The Human Membrane Cofactor CD46 Is a Receptor for Species B Adenovirus Serotype 3

Dominique Sirena,† Benjamin Lilienfeld,† Markus Eisenhut,‡ Stefan Kälin, Karin Boucke, Roger R. Beerli, Lorenz Vogt, Christiane Ruedl, Martin F. Bachmann, Urs F. Greber, and Silvio Hemmi

Institute of Molecular Biology and Institute of Zoology, University of Zürich, CH-8057 Zürich, and Cytos Biotechnology AG, CH-8952 Schlieren-Zürich, Switzerland

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To date, 51 human adenovirus (Ad) serotypes have been identified and classified into six species, A to F (47). The major Ad vectors currently used in clinical applications are derived from species C Ad serotype 2 (Ad2) and Ad5. Their biology is very well characterized (for reviews, see references 27, 33, and 48). Some species B Ads are capable of infecting cells refractory to the well-characterized species C Ads and have a low seroprevalence (34, 46). These characteristics make the species interesting vectors for gene therapy approaches (14). We demonstrate here that the membrane cofactor CD46 is a receptor for species B Ad3. Our data indicate that CD46 directly binds to the Ad3 fiber knob with subnanomolar affinity and mediates dose-dependent viral transgene expression and cytopathic effects (CPE) in rodent and human cells.

MATERIALS AND METHODS

Virus. The molecular identity of the Ad3 stock (prototype strain GB, kindly provided by the late T. Adriani, Medizinische Hochschule Hannover, Hannover, Germany) was verified by DNA restriction analysis (1) and DNA sequencing. This Ad3 stock has a single point mutation in the fiber gene resulting in a Tyr-to-Ser change at position 218 of the fiber head (Y218S), relative to the published sequence (39). Ad3 was grown, isolated, radiolabeled, or fluorochrome labeled as described for Ad2 (12, 30). CsCl-purified 3H-labeled Ad3 had a specific radioactivity of ~2.14 × 10^6 dpm/μg. Purified virus was judged to be homogeneous by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and negative-staining electron microscopy (EM) analyses. Alexa-488- and Texas red (TR)-labeled Ad3 had the same infectivity as the unlabeled parental virus.

Construction of Ad5-based AdCMV-eGFP, derived from an E1/E3 deletion mutant, has been described elsewhere (28). The chimeric Ad3 fiber-Ad5-based AdCMV-eGFP vector (Ad5/F3) was constructed in a manner similar to that described for Ad5/F35 (37). Briefly, Ad5/F3 contained 45 amino acids of the Ad5 tail fused to 86 amino acids of the Ad3 shaft and 187 amino acids of the Ad3 knob by overlapping PCR (fiber swapping). The chimeric fragment was introduced into transfer plasmid pBL-EcofrgAd5, containing 6,729 bp of the right end of Ad5, including the fiber gene and inverted terminal repeat sequence, by using the unique NdeI and AflII sites. The chimeric fragment was introduced into transfer plasmid pBL-EcofrgAd5, containing 6,729 bp of the right end of Ad5, including the fiber gene and inverted terminal repeat sequence, by using the unique NdeI and AflII sites.
Isolation of CD46 cDNAs encoding the Ad3 receptor. To identify a cell surface receptor(s) for species B1 Ads, we used the Sindbis virus-based cDNA expression system (19) to screen for the binding of Ad3 particles labeled with the fluorophore Alexa-488 to BHK cells. The cDNA library was made from the virus-infected K562 cells. Viral stocks were prepared and cloned into pCDNA3 (Invitrogen). For bulk cultures, plates were infected at multiplicities of infection (MOIs) of 3.3, 10, and 30, washed at 2 h p.i., and the mixture was incubated under constant agitation for 2 h. The resulting cell culture medium. Cells were diluted to 6.7 × 10^5 cells/ml, and 105 cells were incubated with monoclonal and polyclonal immunoreagents and blocking antibodies specific for CD46, anti-human CAR antibody E1-1 (8), and soluble CD66c-Fc (comprising 295 amino acids of the mature extracellular domain fused to 232 amino acids of the human immunoglobulin G1 (IgG1) Fc domain, including the hinge, CH2, and CH3 regions), and CAR-Fc (8) or a 30-fold excess of unlabeled Ad3 on ice for 15 min. Subsequently, 5 µg of Ad3-Alexa-488 or Alexa-488 Alexa-488 H-linked Ad3 was added in a total volume of 200 µl on ice; after 1 h, the cells were washed and analyzed for bound virus by cytofluorometry or liquid scintillation counting (28). The data were normalized to the amounts of virus bound in the absence of inhibitors and are presented as the mean ± standard deviation. Statistical evaluation was performed with Student's t test. Experiments to assess CE were carried out by using microtiter plate assays with triplicate input samples. Serial 10-fold dilutions of various virus stocks were plated in a volume of 50 µl in a 96-well dish, starting with a concentration of 6 × 10^3 viral particles/ml. All dilutions were prepared in cell culture medium. Cells were diluted to 6.7 × 10^5/ml, and 105 µg of this suspension was added per well (MOIs ranged from 3 × 10^2 to 3 × 10^5). Attached cells were fixed with methanol at 72 h p.i. and stained with crystal violet.

