Comparative analysis of virus–host cell interactions of haemagglutinating and non-haemagglutinating strains of coxsackievirus B3

Andreas Pasch, Jan-Heiner Küpper, Antje Wolde, Reinhard Kandolf and Hans-Christoph Selinka

Department of Molecular Pathology, Institute for Pathology, University of Tübingen, Liebermeisterstr. 8, D-72076 Tübingen, Germany

Decay-accelerating factor (DAF/CD55), and coxsackievirus–adenovirus receptor (CAR) have been identified as cellular receptors for coxsackie B viruses (CBV). To elucidate the interplay of DAF and CAR on the cell surface, virus–receptor interactions of two coxsackieviruses of serotype B3 (non-haemagglutinating CBV3 and haemagglutinating CBV3-HA strain) were analysed. Binding assays revealed clear differences between these viruses with regard to their interactions with DAF and CAR. However, only the combination of anti-DAF and anti-CAR antibodies resulted in complete inhibition of virus binding for both strains. In plaque-reduction assays, anti-DAF antibodies had no effect, whereas CAR-specific antibodies significantly reduced productive infection of HeLa cells by both viruses. Interestingly, a synergistic inhibitory effect of anti-DAF and anti-CAR antibodies was also observed with regard to infection. These findings support the model of preferential interactions of both strains of CBV3 with closely associated DAF and CAR proteins on HeLa cells, despite displaying clear differences in their binding phenotypes.

The six serotypes of coxsackie B viruses (CBV1–6) are members of the enterovirus group of the family Picornaviridae. They are causative agents of a broad spectrum of clinically relevant diseases including acute and chronic myocarditis, meningitis and possibly autoimmune diabetes (Fohlman & Friman, 1993; Kandolf et al., 1985; Klingel et al., 1992, 1998). Decay-accelerating factor (DAF/CD55), a 70 kDa glycosyl-phosphatidylinositol-anchored membrane protein, known as a cellular receptor or co-receptor for a number of different enteroviruses (Bergelson et al., 1994; Karnauchow et al., 1996; Shafren et al., 1997a, b), has also been identified as a CBV-specific binding protein (Reagan et al., 1984; Shafren et al., 1995, 1998). However, binding of CBV3 to DAF does not necessarily mediate infection of cells (Shafren et al., 1995). In contrast, the coxsackievirus–adenovirus receptor (CAR) protein, a member of the immunoglobulin superfamily with a molecular mass of 46 kDa, has been shown to be the major prerequisite for infection of cells with CBV3 (Mapoles et al., 1985; Bergelson et al., 1997a, 1998; Tomko et al., 1997; Selinka et al., 1998). Upon transfection with CAR cDNA, non-infectable hamster CHO cells become susceptible towards infection with CBV (Bergelson et al., 1997a; Tomko et al., 1997). Moreover, even CBV strains with strong DAF-binding properties may require the CAR protein to mediate lytic infection (Shafren et al., 1997b). It has been suggested that DAF and CAR are closely associated (Hsu et al., 1988) or even components of a putative CBV3 receptor complex (Shafren et al., 1997b).

With regard to these findings, a study was performed to elucidate the interplay of DAF and CAR on HeLa cells. To address this topic, we selected two different strains of serotype CBV3, one of which (CBV3-HA; ATCC VR-688) is able to haemagglutinate human group O erythrocytes, whereas the other, cDNA-generated CBV3 (Kandolf & Hofschneider, 1985; Klump et al., 1990), is not. Comparing virus–receptor interactions of these strains of CBV3, we present data supporting the model of a putative DAF/CAR complex on human HeLa cells.

