isolate to be described was CB3-

RD, a variant originally obtained by passage of the prototype strain CB3-Nancy in rhabdomyosarcoma (RD) cells (33). CB3-RD is closely related to the Nancy strain, which does not bind DAF; there are only six amino acid differences in the 880-residue capsid region, and the RD phenotype is reported to depend on only two amino acid differences within capsid protein VP2 (26). CB3-RD interacts with both DAF and CAR. It infects Chinese hamster ovary (CHO) cells expressing human CAR (CHO-CAR) but does not infect CHO cells expressing DAF (CHO-DAF), despite its avidity for DAF on the cell surface.

To define the block to infection following virus attachment to DAF, we have examined the early events in CB3-RD infection. We find that, although CAR induces A-particle formation, DAF does not; the results suggest that DAF—in its interaction with CB3-RD and possibly with other picornaviruses as well—is incapable of triggering the conformational changes that lead to release of viral RNA into the cytoplasm.

Both CAR and DAF mediate tight binding. In the cold, CB3 attached to the HeLa cell surface remains reversibly bound and can be released with an acid wash (8). At 37°C, CB3 undergoes eclipse, becoming resistant to acid elution (47). To determine if virus undergoes eclipse after binding to CAR and DAF, we examined the interaction between ³⁵S-labeled CB3-RD and CHO-CAR and CHO-DAF cells. CHO-CAR and CHO-DAF cell monolayers were incubated with ³⁵S-labeled CB3-RD at 4°C, washed to remove unbound virus, heated to 37°C, and after various intervals treated with an acidic buffer (50 mM glycine, pH 1.5). Virus bound to both CHO-DAF and CHO-CAR cells rapidly became resistant to elution by acidic buffer (Fig. 1), suggesting that the postattachment block to infection is not secondary to DAF's incapacity to induce the tight binding associated with early viral eclipse.

A-particle formation induced by soluble CAR but not by soluble DAF. Many picornaviruses undergo conformational changes (A particle formation) when exposed to cell-associated or soluble receptors at 37°C (11, 14, 22, 24, 32). To test whether interaction with CAR and DAF induces a conforma-

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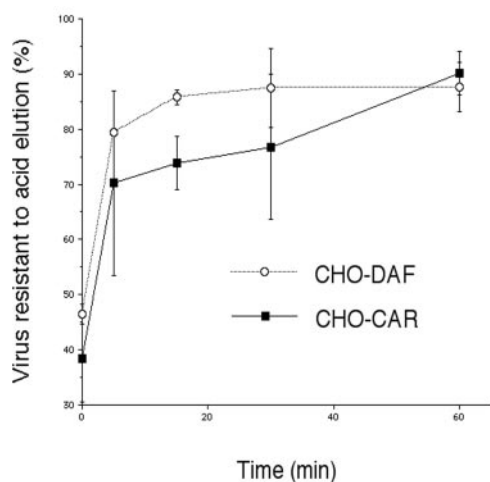


FIG. 1. DAF and CAR mediate tight binding. CHO dhfr cells were stably transfected with CAR and DAF cDNA and sorted for homogeneous expression, as described for the generation of VLA-2-expressing CHO cells in reference 4. Adherent CHO-CAR and CHO-DAF cells were suspended with 2.5 mM EDTA in phosphate-buffered saline. Cells (5×10^6) in 200 μ l of virus binding buffer (VBB; Hanks' balanced salt solution containing 10 mM $MgCl_2$, 10 mM HEPES, 4 mM $CaCl_2$, and 4% bovine calf serum) were incubated for 1 h at 4°C with [^{35}S]methionine- and [^{35}S]cysteine-labeled CB3-RD (30,000 cpm; prepared as described in reference 4) and then washed three times with VBB to remove unbound virus. CB3-RD bound to CHO-CAR cells at 3,748 cpm; the level of CB3-RD binding to CHO-DAF cells was 16,269 cpm. Cells were heated at 37°C for various periods and then treated with 1 ml of 50 mM glycine (pH 1.5) or with VBB as a control. Cells were washed with VBB and then lysed, and cell-bound radioactivity was determined by liquid scintillation counting. Results are expressed as the percentages of bound virus that remained cell associated following acid elution from the formula (counts per minute for virus bound after acid wash/counts per minute for virus bound after VBB wash) \times 100% and are means \pm standard deviations for triplicate samples.

