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FULL PAPER

Gallus gallus coxsackievirus and adenovirus receptor facilitates the binding of fowl adenovirus serotype 1 in chickens

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Abstract

Coxsackievirus and adenovirus receptor (CXADR) is an integral membrane protein that serves as a receptor for coxsackie B viruses and adenovirus types 2 and 5. Previous studies demonstrated that Fowl adenovirus (FAV) can also utilize *Homo sapiens* CXADR to infect cells. FAV is a double-stranded DNA virus of the family *Adenoviridae*. FAV causes inclusion body hepatitis and hydropericardium syndrome in chickens. In addition, FAV serotypes 1 and 8 have recently been shown to cause gizzard erosion in chickens. These chicken diseases and growth insufficiency caused by FAV infection result in great economic loss. Thus, identifying and characterizing the viral receptor would further enhance our understanding of the mechanisms underlying virus infection and histocompatibility. Here, in order to determine the FAV receptor in chickens, we investigated the effect of the recently identified *Gallus gallus* CXADR (ggCXADR) on FAV infection. Overexpression of ggCXADR in CHO cells resulted in increased FAV binding and expression of early FAV genes. However, the propagation of infectious viruses in CHO cells expressing ggCXADR was not detected. These findings provide the basis for further studies aimed at elucidating the infection mechanism of FAV. Further research is required to characterize the additional host factors involved in FAV infection and life cycle.

Key Words: CXADR, Fowl adenovirus, Receptor

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Introduction

Adenoviruses, which belong to the family *Adenoviridae*, contain a double-stranded DNA viral genome within a non-enveloped icosahedral capsid[14]. *Adenoviridae* contains four genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus*. Fowl adenovirus (FAV), which belongs to the genus *Aviadenovirus*, can infect chickens and is classified into at least twelve serotypes[11]. FAV has a broad range of avian hosts and infects culture cells of avian origin[25,32]. In particular, FAV is often isolated from the intestinal tract and the respiratory organs of chickens[7]. FAV causes inclusion body hepatitis and hydropericardium syndrome in chickens[1,30] and FAV serotypes 1 and 8 can cause gizzard erosion[18,24]. Moreover, FAV infection contributes to growth insufficiency and low egg-laying rate, thus FAV infection is responsible for significant economic loss for the chicken industry[14]. Human adenoviruses, especially types 2 and 5 (Ad2, Ad5) are used as viral vectors[22,28]. The Ad5 vector exhibits high transduction efficiency and can be used to effectively transduce many species of non-dividing cells. In addition, Ad5 integration events are lower than in other viral vectors derived from retroviruses or lentiviruses. However, continual administration of adenovirus vectors of the same serotype can lead the induction of a host immune response. Therefore, the FAV serotype 1 CELO vector that does not share cross-immunity with Ad5 was developed for use in individuals who possess neutralizing antibodies against human adenoviruses. The CELO vector carrying herpes simplex virus thymidine kinase demonstrates anticancer activity in human tumor derived culture cells[23]. In addition, the FAV vector can be propagated in eggs, thus facilitating efficient large-scale production.

Ad2 and Ad5 invade susceptible cells through the interaction between the coxsackievirus and adenovirus receptor (CXADR) and the fiber protein on the viral penton base[3,27]. The binding of Ad5 and CXADR is mediated via the virus fiber knob. The penton base Arg-Gly-Asp (RGD) motif associates with the αvβ3 and αvβ5 integrins, which act as co-receptors of Ad5, and Ad5 is then incorporated into the cells by endocytosis[29]. Following entry into the cells, the conformation of the virus penton base is altered by the acidic environment in the endosome and the virus is released from the endosome via a pH-dependent mechanism[4]. FAV serotype 1 CELO also utilizes CXADR for entry into susceptible cells; FAV CELO uses two fiber proteins, a long fiber and a short fiber on the penton base, for entry into the cells[26]. It is known that FAV CELO binds to *Homo sapiens* CXADR (hsCXADR) via the long fiber[26]. A previous study showed that recombinant FAV CELO deficient in the long fiber protein could infect chicken cells but not human cells, although wild type FAV CELO could infect both types of cells[26]. It has been hypothesized that the short fiber of FAV CELO binds to a receptor specifically expressed in chickens. Thus, FAV infection is dependent on the expression of CXADR, and/or a yet unidentified endogenous FAV-specific chicken receptor. Recently, a homolog of hsCXADR was identified in chickens [GenBank:XM_416681]. Transgenic analysis revealed that this *Gallus gallus* CXADR (ggCXADR) rescued embryonic-lethal CXADR-deficient mice via expression in cardiomyocytes[9]. These findings suggest that ggCXADR is expressed as a protein and may have a function in chickens. However, although other CXADR viral receptors have been characterized, the relationship between ggCXADR and FAV and the FAV receptor in chickens have not been studied.