**RESULTS**

**Isolation of CD46 cDNAs encoding the Ad3 receptor.** To identify a cell surface receptor(s) for species B1 Ads, we used the Sindbis virus-based cDNA expression system (19) to screen for the binding of Ad3 particles labeled with the fluorophore Alexa-488 to BHK cells. The cDNA library was made from human K562 chronic myelogenous leukemia cells, which efficiently bound Ad3-Alexa-488 (Fig. 1A, left panel). In contrast, rodent BHK cells did not bind significant amounts of Ad3, in

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agreement with the earlier notion that the receptor for species B Ads is not conserved between rodents and primates (13, 14, 36) (Fig. 1A, right panel). About 0.0024% of Sindbis virus-infected cells expressing cDNAs also bound fluorescent Ad3-Alexa-488 (Fig. 1B), whereas cells infected with Sindbis virus lacking cDNA inserts did not (Fig. 1C). Rescreening resulted in more than 30 positive clones binding Ad3; one of these is shown in Fig. 1D. Of 30 positive clones, 28 contained cDNA fragments of about 3 kb encoding CD46; the other 2 clones represented cloning artifacts. It is known that multiple splice variants of the Ser/Thr-rich domain-encoding exons and cytoplasmic tail exons give rise to four major splice variants, C1, C2, BC1, and BC2, and to additional minor variants (21). Among the 10 inserts that were completely sequenced, the major splice variants C2 and BC1 were found five times and two times, respectively, and the variants BC2 and C1 were found once each. The rare form B1 was found once, whereas the rare forms ABC1 and ABC2, typically expressed in cancer cells, were not recovered.

**Ad3 binds with a high affinity to BHK-CD46 cells through direct fiber head-CD46 interactions.** BHK cells stably expressing the BC1 form (cDNA clone 54) were generated. CD46 expression levels of bulk cultures (BHK-CD46-cl54 bulk cells; data not shown) or clonal BHK-CD46-cl54-A1 cells expressed CD46 at intermediate levels, compared to human cell lines, such as K562, HeLa, and A549 (Fig. 2A). The binding of 

\[ ^{3} \text{H-} \text{thymidine-labeled Ad3 to BHK-CD46-cl54 bulk cells was dose dependent and saturable, compared to background binding in the presence of 2 nM Ad3 DF (10) and to parental BHK cells (Fig. 2B). One DF consists of 12 penton base pentamers, each of which contains one trimeric fiber molecule with a distal fiber head at the outside. Scatchard plots of Ad3 binding showed parallel slopes with extrapolated } K_d \text{ values of 0.3 nM for both BHK-CD46 transfectants and HeLa cells (Fig. 2C). The number of Ad3 binding sites on BHK-CD46-cl54 bulk cells was } 2.4 \times 10^7 \text{, and that on human cervical carcinoma HeLa cells was } 4.4 \times 10^7 \text{, consistent with earlier experiments with human nasopharyngeal carcinoma KB cells and human lung carcinoma A549 cells (6). Competition with the recombinant Ad3 fiber knob (7) eliminated more than 75% of Ad3 binding to HeLa cells (mean of two experiments; data not shown), in agreement with earlier experiments (43). Further, CD46ex-Fc was able to pull down both the recombinant Ad3 fiber head and DF but not DP (Fig. 2D). DF was not recognized by CARex-Fc, indicating specific binding of DF to CD46ex-Fc (data not shown). In addition, CD46ex-Fc also pulled down Ad3 but not Ad2 or the fiber head of Ad2, further confirming the specificity of the interaction of CD46 with Ad3 (data not shown). These results indicate that CD46 expression on nonpermissive rodent cells mediates high-affinity and saturable Ad3 binding through a direct interaction of the extracellular CD46 domain with the Ad3 fiber head.