tional change in CB3-RD, we first prepared (in a baculovirus system) recombinant soluble forms of the human CAR and DAF extracellular domains. DNA encoding the extracellular domain of human DAF (ecDAF) and the extracellular domain of human CAR (ecCAR) was amplified with PCR primers that added six C-terminal histidine residues and inserted into a transfer plasmid vector (pVT-Bac) to permit expression in a baculovirus system (40). ecCAR extended from amino acid residues 20 to 230 (LSITTP...LRLNVV) (2), and ecDAF included amino acid residues 35 to 285 (DCGLPP...PPECRG) (6). Generation of recombinant baculovirus, protein expression in baculovirus-infected Sf9 cells and protein purification by nickel affinity chromatography were performed as previously described (45). His-tagged soluble CAR and soluble DAF were collected with a step gradient of imidazole, and the concentrations of purified recombinant proteins were determined by using the Bio-Rad protein assay. The purity and integrity of soluble proteins were assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue (not shown).

Soluble CAR inhibited the binding of CB3-RD to CHO-CAR cells, and soluble DAF inhibited the binding of CB3-RD to CHO-DAF cells, in a dose-dependent fashion (Fig. 2); these preparations of soluble CAR and soluble DAF were thus both

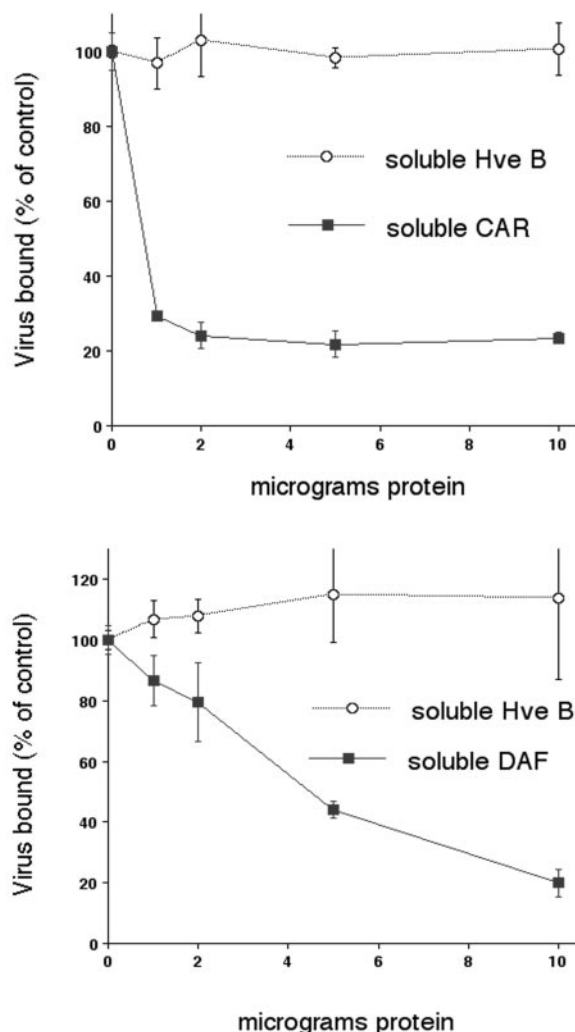


FIG. 2. Soluble CAR and DAF interact with virus. CHO-CAR (top) and CHO-DAF (bottom) cell monolayers in 24-well plates were incubated with ^{35}S -labeled CB3-RD (20,000 cpm/well in 200 μ l of virus binding buffer) for 4 h at room temperature in the presence of soluble CAR (top), soluble DAF (bottom), or control protein HveB. CB3-RD bound to CHO-CAR monolayers at 1,275 cpm; CB3-RD bound to CHO-DAF monolayers at 6,398 cpm. Monolayers were washed three times with phosphate-buffered saline to remove unbound virus and then dissolved with 0.5 ml of Solvable (Packard) and analyzed for cell-bound radioactivity. At each protein concentration, virus bound is expressed as the percentage of the total virus bound to control monolayers incubated with no soluble receptor from the formula (counts per minute for virus bound at a given protein concentration/counts per minute for virus bound when no protein was added) \times 100%; values are means \pm standard deviations for triplicate samples.

capable of interacting with CB3-RD. A control protein, the soluble ectodomain of the herpesvirus entry mediator B (HveB) (44), had no effect on virus binding. The higher concentration of soluble DAF required to inhibit binding may suggest a relatively lower affinity of this CB3 isolate for soluble DAF than for soluble CAR.