To investigate the different binding properties of haemagglutinating and non-haemagglutinating CBV3, virus-overlay protein-binding assays (VOPBAs) were performed. Octyl-glucoside-solubilized cellular proteins of human HeLa and hamster CHO cells, prepared as described by Krah (1989), were separated by 10% SDS–PAGE, and subsequently blotted onto PVDF membranes. The filters were exposed to $^{35}$S-labelled viruses (300,000 c.p.m.) for 2 h and membrane-bound radioactivity was detected by autoradiography. As shown in Fig. 1(a), the cDNA-generated CBV3 bound to a single protein of 46 kDa whereas the haemagglutinating CBV3-HA attached to two proteins with molecular masses of 46 kDa and 70 kDa. Using the protocol of Xu et al. (1995) for VOPBAs, as well as sucrose gradient-purified preparations of infectious CBV

Author for correspondence: Hans-Christoph Selinka. Fax +49 7071 29 5334. e-mail hans-christoph.selinka@med.uni-tuebingen.de
virions (160S) depleted of highly abundant 125S provirions, the previously described 100 kDa binding protein for CBV (Raab de Verdugo et al., 1995) was not observed in the present study. So far, the role of 125S provirions interacting with a 100 kDa membrane protein for the pathogenesis of infectious disease is not known. The VOPBA presented in Fig. 1(a) demonstrates that infectious virions of the cDNA-generated CBV3 strain primarily bind to CAR (46 kDa), whereas virions of the haemagglutinating CBV3-HA variant recognize determinants of DAF (70 kDa) and CAR proteins.

Following demonstration of different binding properties of haemagglutinating and non-haemagglutinating CBV3 to immobilized cellular membrane proteins, attachment of these viruses to viable cells was investigated. Prior to the binding assay, the phenotype and level of CAR and DAF expression on human HeLa cells, human rhabdomyosarcoma (RD) cells and hamster CHO cells were assessed by flow cytometry using CAR-specific (RmcB) and DAF-specific (IF7) antibodies (Fig. 1b). Both antibodies recognize epitopes (within d1/d2 of CAR; SCR2 of DAF) located within or near the CBV-binding sites (Bergelson et al., 1995; Shafren et al., 1995). High levels of CAR and DAF were expressed on HeLa cells, whereas human RD cells, known for constant expression of DAF (Reagan et al., 1984; Bergelson et al., 1995; Shafren et al., 1997b) were clearly CAR-negative. Neither CAR nor DAF expression was detected on CHO cells. For analysis of virus binding, these cells (1 × 10⁶) were incubated with ³⁵S-labelled viruses (20,000 c.p.m.) for 60 min at room temperature. After removal of unbound virus, radioactivity adherent to HeLa, RD and CHO cells was detected in a liquid scintillation counter. As shown in Fig. 1(c), CBV3-HA significantly bound to CAR-negative RD cells, whereas CBV3 did not adhere to these cells. Both viruses bound to HeLa cells but did not bind to CAR-negative/DAF-negative CHO cells. Since HeLa cells express both DAF and CAR on their surface, this cell line was chosen to further investigate the respective roles of DAF and CAR in binding of the different CBV3 variants.

To perform binding inhibition assays, HeLa cells were preincubated for 30 min with receptor-specific antibodies prior to exposure to [³⁵S]methionine-labelled virions. As shown in Fig. 2(a), adherence of the non-haemagglutinating CBV3 strain to HeLa cells was significantly reduced in the presence of antibody RmcB as well as antibody IF7 (1:100 dilutions of ascites fluids), indicating a steric inhibitory effect of anti-DAF antibodies towards this non-DAF-binding strain. By combining anti-DAF and anti-CAR antibodies, binding was completely suppressed in an additive manner. Whereas attachment of the haemagglutinating CBV3-HA was reduced to 40% by use of anti-DAF antibodies, binding of this virus was not significantly inhibited using a 1:100 dilution of RmcB. Although anti-CAR antibodies alone did not cause any detectable inhibitory effect towards haemagglutinating CBV3-HA, they augmented the inhibitory effect of anti-DAF antibodies, resulting in complete...
Interaction of CBV3 strains with DAF/CAR receptors