Here, in order to elucidate the function of ggCXADR in FAV infection, we determined the tissue distribution of ggCXADR mRNA in chickens, and assessed the FAV titer released from Chinese hamster ovary (CHO) cells transfected with a ggCXADR expression plasmid. In addition, the level of FAV surface binding to CHO cells expressing ggCXADR was also examined.

Materials and Methods

Cells: CK cells (primary chicken kidney cells) were cultured in minimum essential media (MEM; Nissui-pharm, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS). The CHO cell line was cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F-12) supplemented with 10% FCS. CK cells and CHO cells were used as FAV permissive cells and non-permissive cells, respectively. The cell line stably expressing ggCXADR was established by transfecting CHO cells with pTAR/ggCXADR, (see below) (CHO/ggCXADR), or empty pTargeT vector (CHO/empty), using a limiting dilution method with G418 (Wako Pure Chemical Industries, Osaka, Japan) selection, and maintained by G418 in DMEM/F-12 with 10% FCS. Transfection was performed using Lipofectamine LTX with Plus Reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions.

PCR and Plasmid construction: Total RNA were isolated from chicken tissues using the TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany). Total RNA was reverse transcribed to generate cDNA using the MMLV RT (Life Technologies, Gaithersburg, MD) enzyme with oligo(dT). PCR reactions were performed with ExTaq polymerase (TAKARA BIO, Shiga, Japan), using ggCXADR specific primers (F: 5'-CCACCA TGGAACCGCCGCCGTTGG-3', R: 5'-TTATACC ACTGTTATGTCAC-3'), DBP specific primers (F: 5'-ACCTCGTACCGTGGAGTT-3', R: 5'-GGT AAAGCGCCTTCGTCCAGT-3'), Hexon specific primers (F: 5'-ACTACACTCAGACCCTGAGTTA-3', R: 5'-CTCGGAGTTGAGCGTTC-3'), and β-actin specific primers (F: 5'-CTAAGGCCAACCGTGAA AAG-3', R: 5'-ACCAGAGGCATACAGGGACA-3'). The ggCXADR specific primers were designed to span introns, thus the PCR products amplified from the genomic DNA could be distinguished from the mRNA. The ggCXADR ORF sequence was amplified from chicken gizzard cDNA and purified using SUPREC-02 cartridges (TAKARA BIO, Shiga, Japan). Following purification, the PCR product was cloned into the pTargeT Vector (Promega, Madison, WI, USA) under control of the CMV promoter, resulting in plasmid pTAR/ ggCXADR. The nucleotide sequence of pTAR/ ggCXADR was confirmed by DNA sequencing.

Virus infection: FAV serotype 1 strain JM1/1 was isolated from a chicken exhibiting gizzard erosion[31]. Parental CHO cells or CHO/ggCXADR were seeded onto six well plates and infected with FAV (MOI = 1). Culture supernatants were collected at 24 h, 48 h, and 72 h post infection. The virus titer was calculated using the $TCID_{50}$ assay using CK cells. To confirm FAV binding to ggCXADR expressing CHO cells, CHO/ggCXADR or CHO/empty were seeded onto six well plates 48 h prior to infection. For transient expression experiments, pTAR/ggCXADR or empty plasmid were transfected into the CHO cells using Lipofectamine LTX 24 h prior to infection. FAV was incubated with the cells for 1 h at 4°C (MOI = 5, copy number was 10^5 copies), then cells were washed with serum-free MEM four times. Subsequent to washing, total DNA were extracted from the cells using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA).

Real-time PCR: The FAV DNA-binding protein (DBP) gene was cloned into the pCR2.1 vector to create a pCR/DBP plasmid standard. To measure the FAV binding to the cells, real-time PCR reactions were carried out using FAV specific primers (F: 5'-ACCTCGTACCGTGGAGTT-3', R: 5'-GGTAAAGCGCCTTCGTCCAGT-3') and SYBR Premix ExTaq (TAKARA BIO, Shiga, Japan) according to the manufacturer's protocols.