To examine receptor-induced conformational changes, we incubated ^{35}S -labeled CB3-RD at 4°C for 1 h with soluble CAR, soluble DAF, or control HveB protein, raised the tem-

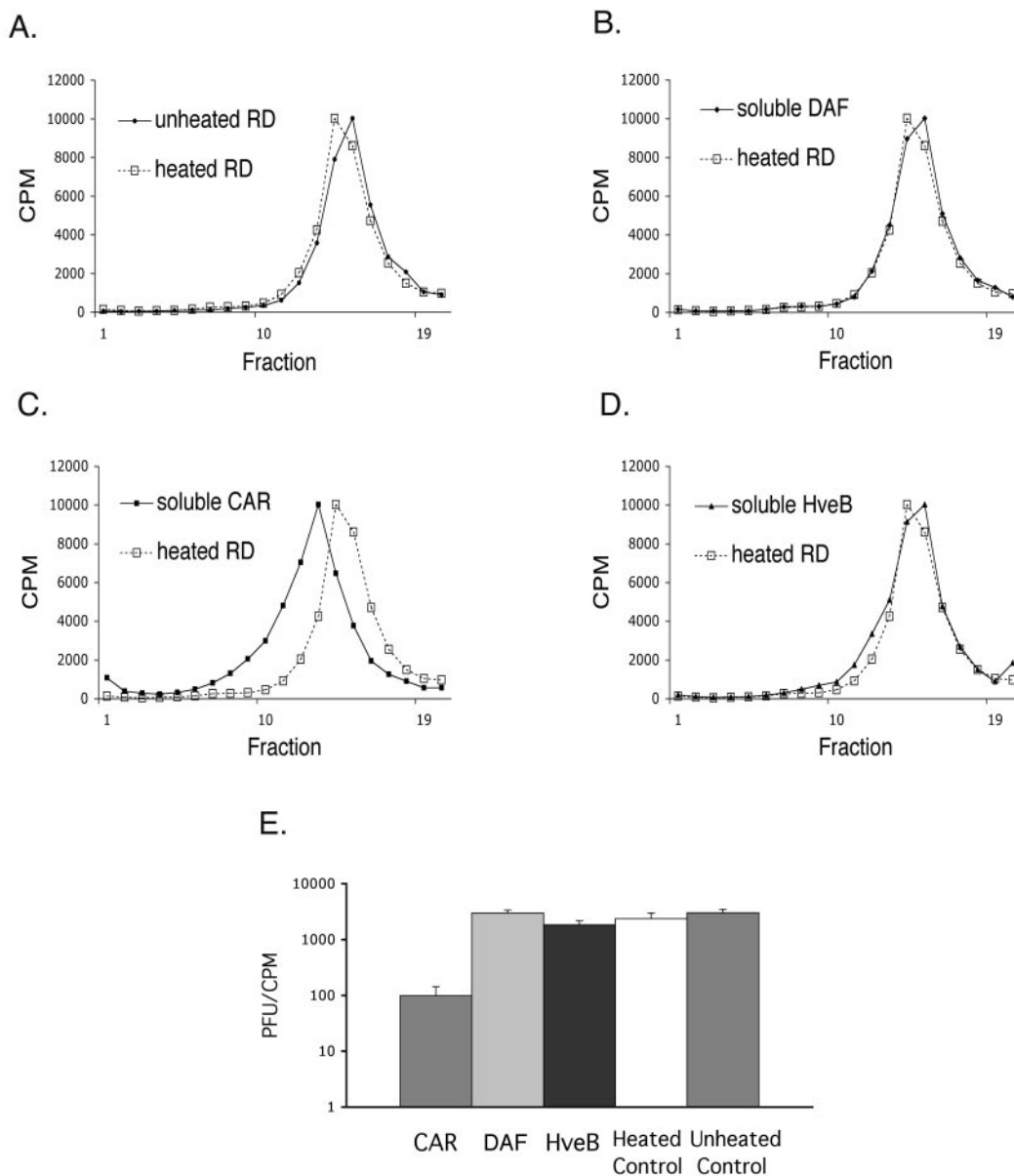


FIG. 3. Soluble CAR induces A-particle formation. ³⁵S-labeled CB3-RD (60,000 cpm) purified by cesium gradient centrifugation (29) was incubated with recombinant protein (5 μg) in 200 μl of minimal essential medium for 30 min on ice to allow binding and then transferred to 37°C for 30 min. A control sample with no protein was left unheated, because in preliminary experiments virus purified on sucrose rather than cesium gradients had appeared heat labile. (A to D) Virus was overlaid on 15 to 30% sucrose gradients and centrifuged at 45,000 rpm for 65 min at 4°C in a Beckman SW55Ti rotor. Fractions (250 μl) were collected from the top of the gradient (fraction 1), and radioactivity was measured. (E) Infectivity of virus recovered from peak gradient fractions (CAR fraction 13, DAF fraction 15, HveB fraction 15, unheated control virus fraction 15, and heated control virus fraction 14) was analyzed by plaque assay on HeLa cell monolayers. Infectivity is expressed as PFU per counts of radioactive virus per minute. Values are means ± standard deviations for triplicate samples.