**Colocalization of Ad3 with CD46 during viral entry.** We next analyzed Ad3 interactions with BHK-CD46 cells and human epithelial cells (HeLa cells) positive for CD46 and permissive for Ad3 by light microscopy and EM. TR-labeled Ad3 bound homogeneously to the cell surface at 4°C, and a large fraction colocalized with CD46, as determined by indirect immuno-fluorescence and single-section CLSM (Fig. 3A and 4A). Over-
lapping staining of CD46 and staining of TR-labeled Ad3 was particularly prominent in basal and peripheral regions of BHK-CD46 and HeLa cells, consistent with basolateral surface expression of CD46 in certain polarized monkey and human cell lines (23) and both apical and basolateral expression in differentiated human airway epithelial and tracheal tissues (41). Ad3-CD46 colocalization was confirmed by high-resolution indirect immunogold EM depicting gold particles attached to goat anti-mouse IgG near Ad3 and occasionally elsewhere on the cell surface (Fig. 3B and 4B). No gold particles were observed in the absence of anti-CD46 antibodies (data not shown). Upon warming, Ad3 was found in the cytosol and endosomal vesicles of BHK-CD46 cells, partly colocalized with CD46 (Fig. 3A, e to g, and Fig. 3B, b). Similar results were obtained with HeLa cells (Fig. 4A, d to f).

FIG. 2. Ad3 binds with a high affinity to CD46-expressing cells through the fiber head. (A) Cytofluorometric analysis of CD46 expression levels in human and hamster cell lines. White histograms show staining with isotype control antibodies, and shaded histograms show CD46-specific staining. The cells used were human K562, HeLa, and A549 cells, parental BHK cells, and stably transfected BHK cells expressing the BC1 isoform of CD46. (B) Binding isotherms of Ad3 incubated with BHK-CD46-cl54 bulk cells or parental BHK cells. Nonspecific binding was determined in the presence of Ad3 DF. Error bar depicts standard error of the mean. (C) Scatchard analysis of Ad3 binding to BHK-CD46-cl54 bulk cells and HeLa cells. The number of binding sites per cell and the $K_d$ values were calculated from the bound and the free virion concentrations determined by subtracting the bound virus from the input virus. (D) Direct interaction of the Ad3 fiber head with CD46ex-Fc. CD46ex-Fc was incubated without any addition (lane 1), with the purified fiber head (lane 2), with DF (lane 3), and with DP (lane 4). CD46ex-Fc was pulled down by protein G (Prot G)-Sepharose, and the SDS eluates were analyzed by SDS-PAGE and silver staining.
FIG. 3. Ad3 colocalizes with cell surface CD46 of BHK-CD46-cl54-bulk cells. (A) CLSM analyses of TR-labeled Ad3 (red) and CD46 stained with non-function-blocking antibody MCI20.6 (green). Single sections were taken at 0 min (a to d) and at 5 min (e to h) p.i., including 4',6'-diamidino-2-phenylindole (DAPI)-stained sections (d and h). Scale bars, 5 μm. (B) Transmission EM (TEM). (a) Immunogold staining of CD46 at 0 min p.i. Large arrows indicate Ad3 associated with protein A-gold directed to anti-CD46; the small arrow indicates a gold particle not associated with Ad3. (b) Ad3 internalization at 0 min p.i. Arrowheads indicate clusters of cytosolic Ad3; the small arrow indicates endosomal Ad3. Scale bars, 200 nm.

