Acta virologica **55**: 93 – 99, 2011 doi:10.4149/av_2011_02_93

Large-scale production of functional recombinant CAR in a baculovirus-insect cell system

M. ZHOU, B. SHI, P WANG, J. GU, Z. LI*

State Key Laboratory of Oncogenes and Related Genes, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai Cancer Institute, Shanghai 200032, P.R. China

Received May 3, 2010; accepted March 15, 2011

Summary. – Coxsackievirus group B and adenovirus receptor (CAR) is a major receptor for the adenovirus groups that has drawn overall attention over the past decade. Although this protein could potentially be used as an agent for the blocking of adenovirus infection, large-scale production of highly purified human CAR in eukaryotic expression system has not been reported. In the present study, we showed the construction of recombinant baculovirus highly-expressing the extracellular domain of human coxsackievirus-adenovirus receptor (exCAR) in High Five insect cells. The recombinant exCAR was recovered from the cell culture medium as a secreted soluble protein and purified by Ni-NTA affinity chromatography. The final yield of recombinant exCAR was about 8–10 mg/l of supernatant with the purity of 96.3%. Binding activity assay showed that the recombinant exCAR exhibited an intact ability of binding to the knob domain of the adenovirus type 5 fiber protein (Ad fiber knob) displayed by T7 phage. These results showed that the recombinant human exCAR produced in insect cells and purified by Ni-NTA chromatography retained its ability to bind to the Ad fiber knob and could potentially be used in therapy of adenovirus infection.

Keywords: CAR; recombinant baculovirus; High Five cells; Ni-NTA chromatography

Introduction

Adenoviruses are double-stranded DNA viruses that are frequently associated with the acute respiratory diseases, gastroenteritis, ocular infections, and myocarditis in children and adults (Nemerow, 2000; Bowles *et al*., 2003). Human adenoviruses occur in six species namely human adenovirus A, B, C, D, E, and F. Individual viruses within these species are distinguished by serotype (altogether 51 serotypes are recognized to date) (Leen and Rooney, 2005). Depending on the infecting serotype, the adenoviruses may cause fatal infection by inducing a severe inflammation (Pham *et al*., 2003; Savon *et al*., 2008; Henquell *et al*., 2009). Unfortunately, the adenoviruses infection can be managed only by treating symptoms and complications of the infection due to the lack of virus-specific therapy.

Previous studies showed that the adenovirus infection was dependent on the initial virus binding to the cell surface receptors and subsequent interaction with $\alpha_v\beta$ integrins that facilitates internalization via receptor-mediated endocytosis (Nemerow, 2000). There are several cellular receptors that were suggested to be involved in the attachment procedure: (1) CAR for adenovirus species A, C, D, E, and F, (2) CD46 for species B, (3) major histocompatibility complex class I a2 subunit for species C, and (4) sialic acid for the human adenovirus 37 (Boulanger, 1999; Arnberg *et al*., 2000; Carson, 2001; Gaggar *et al*., 2003).

CAR is the major receptor for adenovirus serotypes. It is a transmembrane glycoprotein of 46 kDa that belongs to the immunoglobulin superfamily of cell surface molecules (Tomko *et al*., 1997; Schneider-Schaulies, 2000). Human CAR gene is located on the chromosome 21q11.2 and composed of seven exons (Bowles *et al*., 1999). The ORF encodes

*Corresponding author. E-mail: Zonghaili@shsmu.edu.cn; fax: +86-21-64432027.

Abbreviations: Ad fiber knob = knob domain of adenovirus type 5 fiber protein; CAR = coxsackievirus group B and adenovirus receptor; EGF = epidermal growth factor; exCAR = extracellular domain of human coxsackievirus-adenovirus receptor; HRP = horse radish peroxidase; MAb = monoclonal antibody; LR = lambda recombination reaction; p.i. = post infection

365 aa comprising 19 aa signal peptide, 218 aa extracellular domain, 21 aa transmembrane domain, and 107 aa intracellular domain.