Western blotting: CHO cells were seeded into 24 well plates 24 h prior to transfection. Then, the cells were transfected with the pTAR/ggCXADR plasmid using Lipofectamine LTX with Plus reagent. One-day post transfection, the cells were collected in sample buffer [62.5 mM Tris-HCl

(pH 6.8), 5% 2-mercaptoethanol, 2% sodium lauryl sulfate, 5% sucrose, 0.005% bromophenol blue] and boiled at 98°C for 5 min. The collected samples were subjected to SDS-PAGE using 10% acrylamide-gels and western blotting analysis performed. The proteins were transferred onto a polyvinylidene fluoride membrane that was then blocked in 5% skim milk powder in phosphate buffered saline (PBS) (pH 7.4)-0.1% Tween-20. Following blocking, the membrane was incubated with a mouse anti-CXADR antibody (LS-C313489; LifeSpan BioSciences, Seattle, Washington, U.S.A), diluted 1 : 1,000 in PBS-0.1% Tween-20 with 5% skim milk powder for 1 hour at room temperature, and then washed 3 × 10 min. The membranes were then reacted with a horseradish peroxidase-coupled goat anti-mouse antibody (Bio Rad, Hercules, California, U.S.A) and visualized with ECL prime Western Blot Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, England) according to the manufacturer's instructions.

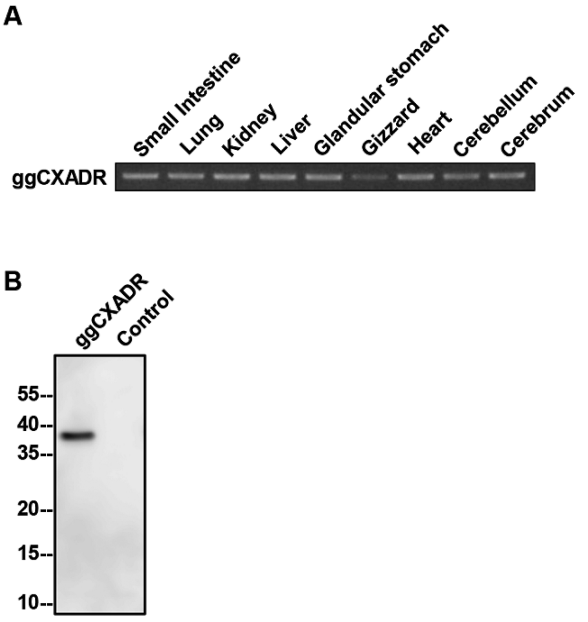


Fig. 1. ggCXADR expression in chicken tissues and in transiently transfected CHO cells. (A) Total RNA was harvested from each tissue and reverse transcribed with oligo (dT). cDNAs were amplified by PCR using ggCXADR specific primers. (B) CHO cells were transfected with a ggCXADR expression plasmid. Twenty-four hours post transfection, the cells were collected and the expression of ggCXADR was detected by western blotting using an anti-CXADR antibody. Non-transfected CHO cells were used as a negative control.

Results

ggCXADR is expressed ubiquitously in chicken tissues

To determine the expression of ggCXADR mRNA in chickens, we performed reverse transcription PCR assays with ggCXADR specific primers using total RNA harvested from the cerebellum, cerebrum, heart, lung, gizzard, glandular stomach, small intestine, kidney, and liver tissues of chicken. Our results demonstrate that ggCXADR mRNA was expressed in all tissue samples examined (Fig. 1A).

FAV does not propagate efficiently in ggCXADR-expressing CHO cells

To examine whether non-permissive CHO cells become sensitive to FAV infection upon expression of ggCXADR, we constructed a ggCXADR expression plasmid by cloning the ggCXADR ORF sequence (1077 bp, predicted molecular weight: 38 kDa) amplified from chicken gizzard cDNA into the pTargeT vector. The expression of the ggCXADR protein was confirmed by western blot analysis (Fig. 1B). To establish CHO cells stably expressing ggCXADR, CHO cells were transfected with the ggCXADR expression plasmid and selected using G418. CHO/ggCXADR and parental CHO cells were infected with FAV (multiplicity of infection, MOI = 1) and the supernatants were collected 24 h, 48 h, and 72 h post infection. The viral titer in the supernatants was measured using the 50% tissue culture infectious dose ($TCID_{50}$) method with primary chicken kidney (CK) cells. The results presented in Table 1 show that no infectious viruses were detected in CHO cell supernatants at any of the time points. We detected a low viral titer in the supernatant of CHO/ggCXADR at 48 h post infection (Table 1).