FIG. 4. Ad3 colocalizes with cell surface CD46 of HeLa cells. (A) CLSM analyses of TR-labeled Ad3 (red) and CD46 stained with non-function-blocking antibody MCI20.6 (green). Single sections are shown at 0 min (a to c) and at 45 min (d to f) p.i. Scale bar, 5 μm. (B) Transmission EM showing immunogold staining of CD46 at 0 min p.i. Arrows indicate Ad3 associated with protein A-gold directed to anti-CD46; the small arrow indicates a gold particle not associated with Ad3. Scale bar, 200 nm.
CD46 expression mediates binding, gene expression, and CPE. We further tested whether Ad3 binding to BHK-CD46 cells was specific for CD46 by using eight different anti-CD46 antibodies (Table 1) and CD46ex-Fc. Six monoclonal antibodies and one polyclonal antibody to CD46 strongly inhibited either Ad3-Alexa-488 or $^3$H-labeled Ad3 binding at concentrations that gave saturating antibody binding to BHK-CD46 cells (Fig. 5, left panel, and data not shown). Monoclonal antibodies M75, 13/42, and MEM-258, recognizing epitopes on short consensus region (SCR) 1 (SCR1), SCR2, and SCR4, blocked Ad3 binding by more than 90%. Antibodies GB-24, E4.3, and Tra-2.10, directed to SCR1 and SCR3/4, inhibited binding at intermediate levels of 85, 65, and 62%, respectively. In contrast, antibody MCI20.6, recognizing an SCR1 epitope, and anti-CAR antibody E1-1 had no significant effect on Ad3 binding. The soluble CD46ex-Fc protein inhibited binding in a dose-dependent manner (data not shown), with a maximal inhibition of 43%, whereas the CARex-Fc protein had no effect. The specificity of this assay was further confirmed by including a 30-fold excess of cold Ad3, reducing labeled Ad3 binding by $>96\%$. The anti-CD46 antibodies also inhibited Ad3 binding to K562 cells, albeit to a lesser extent than to BHK-CD46 cells (Fig. 5, right panel). Notably, antibodies M75, 13/42, and MEM-258, which were the most effective blockers of Ad3 binding to BHK-CD46 cells, also conferred maximal blocking in K562 cells amounting to about 40%. The use of a mixture of all seven blocking antibodies did not increase the blocking of Ad3 binding (data not shown). Likewise, CD46ex-Fc did not inhibit Ad3 binding to K562 cells and, surprisingly, somewhat enhanced Ad3 binding (data not shown). This result may be related to the capacity of CD46ex-Fc to form dimers, but additional experiments are required to clarify this observation.

We next measured Ad3-mediated gene delivery to BHK-CD46 transfectants by using Ad5/F3 (Fig. 6A). Ad5/F3-mediated eGFP expression in hematopoietic human K562 and THP-1 cells was 10- to 20-fold higher than eGFP expression from an Ad5 vector, confirming the functionality of Ad5/F3. This finding was in agreement with earlier reports (42). In BHK-CD46-cl54-A1 cells, the eGFP expression of Ad5/F3 increased in a dose-dependent manner to a maximum of 16-fold, compared to that in BHK cells and CAR-expressing BHK cells (BHK-CAR cells). In BHK-CD46-cl28 bulk cells (an additional BC1 cDNA clone) and clone 54 cells, expression increased to an intermediate level of sevenfold (data not shown), correlating with a twofold-lower level of CD46 expression in these cells than in BHK-CD46-cl54-A1 cells. The transgene expression of Ad5/F3 in human A549 cells was about 30-fold higher than that in clonal BHK-CD46 cells. As expected, BHK and BHK-CD46 transfectants remained refractory to Ad5-mediated eGFP expression, whereas eGFP expression from Ad5 in stable BHK-CAR cells was increased about 23-fold, compared to a 300-fold increase in A549 cells.

