Defining CAR as a cellular receptor for the avian adenovirus CELO using a genetic analysis of the two viral fibre proteins

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The coxsackievirus and adenovirus receptor (CAR) is a high affinity receptor used by adenoviruses, including adenovirus type 5 (Ad5). The adenovirus fibre molecule bears the high affinity cell binding domain of Ad5, allowing virions to attach to CAR. The avian adenovirus CELO displays two fibre molecules on its capsid and it was logical to expect that the cell binding functions of CELO might also reside in one or both of these fibres. We had previously shown that the cell binding properties of CELO resemble Ad5, suggesting that the two viruses use similar receptors. Experiments with CAR-deficient CHO cells and CHO cells modified to express CAR demonstrated that CELO has CAR-dependent transduction behaviour like Ad5. Mutations were introduced into the CELO genome to disrupt either the long fibre 1 or the short fibre 2. A CELO genome with fibre 2 disrupted did not generate virus, demonstrating that fibre 2 is essential for some stage in virus growth, assembly or spread. However, a CELO genome with disrupted fibre 1 gene produced virus (CELOdF1) that was capable of entering chicken cells, but had lost both the ability to efficiently transduce human cells and the CAR-specific transduction displayed by wild-type CELO. The ability of CELOdF1 to transduce chicken cells suggests that CELOdF1 may still bind, probably via fibre 2, to a receptor expressed on avian but not mammalian cells. CELOdF1 replication was dramatically impaired in chicken embryos, demonstrating that fibre 1 is important for the in vivo biology of CELO.

Introduction

This manuscript describes efforts to understand the functions of the two fibres of the avian adenovirus CELO, the prototype member of the fowl adenovirus type 1 species. Early observations of CELO by electron microscopy revealed a two-fibred capsid (Laver et al., 1971; Gelderblom & Maichle-Lauppe, 1982), clearly different from the capsids of human adenoviruses 2 and 5, which present a single fibre. Subgroup F adenoviruses (Ad40 and 41) also display two types of fibres on their capsids (Kidd et al., 1993; Davison et al., 1993). However, with Ad40/41, only one type of fibre is inserted in each penton, whereas with CELO, each penton displays one short and one long, bent fibre (Laver et al., 1971; Gelderblom & Maichle-Lauppe, 1982; Hess et al., 1995).

The coxsackievirus and adenovirus receptor (CAR) is the high affinity receptor used by adenovirus serotypes 2 and 5 in subgroup C (Bergelson et al., 1997, 1998; Tomko et al., 1997; reviewed in Bergelson, 1999). CAR is also used as a receptor for a variety of mastadenovirus serotypes (Roelvink et al., 1998). Adenovirus subgroup C transduction, where transduction is defined as the ability of a virus to bind to, enter and direct expression of a transgene in a target cell, correlates well with CAR expression in cultured cells (Hemmi et al., 1998; Nalbantoglu et al., 1999; Li et al., 1999a, b; McDonald et al., 1999; Rebel et al., 2000; Turturro et al., 2000; Pearson et al., 1999). However, additional barriers to adenovirus exist in vivo and CAR expression is not the sole determinant of adenovirus subgroup C transduction (Walters et al., 1999; Schachter et al., 1999; Fechner et al., 1999; Pickles et al., 2000). There is also evidence that adenovirus subgroup C can employ other cell surface molecules during entry, including integrins (reviewed in Nemerow & Stewart, 1999), MHC class I molecules (Hong et al., 1997) and proteoglycans (Dechecchi et al., 2000). Other adenovirus serotypes may use other receptors. Thus, CAR-independent transduction is observed with adenovirus type 35 for CD34+ cells (Shayakhmetov et al., 2000) and the fibre of subgroup D adenovirus (e.g. Ad17) may have a useful receptor...
on lung and nervous system targets (Zabner et al., 1999; Chillon et al., 1999). Subgroup B adenoviruses appear to use a receptor distinct from CAR (Defer et al., 1990; Roelvink et al., 1998). The structure of CAR and the details of CAR–fibre interaction are now available (van Raaij et al., 1999; Freimuth et al., 1999; Santis et al., 1999; Kirby et al., 1999, 2000; Roelvink et al., 1999; Bewley et al., 1999; Tomko et al., 2000).