With the development of adenovirus infection therapy and adenovirus-based cancer gene therapy, much attention has been paid to the role and mechanism of receptors in the adenovirus infection especially the CAR (Zhang *et al.*, 2008). Recently, the receptor trap therapy has been demonstrated that may be used as a potential therapeutic agent using soluble viral receptors, which block the action of the intracellular entry of the virus (Lim *et al.*, 2006). Therefore, the production of soluble receptors is a prerequisite.

Human CAR has been expressed in *Escherichia coli* system as an insoluble protein (Wu *et al.*, 2004; Zhang *et al.*, 2009). It was also expressed in the mammalian cell expression system, but the production seemed rather low (Lim *et al.*, 2006). To target adenovirus infection to the epidermal growth factor (EGF) receptor overexpressed cancer cells, the ectodomain of human CAR fused to EGF was also expressed successfully in the insect cells (Dmitriev *et al.*, 2000). However, a large-scale production of highly purified human exCAR has not been reported yet.

To investigate the feasibility for large-scale production of highly purified exCAR, we cloned the exCAR sequence into baculovirus DNA and produce human exCAR in a baculovirus-insect cell culture system. We optimized the expression of the exCAR protein in High Five cells and purified exCAR by Ni-NTA affinity chromatography. To estimate the binding capacity of the recombinant exCAR, ELISA of the binding of exCAR to T7-Ad fiber knob phage was performed. We found baculovirus-insect cell culture system is efficient for large-scale production of highly purified exCAR. We also showed that the recombinant exCAR retained its ability to bind to the Ad fiber knob displayed by T7 phage.

Materials and Methods

Cells. Sf9 cells were grown at 27°C in Grace´s medium (Invitrogen) supplemented with 10% FCS. High Five cells were cultured at 27°C in Express-Five SFM medium (Invitrogen) supplemented with 2% FCS and 18 mmol/l glutamine.

Recombinant baculovirus. All procedures were performed according to the manufacturer's protocol (BaculoDirect™ Baculovirus Expression System, Invitrogen) unless otherwise stated. Cloning of the CAR sequence was accomplished by PCR amplification using the pcDNA3.1-CAR plasmid (a generous gift from prof. Qian Huang) as a template. Specific primers for cloning PCR products into a Gateway entry vector pENTR/D-TOPO were designed according to the directional cloning protocols (pENTR™ Directional TOPO® Cloning Kits, Invitrogen). This expression vector allowed the expression of gene products fused with his tag. Primers with sequences 5'-ACCATGGCGCTCCTGCTGTGCTT-3' (forward primer) and 5'-TCCAGCTTTATTTGAAGGAGGGACA-3' (reverse primer) were used to amplify region corresponding to the ectodomain of CAR protein (nts 1–711 corresponding to aa 1–237 of the CAR protein). After the PCR products were confirmed by sequencing, the TOPO cloning reaction was performed to generate an entry clone (pENTR/D-TOPO-exCAR).

The lambda recombination reaction (LR) was then performed according to the manufacturer´s protocol to transfer the CAR gene from the entry clone to the BaculoDirect™ Linear DNA. For the production of recombinant baculoviruses, Sf9 cells (8 x 10^5) were cultured in six-well plate and transfected with baculovirus DNA (LR reaction) using Cellfectin® Reagent (Invitrogen). After transfection procedure, the cells were incubated in complete culture medium with 100 μmol/l ganciclovir (InvivoGen) at 27°C until cell lysis. Culture supernatants were collected as a viral stock. Viral DNA was extracted from the culture medium and analyzed by PCR with the polyhedrin forward primer 5′-AAATGATAACCATCTCGC-3′ and V5 reverse Primer 5′-ACCGAGGAGAGGGTTAGGGAT-3′. Positive clones were analyzed for the protein expression in Sf9 cells. Recombinant baculoviruses were amplified for 4 rounds in Sf9 cells. Viral titers were determined by plaque assay according to the manufacturer´s protocol.