Fig. 2. Antibody-induced inhibition of virus binding and infection. (a) Inhibition of virus binding by preincubation of HeLa cells with antibody IF7 (anti-DAF), RmcB (anti-CAR) or a combination of IF7 and RmcB. Binding of CBV3 and CBV3-HA was completely abolished in the presence of both RmcB and IF7 antibodies. A Hantaan virus-specific control antiserum did not impair binding of the tested CBV3 strains. Results are expressed as mean values (plus SD) of cell-bound radioactivity and are representative of three independent experiments. (b) Plaque phenotypes of CBV3 and CBV3-HA on HeLa cells. (c) Reduction of CBV3 and CBV3-HA plaque formation on HeLa cells in the presence of anti-DAF (IF7) and anti-CAR (RmcB) antibodies. Compared to the presence of IF7 or RmcB alone, simultaneous application of both antibodies (dilution 1:1000 in DMEM) resulted in synergistic protective effects towards both CBV3 and CBV3-HA strains (viable cells after 48 h post-infection were stained with crystal violet).

inhibition of virus binding in the presence of both anti-CAR and anti-DAF antibodies (Fig. 2a). For further analysis of this antibody-mediated inhibitory effect, plaque reduction assays were performed. As shown in Fig. 2(b), CBV3 and CBV3-HA exhibited clear differences in their plaque phenotypes. Infection of HeLa cells with the haemagglutinating CBV3-HA revealed a small plaque phenotype with plaques only 30–50% of the diameter of those of CBV3, without affecting the growth characteristic and titre of this virus (see Fig. 3a). Minimal amounts of CAR-specific and DAF-specific antibodies (dilution 1:1000) were used to study the interplay of haemagglutinating and non-haemagglutinating CBV with CAR and DAF with regard to infection of HeLa cells (Fig. 2c). Cells were grown in 24-well plates, preincubated with antibodies IF7, RmcB or a combination of both antibodies, followed by exposure to CBV3 or CBV3-HA. Monolayers were overlaid with agarose and surviving cells were stained with crystal violet after 48 h incubation at 37 °C. Regarding infectivity, plaque formation by both viruses required 10-fold higher virus titres in the presence of minimal amounts of CAR-specific RmcB antibodies (Fig. 2c). In contrast to the study of Shafren et al. (1997b) with CBV3/New, the presence of DAF-specific antibody IF7 alone did not cause any inhibitory effect on CBV3 and CBV3-HA infections. However, in accordance with the CBV3/New study (Shafren et al., 1997b), the combination of both IF7 and RmcB resulted in a synergistic inhibitory effect with respect to infection of HeLa cells with both CBV3 strains. These data further support the model that DAF and CAR are closely associated on the membrane of human HeLa cells.

Binding of enteroviruses to cells expressing specific receptors triggers changes in the virion and a significant fraction of attached virus particles is subsequently released in a conformationally altered form, designated A-particles (Crowell & Philipson, 1971; McGeady & Crowell, 1979; Powell et al., 1997). Such A-particles have lost viral protein 4 (VP4), are non-infectious and exhibit a reduced sedimentation rate (135S, in contrast to 160S of complete virions) in sucrose gradients. The amount of CBV3 and CBV3-HA virions converted into A-particles was determined in supernatants of CBV-infected HeLa cells. Radioactively (35S) labelled CBV3 and CBV3-HA (140 000 c.p.m. each) were allowed to bind to 1 × 10^7 HeLa cells at room temperature. After removal of unbound virus, cells were incubated at 37 °C for 1 h and culture supernatants were spun through a 5–30% sucrose gradient (25 400 g, 80 min at 4 °C). As determined by liquid scintillation counting, virus–host cell interactions of both virus strains resulted in generation of 135S A-particles (Fig. 3b). Since comparable amounts of conformationally changed virus particles were obtained with both virus strains, these data suggest that formation of 135S A-particles may be primarily dependent on CAR, the host cell protein involved in specific interactions with both CBV3 and CBV3-HA. To pursue this hypothesis, formation of A-particles of CBV3 and CBV3-HA was also investigated in the presence of receptor-specific...
Fig. 3. Common features of virus–host cell interactions. (a) Single-step growth curves of CBV3 and CBV3-HA in HeLa cells. CHO cells are non-permissive towards both CBV strains. (b) Formation of receptor-mediated altered virus particles (A-particles; 135S) by specific interactions of CBV3 or CBV3-HA virions (160S) with HeLa cells. After 60 min incubation at 37°C, particles were separated in 5–30% linear sucrose gradients. Despite different binding properties, comparable amounts of A-particles were obtained with both virus strains. As sedimentation markers, 35S-labelled virions (160S) and provirions (125S) were spun in parallel (dotted lines). (c) Formation of 135S particles of CBV3 and CBV3-HA in the presence of receptor-specific antibodies during virus binding (at 4°C) and/or virus internalization (37°C). Both CAR and DAF antibodies compete for virus binding and subsequent A-particle formation. During virus internalization at 37°C, only the presence of CAR-specific antibodies significantly reduced the level of non-infectious A-particles released into the culture supernatant.