The adenovirus fibre molecule bears the high affinity cell binding domain of Ad5, allowing virions to attach to CAR. It was thus logical to expect that the cell binding functions of CELO might also reside in one or both of the fibre molecules present on the CELO virion. It is demonstrated here that CELO, like Ad5, transduces CAR-deficient CHO cells poorly and, as observed for Ad5, transduction is 100-fold greater with CHO cells modified to express CAR. A genetic analysis was performed to determine if one or both of the CELO fibres plays a role in CAR binding. Mutations were introduced into the CELO genome to disrupt either the long fibre 1 or the short fibre 2. A CELO genome with disrupted fibre 2 did not generate virus, suggesting that fibre 2 is essential for some stage of virus propagation. However, a CELO genome with the fibre 1 gene disrupted produced virus (CELOdF1) that was capable of infecting chicken cells, but had lost its ability to efficiently infect all tested human cells. An analysis of CHO and CHO-CAR transduction demonstrated that removal of fibre 1 resulted in loss of the CAR-specific transduction and CHO-CAR transduction demonstrated that removal of fibre 1 resulted in loss of the CAR-specific transduction displayed by wild-type CELO. The ability of CELOdF1 to infect chicken cells suggests that CELOdF1 may still bind to a receptor expressed specifically on avian cells, probably via fibre 2.

Methods

- **Deletion of fibre 1 from the CELO genome (CELOdF1).** The CELO HindIII fragment from nt 27061 to 33921 was cloned into a modified pSP65 plasmid containing no Spl site to generate pTPK91. The CELO fibre 1 sequences between the unique Spl (28094) and HpaI (30503) sites were replaced by a chloramphenicol-resistance cassette to generate pTPK92. The plasmid pTPK93, which contains the CELO Smal fragments from nt 26150 to 36818 cloned into a PUC19 derivative, was linearized at the unique MluI site and recombined in E. coli B/J5183 with the HindIII fragment from pTPK92 to generate pTPK94. Finally, the Smal fragment bearing the disrupted CELO fibre 1 region from pTPK94 was introduced into partially digested (AflII) pAIM46 (Michou et al., 1999) by homologous recombination in E. coli B/J5183 to generate pCELOTPK10, a plasmid with ampicillin resistance bearing a Spl-flanked CELO mutant genome with the following features: CELO nt 1–28094, a CMV promoter, and an internal luciferase expression cassette; CELO nt 28849 in CELO genome) were used.

- **PCR analysis of the fibre 1 deletion.** Template DNA was prepared by proteinase K treatment of LMH infected with passage 3 material in 6-well plates. Control DNAs were plasmids carrying either wild-type or truncated fibre 1. Oligonucleotide pTPK57 (5′ AAGCAGAGGTGTCCACCTGAATT-3′, which hybridizes at position 29399 in the CELO genome and introduces an MluI site, underlined) and pAIM53 (TCCTGCGGCACCTGAATTTC, which hybridizes at position 30562 in the CELO genome and introduces an Ascl site, underlined). Meanwhile, CELO genome plasmid pAIM69 (which bears a deletion of CELO nt 41520–43682 and an insertion of a luciferase expression cassette; Michou et al., 1999) was digested with HpaI, the internal HpaI fragments were removed and the fragment bearing the bacterial plasmid sequence and the two CELO ends was religated to generate pAIM73. The OAIM37/38 PCR product was digested with MluI and Ascl and subcloned into pAIM73 cut with MluI. The plasmid obtained, pAIM76, has the following features: CELO left end nt 1–4868, CELO right end nt 29399–30563, nt 31326–41730, luciferase expression cassette and CELO right extremity 43685–43812.

- **Other viruses.** The luciferase-expressing viruses with wild-type capsid have been described previously (Michou et al., 1999). These include the Ad5-based (AdLuc) or CELO-based CELOwt (CELO with wild-type capsid, AIM46). Both viruses contain the same CMV/β-luciferase/β-globin cassette that is present in the CELOdF1 and CELOdF2 genomes.