Production and purification of exCAR. In preliminary assays, Sf9 cells and the culture medium were collected on the 8th day after transfection. After centrifugation at 4,000 x g for 10 mins, the culture supernatants were collected and cell pellets were resuspended in the protein extraction reagent with protease inhibitor. The culture supernatants and whole cell lysates were analyzed by 12% SDS-PAGE and Western blot analysis.

To optimize the expression of the exCAR protein, High Five cells were infected with the recombinant baculovirus stock at different MOIs (0.5~10 PFU/cell). Culture supernatants were collected at 0, 24, 48, 96, and 120 hrs post infection (p.i.) and analyzed by 12% SDS-PAGE and Western blot analysis.

For purification of exCAR, the supernatants of High Five cells culture collected at 96 hrs p.i. were concentrated 5-to 10-fold by ultrafiltration and dialyzed against PBS. Recombinant exCAR protein was purified by the immobilized metal ion affinity chromatography on Ni-NTA-Sepharose (Novagen). Protein concentration was determined by Bradford protein assay with bovine gamma globulin as a standard (Zor and Selinger, 1996). Purification fractions were analyzed by 12% SDS-PAGE and stained with CBB. The purity of exCAR was determined by the gel image analysis using the software of Bandscan, version 5.0.

Western blot analysis of exCAR. Protein samples were separated by 12% SDS-PAGE and electroblotted to a nitrocellulose membrane. The blots were incubated with blocking buffer (0.1% Tween 20 and 5% non-fat dry milk in PBS) for 2 hrs and then with the anti-CAR monoclonal antibody (MAb, Upstate) overnight. After washing, the blots were incubated with anti-mouse horse radish peroxidase (HRP)-conjugated antibody (Upstate) for 1 hr and treated with SuperSignal West Pico chemiluminescent substrate (Pierce) for 5 mins, exposed to X-ray film, and developed 5 mins later.

T7-Ad fiber knob phage. T7 phage was used to display Ad fiber knob protein. The knob domain was amplified using plasmid psfiber (a generous gift from prof. Qian Huang) as a template (forward primer: 5′-CTGGTAAGGAGGTACCGATGGGTGCCATTACA GTAGGAAACAAAA-3′, reverse primer: 5′-CCCAAGCTTTTA TTCTTGGGCAATGTATGAAAAAGTG-3′). A Gly-Ser link [(G3S)3 (G4S)5] sequence and a ribosome-binding site (RBS, 5′-GGTAAGGAGGTACCG-3′) downstream of the link were obtained by DNA synthesis, then annealed with the knob sequence and cloned into the pGEM-T-easy vector. The link-RBS-knob sequence was digested (*Eco*RI and *Hind*III, Takara) from pGEM-T-easy Knob and inserted into T7Select10-3b cloning vector (Novagen). The ligation mixture was packaged using the commercial packaging extracts (Novagen) and amplified using *E. coli* BLT5403 strains. The T7-knob phage was purified by 20% PEG in 0.4 mol/l NaCl precipitation and then suspended in TBS. The Ad fiber knob protein displayed by T7 phage was analyzed by a native gel electrophoresis and Western blot analysis. Anti-knob MAb was prepared as previously described (Wang *et al.*, 2007).

ELISA of binding of exCAR to T7-Ad fiber knob phage. ELISA was performed to analyze the binding activity of recombinant exCAR to the Ad fiber knob. The 96-well ELISA plates were coated with 50 ng of purified recombinant exCAR. After blocking with blocking buffer, the ELISA plates were incubated with serially diluted T7-knob phage at 37°C for 1 hr, while T7 phage was used as a negative control. The plates were incubated with anti-T7 tag antibody (Novagen) for 1 hr and then with HRP-conjugated goat anti-mouse antibody for 1 hr. After washing, the plates were patted dry. ABTS was added to the wells and plates were incubated at 37°C for 30 mins. The A_{405} was taken with a microplate reader (Biorad).