antibodies (Fig. 3c). In the presence of anti-CAR or anti-DAF antibodies (dilution 1:500), during both virus binding (at 4°C) and virus internalization (at 37°C), the amount of 135S particles released into the culture supernatant after 1 h at 37°C was severely reduced. This result is not unexpected, since both anti-DAF and anti-CAR antibodies do affect virus binding to the putative DAF/CAR complex on HeLa cells. In contrast, different results were obtained when receptor-specific antibodies were only present at the post-binding events at 37°C. CAR-specific RmcB antibodies did still lead to reduced levels of released A-particles, whereas the same amounts of 135S particles were measured in the presence or absence of DAF-specific IF7 antibodies. These results suggest participation of CAR but not DAF in post-binding events leading to A-particle formation.

The additive effects of minimal amounts of anti-CAR and anti-DAF antibodies on virus binding and infection presented in this paper favour a model of specific interactions of both haemagglutinating and non-haemagglutinating strains of CBV3 with a DAF/CAR complex on the surface of HeLa cells, with CAR being the infection-mediating component. In this model, the cDNA-generated, non-haemagglutinating CBV3...
primarily binds to CAR whereas the haemagglutinating strain CBV3-HA contacts both components of the putative DAF/CAR complex. The haemagglutinating CBV3-HA virus has the ability to bind to human RD cells but, in contrast to the haemagglutinating CBV3-RD variant of Crowell and colleagues (Reagan et al., 1984), the host range of CBV3-HA is not extended to these cells (data not shown) but is still dependent on CAR. The presence of a DAF/CAR complex on HeLa cells is also supported by recently published data of Martino et al. (1998), reporting the observation that pretreatment of HeLa cells with DAF-binding viruses or antibodies sterically blocks the interaction of adenovirus type 2 with CAR. Data reported for coxsackie A virus 21 (CAV21), which binds to DAF but requires the DAF-adherent ICAM-1 molecule for cell entry (Shafren et al., 1997a), might well be interpreted accordingly.

Since the CBV canyon harbouring the receptor-binding site is not as pronounced as the canyon of polioviruses and rhinoviruses (Muckelbauer et al., 1995), a multicomponent receptor complex may substantially increase the efficiency of virus binding. However, despite different binding properties, the growth characteristics of CBV3 and CBV3-HA did not significantly differ in HeLa cells (Fig. 3a). Therefore, the positive role of DAF as a CBV3 sequestration site, as postulated by Shafren et al. (1997b), could not be verified for the haemagglutinating CBV3-HA strain and may vary within DAF-binding CBV strains. However, in cells or tissues where CAR expression is very low, e.g. smooth muscle cells, the proposed DAF/CAR complex might well select for DAF-binding CBV3-HA, due to its affinity for both components, DAF and CAR. Supporting this model, a high prevalence of DAF-binding CBV3 strains among clinical isolates has been reported (Bergelson et al., 1997b). Therefore, it is tempting to speculate whether a tissue-specific pattern of DAF and CAR molecules might control selection of DAF-binding CBV3 strains in vivo. Consequently, investigations should be pursued to determine the distribution of DAF and CAR molecules in different tissues as well as their interplay with regard to CBV susceptibility and virus resistance.

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References


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