We next measured the CPE of Ad3 in BHK-CD46 cells and found that there is about a 1-log-unit increase in the sensitivity of BHK-CD46 cells over that of native BHK cells (Fig. 6B). Control infections with Ad5 showed no difference in CPE between BHK and BHK-CD46 cells. Human cells were up to 2 log units more sensitive to Ad5 and at least 1 log unit more sensitive to Ad3 than BHK-CAR or BHK-CD46 cells to Ad5 or Ad3, respectively (Fig. 6B), consistent with the eGFP expression data for Ad5/F3 (Fig. 6A). Together, these experiments corroborate the binding data and demonstrate that parental BHK cells are refractory to Ad3 infection, whereas BHK-CD46 transfectants develop Ad3-mediated CPE, implying that CD46 supports Ad3 infection.

FIG. 5. CD46-dependent binding of Ad3 revealed by anti-CD46 antibodies and CD46ex-Fc. BHK-CD46-cl54 bulk cells and human K562 cells were incubated with the indicated antibodies, CD46ex-Fc, or CARex-Fc (control), followed by the addition of either Ad3-Alexa-488 or $^3$H-labeled Ad3. Virus binding was measured by flow cytometry or liquid scintillation counting. The asterisks indicate the level of significance ($P$ values of $<0.05$ [single asterisks] and $<0.005$ [double asterisks] for comparisons with the negative controls E1-1 [anti-CAR] and CARex-Fc, respectively). rab, rabbit.
DISCUSSION

Our expression cloning approach identified a receptor of species B Ad3, the membrane cofactor CD46. The screening experiments indicated that all four major splice variants, BC1, BC2, C1, and C2, and the rare splice form B1 are capable of binding Ad3, similar to what has been described for the binding of measles virus (24). Ad3 binding to rodent cells expressing the most common isoform, BC1, was of high affinity and saturable; the yield was about 2,400 virus binding sites per rodent cell, compared to 4,400 per HeLa cell, consistent with a previous report on HeLa cells (6). We found that Ad3 binding to CD46-expressing rodent cells was inhibited by a panel of anti-CD46 monoclonal and polyclonal antibodies and by a recombinant extracellular domain of CD46, supporting the notion that CD46 represents a major Ad3 binding site in these cells. In human cells, CD46 is also an important Ad3 receptor, as indicated by colocalization data at the light microscopy and EM levels and by antibody inhibition experiments, although the latter were less efficient than those with rodent cells. Notably, Ad3 has the same subnanomolar affinity for BHK-CD46 cells as for HeLa cells and enters and gives rise to transgene expression and CPE in both cell types. Further, in vitro pull-down experiments indicated that Ad3 directly binds the extracellular domain of CD46 through contact with the fiber head. Nonetheless, a recent study had suggested that a particular Ad3 isolate did not bind to Chinese hamster ovary cells expressing the C2 isomor of CD46 (11). We therefore checked the DNA sequence of our Ad3 stock and found that the fiber sequence was identical to the published sequence (39), except for a point mutation (Y218S) in the fiber head. This mutation, however, is unlikely to affect virus binding to CD46, since the recombinant Y218F fiber directly bound to CD46-Fc (like our Y218S Ad3) and Y218 DF abolished the binding of our Ad3 to HeLa and BHK-CD46 cells as efficiently as nolabeled competitor Y218S Ad3. These data strongly argue that all of the splice variants of CD46 can serve as Ad3 binding sites, at least on rodent cells. However, Ad5/F3-mediated gene expression and wild-type Ad3-mediated CPE in rodent cells were lower than those in human epithelial cells (A549 and HeLa cells). Similarly, Ad5-mediated eGFP expression was lower in BHK-CAR cells than in fully permissive A549 cells. It is unknown whether rodent cells lack an unidentified factor for effective Ad-mediated transgene expression, for example, a secondary receptor stimulating endocytosis and nuclear transport (26, 45). It is interesting, however, that the level of Ad3 infection was reduced about threefold in human M21-L12 cells lacking ανβ3- and ανβ5-integrins compared to integrin-positive M21-L4 cells and that infection of M21-L4 cells was inhibited by integrin-specific antibodies and RGD peptides blocking penton base interactions with integrins (25). Whether the association of CD46 with α1-integrins, as reported for HeLa cells (22), facilitates Ad3 infection needs to be investigated. It is
possible that human cells express additional Ad3 receptors unrelated to CD46, as suggested by our finding of relatively weak Ad3 binding inhibition with anti-CD46 antibodies in K562 cells. The nature of such hypothetical binding sites is unclear. It is also conceivable that glycosylation may modulate Ad3 binding. Interestingly, CD46 is heavily N and O glycosylated, depending on the tissue type, and the removal of N-linked but not O-linked glycans or siadic acid abolished the binding of measles virus strain Edmonston, which also uses CD46 as a receptor (17).