Results

Production of exCAR

The extracellular domain of the human CAR protein (237 aa in length) was cloned into the BaculoDirect™ Linear DNA and determined by PCR analysis (data not shown). The recombinant baculovirus bearing the exCAR gene was obtained by recombination of the entry clone pENTR/D-TOPO-exCAR with a baculovirus genomic DNA in Sf9 cells.

After obtaining the recombinant viruses stock, we analyzed the expression of exCAR in Sf9 cells. Under the microscope, the recombinant virus-infected Sf9 cells typically displayed increased cell diameter and nuclei appeared to "fill" the cells (Fig. 1a). The culture supernatants and the intracellular proteins were analyzed by 12% SDS-PAGE and Western blot analysis with a MAb specific to the human CAR protein. Protein exCAR was detected both in the culture supernatants and cell lysates as a band of approximately 33 kDa (Fig. 1b).

To optimize the condition for soluble exCAR expression, High Five cells infected with baculovirus stock at the different

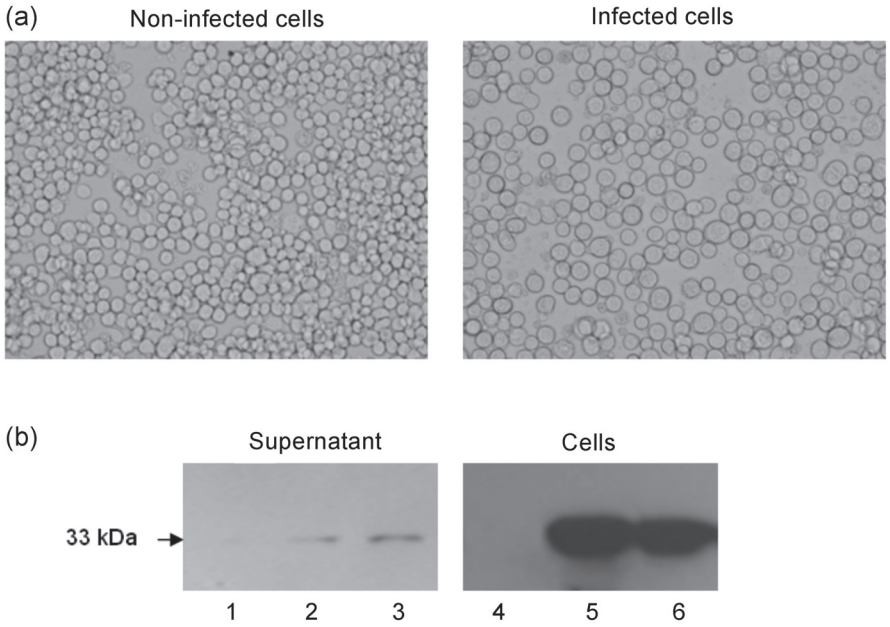

Fig. 1

Expression of exCAR in Sf9 cells

(a) Cell morphology at 72 hrs p.i., magnification 200x. (b) Western blot analysis of recombinant baculovirus-infected supernatants and cells at 72 hrs p.i. (lanes 2–3 and 5–6, respectively) and non-infected supernatant and cells (lanes 1 and 4, respectively).

MOI values(0.5~10)together with the time-course (0~120 hrs) expression of the recombinant exCAR protein in culture supernatants were analyzed by SDS-PAGE and Western blot analysis. High Five cells appeared to increase cell diameter and released from the flask surface after infection with the baculovirus at 24 hrs p.i. (Fig. 2a). Afterwards, the cells stopped the growth at 48 hrs p.i. and started to lyse at 96 hrs p.i. The majority of cells appeared to be lysed at 120 hrs p.i. (Fig. 2a). SDS-PAGE and Western blot analysis results (Fig. 2b and 2c) showed that the expression level of exCAR at the MOI of 1 was higher than that at MOI of 0.5. However, the expression levels of exCAR at MOI from 1 to 10 were almost equal. The best time to collect the culture supernatant was at 96 hrs p.i. (Fig. 2c). At 120 hrs p.i., the production of the soluble exCAR appeared to decrease.