perature to 37°C for 30 min, and then analyzed each sample by sucrose gradient centrifugation. Compared to untreated CB3-RD (or to virus heated without exposure to soluble receptors), CB3-RD exposed to soluble CAR had the reduced sedimentation velocity characteristic of A particles (Fig. 3A to D); exposure to soluble DAF or to the control protein had no effect on the sedimentation profile of CB3-RD. To confirm that the altered sedimentation resulted from a conversion to A particles, not just a transient association with CAR, we recov-

ered virus-containing fractions from the sucrose gradients and measured their specific infectivities (determined as PFU per counts of radiolabeled virus per minute). Virus exposed to soluble CAR showed a marked decrease in infectivity (Fig. 3E); in contrast, virus exposed to soluble DAF retained its infectivity. These data indicate that exposure of CB3-RD to soluble CAR induced A-particle formation, whereas exposure to soluble DAF did not.

A-particle formation is induced by CAR, but not by DAF, on

TABLE 1. Virus elution from CHO-CAR and CHO-DAF cells^a

Virus status	Mean % (\pm SD) of virus for:	
	CHO-CAR cells	CHO-DAF cells
Eluted at 37°C	25 (\pm 2)	5 (\pm 1)
Released from cell pellet by detergent	68 (\pm 2)	48 (\pm 6)
Retained in cell pellet after detergent	3 (\pm 0.3)	36 (\pm 9)

^a CHO-CAR and CHO-DAF cells (2.5×10^6) were incubated with ³⁵S-labeled CB3-RD overnight at 4°C and then washed to remove unbound virus and resuspended in 150 μ l of Dulbecco's phosphate-buffered saline (dPBS). CB3-RD bound to CHO-CAR cells at 4,581 cpm and to CHO-DAF cells at 11,834 cpm. Cells were incubated for 30 min at 37°C and then were pelleted for 2 min at 1,000 rpm (Eppendorf F45-24-11 microcentrifuge rotor), and supernatants containing eluted virus were collected. Cell pellets were lysed with detergent solution (PBS containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) for 10 min on ice; lysates were centrifuged for 2 min at 13,000 rpm, and then the radioactivity released or retained in the cell pellet was determined. Results are expressed as percentages of the total bound virus for triplicate samples. Approximately 10% of the bound virus was not recovered.

the cell surface. To exclude the possibility that the failure of soluble DAF to induce A-particle formation reflected some difference between cell surface and soluble receptors (or some defect in the soluble DAF preparation we used), we also examined virus interaction with CAR and DAF on the cell surface.

Soon after infection, A particles can be detected both within cells and in the cell supernatant (10, 21); elution from the cell surface at physiologic temperatures most likely reflects the incapacity of altered particles to remain bound to receptors. We permitted ³⁵S-labeled CB3 to attach to CHO-CAR and CHO-DAF cells at 4°C, washed the cells to remove unbound virus, and then incubated the cells 37°C for 30 min and measured the amount of virus that eluted into the cell supernatant (Table 1); we also measured the amount of uneluted virus released when cell pellets were lysed with detergent. Approximately 25% of bound virus was recovered in supernatants of CHO-CAR cells, and almost all of the remainder was released after detergent treatment. Virus eluted from CHO-CAR cells had lost infectivity (untreated-virus infectivity, $2,100 \pm 300$ PFU/cpm; infectivity of virus eluted from CHO-CAR cells, 26 ± 14 PFU/cpm). Only a small amount of virus (5%) eluted from CHO-DAF cells at 37°C; an equal amount eluted from CHO-DAF cells kept on ice (not shown). The virus eluted from CHO-DAF cells did not show the loss of infectivity associated with conversion to A particles (untreated-virus infectivity, $2,100 \pm 300$ PFU/cpm; infectivity of virus eluted from CHO-DAF cells, $4,700 \pm 1,800$ PFU/cpm). These results suggest that interaction with DAF on the cell surface did not lead to elution of A particles.