Table 1. The FAV titer in the supernatant of CHO cells expressing ggCXADR or not

	24 h	48 h	72 h
CHO/CXADR	—	$10^{0.25}$	—
CHO/—	—	—	—

($TCID_{50}$/100ul)

Expression of ggCXADR increases FAV binding in CHO cells

Since FAV did not propagate in CHO/ggCXADR cells, we next examined the binding of FAV to cells expressing ggCXADR. CHO/ggCXADR, CHO/empty and CHO cells which transiently transfected with pTAR/ggCXADR or empty vector were inoculated with FAV (MOI = 5) and incubated for 1 h at 4°C. Following incubation, the cells were washed and total DNA was extracted from the cells. FAV copy numbers in the total DNA were measured by real-time PCR (Fig. 2). The copy number of cell-associated viral DNA was significantly higher in cells expressing ggCXADR than in empty plasmid transfected cells. Similar results were obtained for cells stably expressing ggCXADR; CHO/ggCXADR cells exhibited a significantly higher viral DNA copy number than CHO/empty (Fig. 2).

FAV induces the transcription of DBP and Hexon mRNA in CHO cells expressing ggCXADR

Based on these results, we hypothesized that FAV could attach to CHO cells expressing ggCXADR but could not propagate efficiently. Thus, to elucidate the viral infection process in CHO cells expressing ggCXADR we examined viral mRNA expression profiles. Total cell RNA was isolated from FAV-infected cells with or without pTAR/ggCXADR. Subsequent to cDNA synthesis using total RNA, the viral genes were PCR amplified using DBP and Hexon specific primers (Fig. 3). DBP is one of the early genes associated with viral DNA replication. DBP mRNA is expressed during early stages of FAV infection and Hexon mRNA is expressed during late stages of FAV infection. DBP and Hexon mRNA expression was not detected in CHO cells

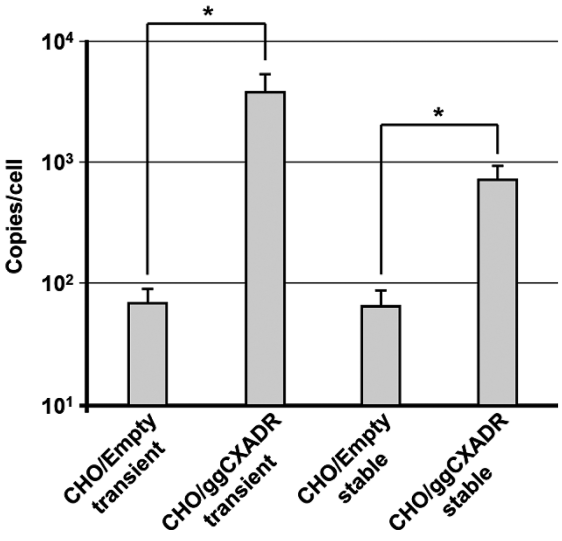


Fig. 2. Copy number of FAV bound to CHO cells expressing ggCXADR. CHO cells transiently expressing ggCXADR, or empty and CHO cells stably expressing ggCXADR, or empty were inoculated with FAV (MOI = 5). One hour post inoculation, the cells were washed and total DNA was extracted from the cells. Virus copy number was quantified by real-time PCR. The standard curve was created using a plasmid containing FAV DBP. Statistical significance was analyzed by the two-tailed t test. *$p < 0.05$

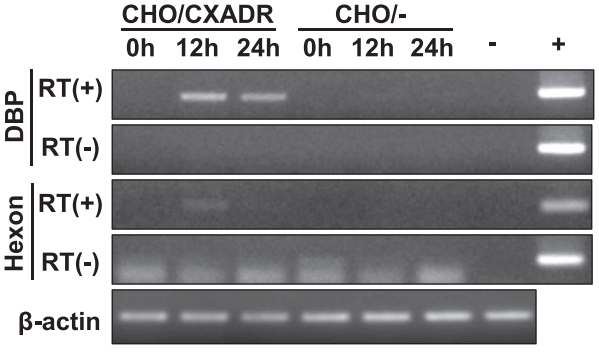


Fig. 3. Expression of FAV mRNA in ggCXADR-expressing CHO or parental CHO cells. Cells were infected with FAV at an MOI of 1. Total RNA was extracted from the cells at three time points. Following DNase treatment, the total RNA was reverse transcribed with oligo(dT). cDNAs were amplified with the indicated gene specific primers. RT (—) sample reactions did not contain the RT enzyme. FAV genomic DNA was used as a positive control (+) and distilled water was used as a negative control (—).

at any time point; however, in CHO cells expressing ggCXADR, DBP mRNA expression was detected at 12 h and 24 h post infection. In addition, low expression of Hexon was detected

at 12 h in CHO cells expressing ggCXADR.