Purification of exCAR

Protein exCAR was purified from the culture supernatants using a Ni-NTA column. After purification, it was examined by SDS-PAGE (Fig. 3). According to the analysis of the purified exCAR fractions, approximately 8–10 mg of exCAR with a purity of 96.3% can be obtained from 1 liter of the High Five cells culture supernatant.

Functional analysis of exCAR

The Ad fiber knob protein fused with T7 capsid 10B displayed by T7 phage was detected by anti-knob MAb. Both monomeric (63 kDa) and trimeric (189 kDa) form of the knob protein were produced (Fig. 4a). ELISA clearly showed that the recombinant exCAR protein was efficiently bound to the T7-knob fusion protein (Fig. 4b).

Discussion

Previous studies showed that the complete CAR ectodomain was insoluble when expressed in the bacteria system (Wu *et al.*, 2004; Zhang *et al.*, 2009). The process of denaturation and renaturation of recombinant proteins was very cumbersome. Additionally, prokaryotic expression system failed to generate glycosylated CAR, what affected both the

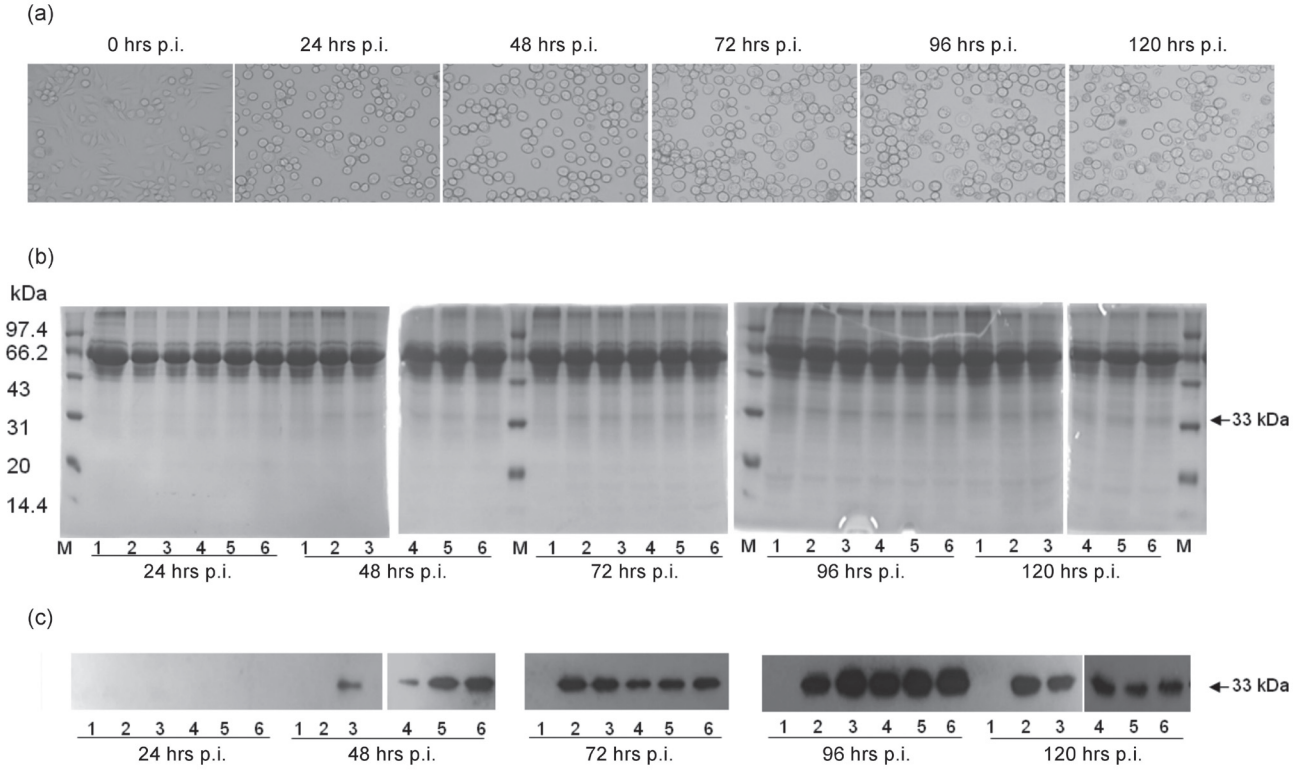

Fig. 2

Expression of exCAR in High Five cells

The cells infected with recombinant baculovirus at various MOI and analyzed at various times p.i. (a) Cell morphology at MOI of 5. (b) SDS-PAGE. (c) Western blot analysis. The cells infected with recombinant baculovirus at MOI of 0.5 (lanes 2), 1.0 (lanes 3), 2.5 (lanes 4), 5.0 (lanes 5) and 10.0 (lanes 6). Non-infected cells (lanes 1). Protein size marker (lanes M).

cell adhesion and adenovirus infection in unique way (Excoffon *et al.*, 2007). In this report, we describe the expression of recombinant extracellular domain of human CAR fused with his tag in the baculovirus-insect cell system. This eukaryotic system was able to carry out complex post-translational modifications. Generally, recombinant proteins produced in this system are correctly folded and glycosylated, what allows obtaining a soluble protein (Golden *et al.*, 1998).