In a separate experiment, to examine both cell-associated and eluted virus, we permitted ³⁵S-labeled CB3-RD to bind to CHO-CAR and CHO-DAF cells in the cold, washed the cells to remove unbound virus, raised the temperature to 37°C for 60 min, and then lysed the cells and analyzed the lysates. CB3-RD bound to cell surface CAR showed the sedimentation velocity characteristic of A particles and lost infectivity (Fig. 4); in contrast, virus released after attachment to cell surface DAF did not differ from untreated virus in either sedimentation rate or specific infectivity. These data indicate that virus bound to

CAR on the cell surface underwent conversion to A particles at 37°C and that virus bound to cell surface DAF did not.

Discussion. Although many enteroviruses interact with DAF, attachment to DAF on the cell surface is not in itself sufficient for infection to proceed. In the work reported here, we have examined the early events that follow attachment of a DAF-binding CB3 isolate to CAR and DAF. Attachment to either receptor permitted virus to become tightly associated with the cell surface and to resist acid elution. However, only interaction with CAR—in soluble form or expressed on the cell surface—led to the formation of A particles. The results suggest that CAR, but not DAF, is capable of triggering the conformational changes that lead to RNA release from the viral capsid.

Many picornaviruses, including CB3 (17, 18), have a narrow depression on the capsid surface, termed the canyon, which has been shown to be the receptor-binding site (7, 17, 20, 35, 46). Beneath the base of the canyon, a hydrophobic pocket is filled by a lipid-like molecule—referred to as the pocket factor (15)—that is thought to help stabilize the viral capsid. Receptor interaction with the canyon may lead to displacement of the pocket factor and destabilization of the capsid (34). Although the binding site for DAF on the CB3 capsid has not been directly identified, genetic evidence (26, 30) and the structures of complexes between DAF and other enteroviruses (5, 19) suggest that DAF binds outside the canyon, where it is unlikely to trigger capsid disruption.

CB3-RD is one of many enteroviruses that fail to infect DAF-transfected rodent cells, despite efficient attachment to DAF on the cell surface. We have tested only CB3-RD, but we suspect that a failure to initiate A-particle formation and uncoating may explain why attachment of all these viruses to DAF does not necessarily lead to infection. Another DAF-binding enterovirus, echovirus 7, is converted to A particles when bound to the HeLa cell surface but not when exposed to soluble DAF (32); although it has not been shown that interaction between echovirus 7 and native DAF on the cell surface cannot trigger the essential conformational changes, it is entirely possible that, like CB3-RD, echovirus 7 and other DAF-binding picornaviruses must interact with other, unidentified receptors before uncoating and infection can occur.

CA21 interacts both with DAF and with intercellular adhesion molecule 1 (ICAM-1). Although DAF itself does not permit infection by many CA21 isolates, the virus infects cells on which DAF has been bound by antibodies (36), and viral variants that infect by a DAF-dependent pathway without antibody treatment have been identified (31). ICAM-1 induces CA21 A-particle formation, but in cells infected by the DAF-dependent mechanism no A particles were detected (36). CA21 attachment to DAF may lead to RNA release without A-particle formation; alternatively, it is possible that the kinetics of the DAF-initiated infectious process may be such that A particles do not accumulate to detectable levels. It has been reported that a CB3 isolate infects by a route that involves attachment to heparan sulfate rather than to CAR (48); the heparan-dependent entry mechanism has not been defined.

In the experiments reported here, we found that virus bound to DAF on the cell surface did not elute and remained in an intact, infectious state. Although CHO-DAF cells do not become infected by CB3-RD, we have observed (J. Shieh and J.