- **Cell lines and culture.** Chinese hamster ovary (CHO) cell lines stably transfected to express human CAR (CHO-CAR) or a control CHO cell line stably transfected with pCDNA3.1 (CHO-pCDNA3.1) were previously described (Bergelson et al., 1998). Primary chicken embryonic kidney and liver cells were prepared as previously described (Chiocca et al., 1996).

The A549 (human lung carcinoma) and TIB73 (murine hepatocyte, BNL CL.2) cell lines were from the ATCC, the chicken hepatocyte LMH cell line (Leghorn male hepatoma) was described previously (Kawaguchi et al., 1987), the chicken fibroblast CEF38. cell line was obtained from Martin Zenke (MDC, Berlin, Germany) and normal human dermal fibroblasts were obtained from Clonetics and were used between passage 5 and 15; all five cell types were cultured in DMEM–10% FCS. The 293 cell line (Graham et al., 1977) was from the ATCC and was cultured in MEMalpha with 10% newborn calf serum.

- **Virus infections.** Infections were performed in 24-well plates with a defined cell number at approximately 70% confluence. Cells were infected with AdLuc or CELOLuc or CELOdF1 at 10000, 3000, 1000, 300 or 100 virus particles per cell. Cell lysates were prepared at 24 h post-infection and luciferase gene expression was measured in equal protein aliquots.

- **Analysis of CELOdF1 and CELOdF2 growth.** The mutant plasmid-borne genomes (pCELOdF1 and pCELOdF2) were linearized with SpeI and transfected into LMH cells in 24-well plates (0.75 μg DNA per well transfected using polyethyleneimine, PEI; Michou et al., 1999). After 1 week, the cells were harvested and freeze-thawed/sonicated to release virus progeny. Aliquots of the resulting lysate (passage 0) were analysed for luciferase activity in triplicate and a second set of aliquots was applied to fresh LMH cells. This process was repeated until passage 3.

- **PCR analysis of the fibre 1 deletion.** Template DNA was prepared by proteinase K treatment of LMH infected with passage 3 material in 6-well plates. Control DNAs were plasmids carrying either wild-type or truncated fibre 1. Oligonucleotide pTPK57 (5′ AAGCAGAGGTGTCCACCTGAATT-3′, which hybridizes at position 29399 in the CELO genome and introduces an MluI site, underlined).
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Fig. 1. (a, b) Diagrams showing the construction of CELO genomes with disrupted fibre 1 or fibre 2. (c) CELO can transduce via CAR. CHO-CAR and CHOpDNA3.1 cells were infected with Ad5 (left panel; AdLuc with wt Ad5 capsid), CELOdF1 (middle panel) or CELOwt (right panel) at $10^2$, $3 \times 10^2$, $10^3$, $3 \times 10^3$ or $10^4$ particles per cell (indicated by the triangle under each set of bars, increasing width indicates increasing virus). Forty-eight hours after infection, cells were lysed and assayed for luciferase activity, expressed as relative light units per µg of cellular protein. The data are the mean of triplicate samples with a standard deviation indicated, background measurement from uninfected cell lysates has been subtracted from all values. Open bars, CHO-pCAR; hatched bars, CHO-pCDNA3.1. (d) CELOdF1 is viable, CELOdF2 is not. As described in Methods, LMH cells were transfected with the luciferase-bearing CELO genomes pCELOdF1 or pCELOdF2. Luciferase values (average of three measurements with a standard deviation indicated) are presented for the lysate after each passage.

- PCR analysis of the fibre 2 deletion. Template DNA was prepared by proteinase K treatment of LMH cells infected with passage 4 material. Control DNAs were plasmids carrying either wild-type or truncated fibre 2. Oligonucleotide OTPK61 (5' TCTTGGGAGTGCTTCTGAAAGG 3', sense oligo hybridizing at positions 30933–30954 in the CELO genome) and OTPK62 (5' TCTGCGGGGTTCAAGAGG 3', antisense oligo hybridizing at positions 31452–31471 in CELO genome) were used.

- Electrophoresis and Western blot analysis of CELO mutants. Electrophoresis was performed on 6% or 10% polyacrylamide...
gels in the presence of SDS and, after transfer to nitrocellulose membranes, viral proteins were analysed by immunoblotting with a polyclonal antibody recognizing CELO capsid proteins (Michou et al., 1999). Antibody binding was revealed with a peroxidase-conjugated secondary antibody (rabbit anti-mouse HRP; Dako) followed by ECL (Amersham).