The Sf9 cells have a higher transfection efficiency, but the High Five cells possess a more potent secretion apparatus (Wickham *et al.*, 1992; Davis *et al.*, 1993). Thus, we optimized the condition for soluble exCAR expression in the High Five cells. No differences concerning the production of soluble exCAR were observed among cell cultures infected at different MOIs (from 1 to 10). These findings were consistent with previous reports (Yamaji *et al.*, 1999). In the baculovirus-insect cell culture system, cells for the production of recombinant proteins are generally cultured in serum-free medium. Often encountered problem is a proteolytic degradation of recombinant proteins by proteases derived from the virus-infected cells in serum-free medium (Martensen and Justesen, 2001; Yamaji *et al.*, 2003). Therefore, during the expression of soluble exCAR in High Five cells, we kept the concentration of 2% FCS in the cultivation medium. The presence of serum helped to protect recombinant protein from the proteolysis and had no adverse effect on its further purification. At 120 hrs p.i., the amount of soluble exCAR in culture supernatants appeared to decrease**,** what might be due to a higher amount of proteases released into the culture supernatant upon lysis of virus-infected cells.

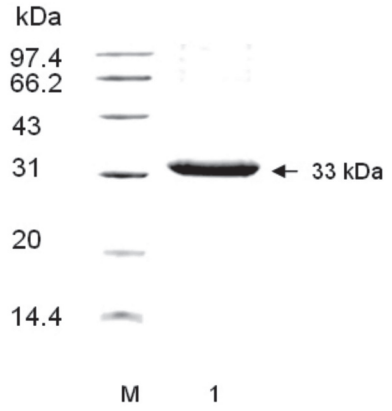

Fig. 3

SDS-PAGE analysis of purified exCAR

ExCAR produced in High Five cells at MOI of 1 at 96 hrs p.i. purified by Ni-NTA chromatography and analyzed by SDS-PAGE. Protein size marker (lane M), exCAR purificate (lane 1).