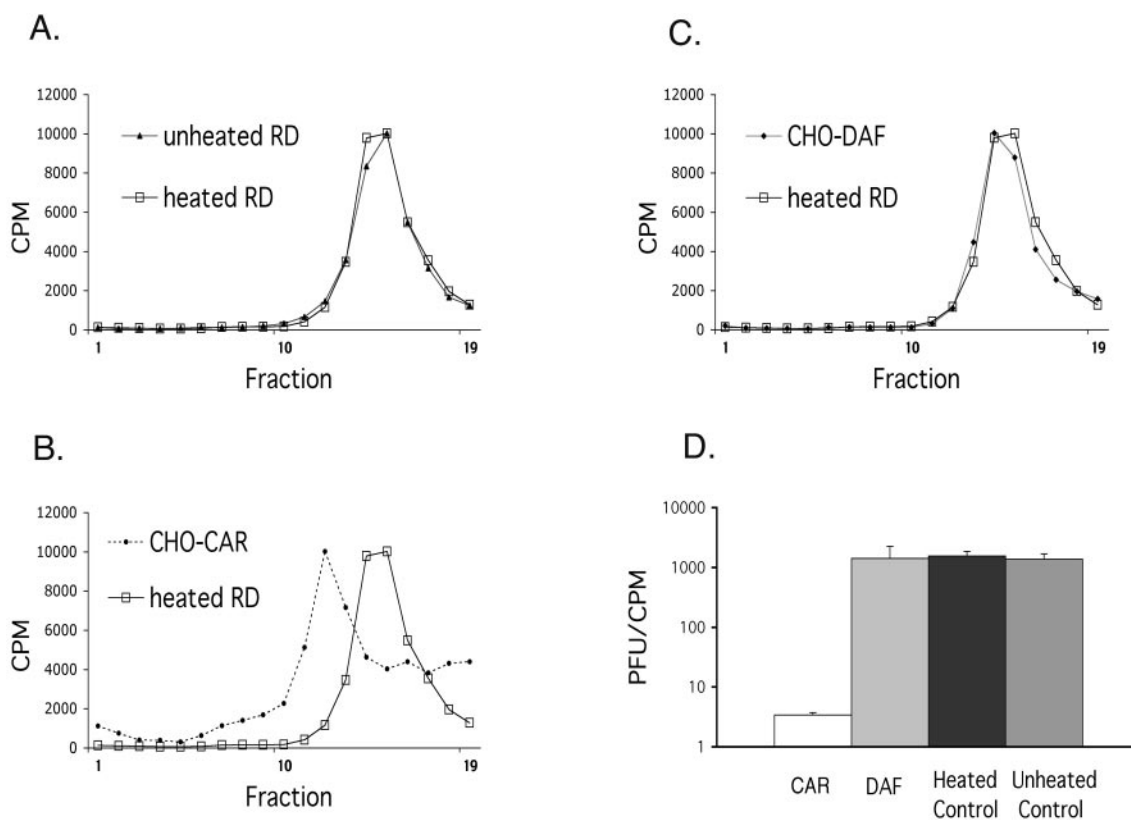


FIG. 4. CAR on the cell surface induces A-particle formation. CHO-CAR and CHO-DAF cells (1.5×10^7) were removed from culture flasks with 2.5 mM EDTA in phosphate-buffered saline (PBS), resuspended in 1 ml of Dulbecco's PBS (dPBS), incubated with cesium gradient-purified ^{35}S -labeled CB3-RD (200,000 cpm) overnight at 4°C , and then washed three times to remove unbound virus and resuspended in 250 μl of cold dPBS. CB3-RD bound to CHO-CAR cells at a level of 17,360 cpm and to CHO-DAF cells at a level of 74,020 cpm. Cells were then incubated at 37°C for 1 h; a control virus sample was left on ice, and an additional control sample was heated with no added cells. (A to C) Cells were lysed with 1% Triton X-100–0.5% deoxycholic acid–0.1% SDS, lysates were centrifuged to remove cell membranes and insoluble debris, and the supernatant was overlaid on a 15 to 30% sucrose gradient and centrifuged at 45,000 rpm for 65 min at 4°C in a Beckman SW55Ti rotor. Fractions (250 μl) were collected from the top of the gradient (fraction 1), and radioactivity was measured. (D) Infectivity of CB3-RD from peak gradient fractions (CHO-CAR fraction 12, CHO-DAF fraction 14, unheated control virus fraction 15, and heated control virus fraction 15) was analyzed by plaque assay on HeLa monolayers. Values are means \pm standard deviations for triplicate monolayers.

Bergelson, unpublished data) that transient expression of DAF on the surface of CHO-CAR cells, which enhances virus attachment to the cell surface, significantly increased their susceptibility to infection. It is thus clear that attachment to DAF is not a dead-end pathway: once bound to DAF, virus is likely to be available for subsequent interactions with CAR that lead to infection.

We previously observed that DAF-binding CB isolates, including CB3-RD, attach to DAF on the apical surfaces of polarized epithelial cells and initiate productive infection despite the apparent sequestration of CAR within tight junctions (39). Although we cannot exclude the possibility that infection of polarized cells is independent of CAR, the failure of DAF to trigger A-particle formation suggests that, once virus has attached to DAF, an interaction with CAR may be essential for infection to proceed.

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