**Results and Discussion**

The similarity of transduction levels of various cell types suggested that Ad5 and CELO might use similar receptors for infection. To test this idea more directly, control cells (CHO-pcDNA3.1) lacking functional levels of CAR and CHO-CAR which have been engineered to express high levels of human CAR were used to examine the role of CAR in CELO transduction. CHO-pcDNA3.1 and CHO-CAR were infected with adenovirus or CELO over a range of multiplicities of infection. Both virus types bear the same CMV-{	extit{luciferase}} expression cassette; thus transduction efficiencies could be measured by monitoring luciferase activity. CELO transduction was found to be approximately 100-fold higher when the target cells expressed CAR (Fig. 1c), similar to the behaviour previously reported for Ad5. The substantial improvement of CELO transduction with CAR-bearing cells demonstrates that CAR can function as a receptor for CELO.

Based on mastadenovirus studies, it was hypothesized that CAR binding would be a property of one or both of the two CELO fibres. To test this hypothesis, CELO genome mutants lacking either fibre 1 (CELOdF1) or fibre 2 (CELOdF2) were constructed within a CELO backbone (Michou et al., 1999) which bears a luciferase expression cassette to facilitate monitoring of virus growth and transduction.

LMH cells were transfected with the linearized plasmid-borne CELO genomes pCELOdF1 (deleted for fibre 1) and pCELOdF2 (deleted for fibre 2). At 1 week intervals, the cells were harvested, freeze–thawed and sonicated to release
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Fig. 4. CELO fibre 1 is important for mammalian cell transduction and fibre 2 interacts with receptor on avian cells. Infectivity of wild-type vs CELOdF1 on various cell lines was determined. Cells were infected with AdLucTPK (wt Ad5 capsid), CELOdF1 or CELOwt (AIM46, wild-type capsid) at $10^2$, $3 \times 10^2$, $10^3$, $3 \times 10^3$ or $10^4$ particles per cell (indicated by the triangle below each set of bars, increasing width indicates increasing virus). Twenty-four hours after infection, cells were lysed and assayed for luciferase activity, expressed as relative light units per µg of cellular protein. The data are the mean of triplicate samples with a standard deviation indicated; background measurement from uninfected cell lysates has been subtracted from all values.

potential viruses and the cleared lysates were used to infect fresh LMH monolayers. Luciferase activity was monitored at each passage.

Luciferase was clearly present at passage 0, indicating successful transfection of the modified viral genomes (Fig. 1d). Luciferase activity was maintained in subsequent passages of CELOdF1, consistent with virus replication and demonstrating that the presence of fibre 1 was not essential for either virus assembly or infection. In contrast, the CELOdF2 genome produced luciferase at passage 0, indicating successful transfection, but luciferase activity was not observed in subsequent passages, indicating that fibre 2 was essential for virus assembly or infection (Fig. 1d). This pattern was observed in several transfection attempts, supporting the conclusion that it is the lack of fibre 2 and not a technical problem that was responsible for the absence of passageable material.

Further evidence that CELO fibre 1 is dispensable for virus growth was obtained by amplifying the CELOdF1 material and isolating virions in a pure form for analysis. The CELOdF1 virus yield was reduced compared to that of CELO with a wild-type capsid. Purified CELOdF1 was obtained at approximately $3 \times 10^9$ particles per $10^7$ cells. CELO with a wild-type capsid
Fig. 5. Growth of CELOwt and CELOdF1 in chicken embryos. The indicated particle numbers of either CELOwt or CELOdF1 were injected into the allantoic cavity of 9-day-old embryos. After a 4 day incubation at 37 °C, the virus present in cleared lysates of allantoic fluid was measured by luciferase transduction in CEF cells. Each value represents the average of at least five independent embryo infections with luciferase transduction measured in triplicate for the allantoic fluid of each embryo.