Since the Ad fiber knob is shown to trimerize and has been successfully expressed on the capsid of phage λ, we employed the T7 phage system to display the Ad fiber knob protein (Fontana *et al.*, 2003). T7 phage display is an effec-

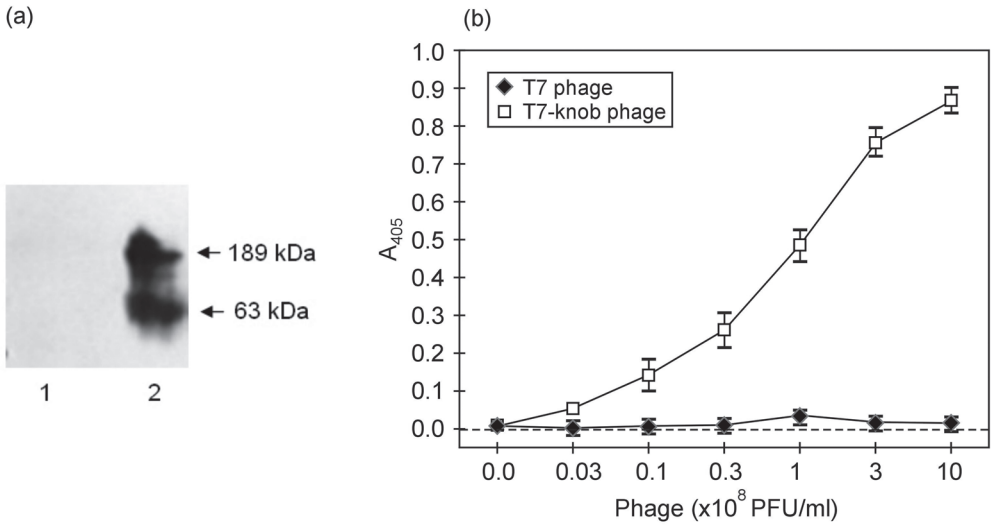

Fig. 4

Ad fiber knob protein displayed by T7 phage and its binding to exCAR

(a) Western blot analysis of Ad fiber knob protein displayed by T7-Ad fiber knob phage. T7 phage, negative control (lane 1), monomeric and trimeric forms of Ad fiber knob protein (lane 2, arrows). (b) ELISA of the binding of exCAR to T7-Ad fiber knob phage or T7 phage (negative control).

tive method for the detection of protein-protein interactions (Ishi and Sugawara, 2008). The BLT5403 strains produce the capsid 10A and T7Select10-3b vector expressed knob proteins fused to the capsid 10B, which allowing the assembly of phage particles displaying the knob protein in 5–15 copies per phage particle. This may facilitate the Ad fiber knob protein to form a trimer and to retain its natural binding activity. T7 phage provided the efficient expression of knob trimer on the capsid and the recombinant exCAR sustained its binding ability to the Ad fiber knob.

In conclusion, we performed recombinant baculovirus expression of the ectodomain of human CAR in insect cells with a high yield of purified protein (8–10 mg/l). Importantly, the recombinant exCAR maintained its binding activity to the Ad fiber knob. This feature could allow its application as a blocking agent in the initiation of adenovirus infection. In addition, recombinant adenoviral vectors are widely used for the gene therapy studies *in vitro* and *in vivo* for their high infection efficiency in a variety of cell types and tissues (Douglas *et al.*, 1999; Koizumi *et al.*, 2003). Therefore, the recombinant exCAR could also be used as a tool for studying the tropism of a modified recombinant adenovirus in the gene therapy.

Acknowledgement. This study was supported by the grant No. 2007AA021203 from the National High Technology Research and Development Program of China.

References

Arnberg N, Edlund K, Kidd AH, Wadell G (2000): Adenovirus type 37 uses sialic acid as a cellular receptor. J. Virol. 74, 42–48. doi:10.1128/JVI.74.1.42-48.2000

Boulanger P (1999): Cell receptors for human adenoviruses. J. Soc. Biol. 193, 77–84.