In terms of CD46 binding and infection, it appears that our Ad3 behaves more like species B Ad11 and Ad35 than Ad7. The binding of both Ad3 and Ad11 to human cells was partially blocked by anti-CD46 antibodies, with 20 to 30% inhibition for Ad11 (35) and about 40% inhibition for Ad3 (this study), or by small interfering CD46 RNAs, which reduced Ad35 binding by about 40% (11). The soluble CD46 receptor also inhibited the transgene expression of Ad5/F35 (11). The binding of Ad7 to human cells was apparently unaffected by anti-CD46 antibodies (35). In contrast, Ad3, Ad7, and Ad11 binding to CD46-expressing rodent cells was inhibited by anti-CD46 antibodies, and infection of CD46 BC1 isofrom-expressing Chinese hamster ovary cells was possible with Ad11 but not with Ad7; these results suggested that Ad7 may bind CD46 but not use it for infection. It is unknown, however, whether Ad7 infects human cells with the same efficiency as CD46-expressing rodent cells. Whether Ad3, Ad7, Ad11, Ad35, and other Ad serotypes all use the same binding site on CD46 remains to be investigated.

The species B Ads are considerable human pathogens. Ad3 and Ad7 are responsible for a significant proportion of Ad infections worldwide and cause infections of the upper respiratory tract, including acute febrile and severe respiratory illness, whereas the less abundant Ad11 and Ad35 cause infections of the urinary tract (34). The identification of Ad3 receptor CD46 has medical implications for the development of new antiviral agents. Besides, species B Ads are interesting gene transfer vehicles, as they use a receptor distinct from the common Ad receptor CAR. Since CAR is expressed on many but not all cell types and is down regulated on many cancer cells, conventional therapeutic Ad vectors have limitations. These limitations can be overcome in part by swapping the fiber protein of species C serotypes with that of species B serotypes. Accordingly, fiber-swapped Ad vectors have an extended tropism compared to species C vectors and are able to efficiently infect hematopoietic and dendritic cells (13, 14, 18, 31, 37). In addition, several species B Ads have a low serum prevalence, which makes them attractive for in vivo applications (46).

Ad3 adds to a growing list of pathogens binding to CD46. For example, CD46 is known to be a receptor for measles virus strain Edmonston, human herpesvirus 6, Neisseria gonorrhoeae, N. meningitidis, Helicobacter pylori, and Streptococcus pyogenes (reviewed in reference 20). CD46 belongs to a family of complement activation regulators (21). The biological role of CD46 is to prevent complement activation on autologous tissue by binding C3b and C4b and by acting simultaneously as a cofactor for proteolytic factor I. CD46 consists of four amino-terminal SCR domains of 60 amino acids each, one to three Ser/Thr-rich domains, a short region of unknown function, one transmembrane domain, and a cytoplasmic tail. The antibody blocking data suggest that Ad3 may bind to all four SCR domains, a suggestion which would be in agreement with the finding that three different receptors of the complement activation regulator family (CD21, CD55, and CD46) can recognize viral particles through two or more SCR domains (for a review, see reference 3). The first two SCR domains of CD46 are trimers (3), indicating that CD46 would be well suited for tight interactions with a trimeric ligand, such as the Ad3 fiber (5). Together with evidence from recent reports suggesting that CD46 is a receptor for species B Ad11 and Ad35 (11, 35), our data corroborate the notion that CD46 is a common receptor for species B Ads. Given that CD46 is expressed on all human cells except for erythrocytes, we expect that viruses using CD46 as a receptor might have evolved broad infectivity tropism, allowing them to utilize cells and infection pathways that are not accessible to other Ad serotypes.

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