(e.g. CELOAIM46; Michou et al., 1999) yields approximately $3 \times 10^{10}$ particles per $10^7$ cells. Analysis of the protein content of purified virions showed that a single protein with the predicted molecular mass of fibre 1 (78 kDa) was present in CELO wild-type virions but absent from virions of CELOdF1 (Fig. 2a, b). Assuming a capsid structure similar to Ad5, a molecular stoichiometry of one fibre 1 to 20 hexons would be expected; the observed ratio of fibre 1 to other capsid proteins is consistent with this (Fig. 2a, b). Analysis of the DNA of CELOdF1 confirmed that the virus had the expected fibre 1 open reading frame deletion (Fig. 2c).

We considered that removal of one fibre might alter the structural properties of the CELO capsid. A striking structural feature of the CELO virion is its elevated temperature stability compared to Ad5 (reviewed in McFerran & Adair, 1977; Michou et al., 1999), thus an examination of the thermal stability of CELOdF1 infectivity might reveal subtle changes in capsid structure. Because all three virus types (Ad5, CELOwt and CELOdF1) can infect chicken cells (see below), the CEF line was used for this analysis. When Ad5, CELOwt and CELOdF1 were examined for heat stability, the behaviour of Ad5 and CELOwt was as previously reported: Ad5 was compromised by exposure to temperatures above 50 °C, while CELOwt required exposure to temperatures above 60 °C to disrupt infectivity (Fig. 3). CELOdF1 behaved like CELOwt, displaying the same profile of heat inactivation (Fig. 3). Thus the removal of fibre 1 did not compromise the capsid structure as measured by heat-resistant infectivity.

It was next determined if the removal of fibre 1 altered the transduction range of CELO. An analysis of CELOdF1 transduction of CHO and CHO-CAR cells showed that the efficient CAR-dependent transduction activity of wild-type CELO was not displayed by CELOdF1 (Fig. 1c), demonstrating that fibre 1 is essential for CELO transduction via CAR. Consistent with this conclusion, CELOdF1 was inactive for transducing any of the mammalian cells tested, including 293, A549, HepG2 and TIB73 (Fig. 4). In contrast, CELOdF1 was capable of transducing a variety of chicken cells, including the CEF and LMH chicken cell lines and primary embryonic chicken hepatocytes and kidney cells, with only modest declines in the efficiency (Fig. 4). Thus, it appears that the CELO fibre 1 is responsible for CAR binding activity of CELO and for the high level transduction of mammalian cells obtained with CELO. Fibre 1-independent transduction, presumably via fibre 2, appears to involve a receptor that is present on chicken but not mammalian cells.
As a further measure of the role of fibre 1 and CAR binding in CELO transduction, the infectivity of CELOwt and of CELOdF1 was compared by measuring virus yield from infected chicken embryos. Embryonic infection via the allantoic cavity involves an initial infection of cells lining the cavity followed by lysis and spread of the virus throughout organs of the developing chicken embryo. CELOwt was especially effective for embryo infection, with full yields of virus obtained with as few as 40 virus particles injected per embryo (Fig. 5). Removal of fibre 1 compromised, but did not completely block, the growth of CELO in embryos, with maximum yields of virus obtained only when $4 \times 10^7$ or more virus particles were injected per embryo (Fig. 5). Thus it appears that the infection and spread of CELO in chicken embryos is primarily facilitated by fibre 1, although inefficient infection and spread can occur in a manner independent of fibre 1.

As a control to demonstrate that the removal of fibre 1 impairs only receptor utilization and does not alter other entry functions of the CELO virion, CELOdF1 was assembled into PEI–DNA complexes which serve to target material via charge interactions with the cell surface (Baker et al., 1997). CELOdF1 was capable of efficient mammalian cell transduction when assembled into such complexes (Fig. 6). Thus the function of CELO fibre 1 can be replaced by artificial methods of cell targeting.

In conclusion, it is demonstrated here that of the two fibre molecules encoded by CELO, the longer fibre 1 is responsible for a CAR-dependent infection pathway. Deletion of fibre 1 is tolerated by the virus presumably because CELO possesses a second mode of cell interaction, likely to involve the shorter fibre 2. Deletion of fibre 1 results in a virus that can still be propagated with reasonable efficiency in avian cells, but which has lost the ability to infect mammalian cells. This virus may provide a useful backbone vector for strategies to target adenovirus to specific mammalian cell types.

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References
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