Discussion

In this study, we demonstrated that ggCXADR mRNA was expressed in the cerebellum, cerebrum, heart, lung, gizzard, glandular stomach, small intestine, kidney, and liver of chickens. Mouse and human CXADR have been shown to be involved in the development of nerves, heart, and lymphatic vessels[2,8,12,15] and a previous study has indicated that ggCXADR also functions in the development of the heart in CXADR KO mice[9]. Embryonic-lethal CXADR KO mice can be rescued by expression of ggCXADR in cardiomyocytes; the cardiomyocytes of rescued KO mice showed normal morphology and function. These results suggest that ggCXADR plays a similar role in the formation of the chicken heart. Whereas mouse CXADR mRNA exhibited high expression in specific tissues[27], ggCXADR mRNA was expressed ubiquitously in chickens, indicating that ggCXADR might have different functions in other tissues.

The results of our overexpression experiment demonstrated that FAV did not propagate efficiently in either CHO cells or CHO cells expressing ggCXADR; however, a higher level of FAV bound to CHO cells expressing ggCXADR than to control CHO cells. Interestingly, a low level of infectious viruses was detected 48 h post infection in CHO cells expressing ggCXADR. Previous results have shown that when HeLa cells were infected with FAV at a multiplicity of 10 PFU, the HeLa cells released 10^5 virus particles per cell at the end of the cycle[10]. Therefore, it is possible that the infectious viruses detected are residual original viral particles. There was no apparent expression of viral mRNA in parental CHO cells following FAV infection. In contrast, ggCXADR-expressing CHO cells exhibited expression of DBP mRNA and low levels of Hexon mRNA. DBP is expressed during the early stages of FAV infection while Hexon is expressed at later stages. Thus, the low expression of Hexon mRNA may indicate that the FAV replication cycle had not been completed. These findings suggest that ggCXADR serves as a receptor of FAV; however, the complete FAV replication cycle requires other cellular factors.

Previous studies have reported that FAV CELO (serotype 1) uses hsCXADR as a receptor. FAV binds to the cell surface via two fibers of different length (short and long) found on the penton base. Wild type FAV CELO can infect human cell lines and CHO cells expressing hsCXADR. Recombinant FAV CELO, which lacks the long fiber, could infect chicken embryo fibroblast (CEF) cells, but not human cell lines and CHO cells expressing hsCXADR[26], suggesting that the CELO long fiber is responsible for binding to hsCXADR. The amino acid homology of ggCXADR and hsCXADR is 57% (data not shown). Human Ad5, which uses hsCXADR as a receptor, can infect CHO cells expressing zebrafish CXADR, which is only 52% identical to hsCXADR[20]. These findings indicate that Ad5 can bind to a wide range of CXADR receptors. FAV might demonstrate similar CXADR binding abilities; the FAV long fiber could bind to both human and chicken CXADR. However, previous findings demonstrate that recombinant FAV lacking a long fiber could infect CEF cells[26]. This result suggests that the short fiber binds to a receptor specifically expressed in chickens. Thus, FAV may possibly bind to ggCXADR via the short fiber.

The interaction of viral and host proteins is essential for virus replication. Although many studies have examined the interaction of human adenovirus proteins and host proteins[5,6,16,19], little is known regarding the interaction of FAV and host proteins[13]. In this study, we confirmed the expression of DBP mRNA in FAV infected CHO cells expressing ggCXADR; DBP is one of the proteins encoded by the E2 genes. During Ad5 replication, the expression of E2 peaks after other early genes[17]; E2 gene expression is promoted by the interaction between the E1 proteins and host

proteins. Thus, it is possible that an FAV E1A-like protein interacted with a host element of the CHO cells and activated E2 gene expression including DBP. This suggests that the viral replication cycle was stopped at some stage after E2 expression resulting in low levels of Hexon mRNA expression. It is also possible that the detected DBP expression was not an activated expression but rather a leaky basal level expression. Defective interactions between host and viral proteins may have prevented the activation of early gene expression, resulting in the low levels of Hexon mRNA detected in this study. Further studies will be required to determine the rate-limiting stage of FAV replication, identify early genes, and investigate host factors that interact with viral proteins.

Many adenoviruses use CXADR as a receptor[21]. A number of studies have reported that FAV could infect CHO cells expressing hsCXADR[26]. However, to date, no studies have demonstrated that chicken CXADR acts as an FAV receptor. FAV contributes to the development of inclusion body hepatitis, hydropericardium syndrome and gizzard erosion in chickens. Hence, FAV is one of the most important pathogens in the chicken farm[1,18,24,30]. Here, we showed that FAV bound to CHO cells expressing ggCXADR and expressed early genes; however, infectious viruses were not released from ggCXADR-expressing CHO cells. These findings suggest that ggCXADR acts as a viral receptor, however, additional host factors are required for the release of infectious viruses in CHO cells.

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