Bowles KR, Gibson J, Wu J, Shaffer LG, Towbin JA, Bowles NE (1999): Genomic organization and chromosomal localization of the human Coxsackievirus B-adenovirus receptor gene. Hum. Genet. 105, 354–349. doi:10.1007/s004390051114

Bowles NE, Ni J, Kearney DL, Pauschinger M, Schultheiss HP, McCarthy R, Hare J, Bricker JT, Bowles KR, Towbin JA (2003): Detection of viruses in myocardial tissues by polymerase chain reaction. Evidence of adenovirus as a common cause of myocarditis in children and adults. J. Am. Coll. Cardiol. 42, 466–472. doi:10.1016/S0735-1097(03)00648-X

Carson SD (2001): Receptor for the group B coxsackieviruses and adenoviruses: CAR. Rev. Med. Virol. 11, 219–226. doi:10.1002/rmv.318

Davis TR, Wickham TJ, McKenna KA, Granados RR, Shuler ML, Wood HA (1993): Comparative recombinant protein production of eight insect cell lines. In Vitro Cell Dev. Biol. Anim. 29A, 388–390. doi:10.1007/BF02633986

Dmitriev I, Kashentseva E, Rogers BE, Krasnykh V, Curiel DT (2000): Ectodomain of coxsackievirus and adenovirus receptor genetically fused to epidermal growth factor mediates adenovirus targeting to epidermal growth factor receptor-positive cells. J. Virol. 74, 6875–6884. doi:10.1128/JVI.74.15.6875-6884.2000

Douglas JT, Miller CR, Kim M, Dmitriev I, Mikheeva G, Krasnykh V, Curiel DT (1999): A system for the propagation of adenoviral vectors with genetically modified receptor specificities. Nat. Biotechnol. 17, 470–475. doi:10.1038/8647

Excoffon KJ, Gansemer N, Traver G, Zabner J (2007): Functional effects of coxsackievirus and adenovirus receptor glycosylation on homophilic adhesion and adenoviral infection. J. Virol. 81, 5573–5578. doi:10.1128/JVI.02562-06

Fontana L, Nuzzo M, Urbanelli L, Monaci P (2003): General strategy for broadening adenovirus tropism. J. Virol. 77, 11094–11104. doi:10.1128/JVI.77.20.11094-11104.2003

Gaggar A, Shayakhmetov DM, Lieber A (2003): CD46 is a cellular receptor for group B adenoviruses. Nat. Med. 9, 1408–1412. doi:10.1038/nm952

Golden A, Austen DA, van Schravendijk MR, Sullivan BJ, Kawasaki ES, Osburne MS (1998): Effect of promoters and signal sequences on the production of secreted HIV-1 gp120 protein in the baculovirus system. Protein Expr. Purif. 14, 8–12. doi:10.1006/prep.1998.0926

Henquell C, Boeuf B, Mirand A, Bacher C, Traore O, Dechelotte P, Labbe A, Bailly JL, Peigue-Lafeuille H (2009): Fatal adenovirus infection in a neonate and transmission to healthcare workers. J. Clin. Virol. 45, 345–348. doi:10.1016/j.jcv.2009.04.019

Ishi K, Sugawara F (2008): A facile method to screen inhibitors of protein-protein interactions including MDM2-p53 displayed on T7 phage. Biochem. Pharmacol. 75, 1743–1750. doi:10.1016/j.bcp.2008.01.020

Koizumi N, Mizuguchi H, Utoguchi N, Watanabe Y, Hayakawa T (2003): Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. J. Gene Med. 5, 267–276. doi:10.1002/jgm.348

Leen AM, Rooney CM (2005): Adenovirus as an emerging pathogen in immunocompromised patients. Br. J. Haematol. 128, 135–144. doi:10.1111/j.1365-2141.2004.05218.x

Lim BK, Choi JH, Nam JH, Gil CO, Shin JO, Yun SH, Kim DK, Jeon ES (2006): Virus receptor trap neutralizes coxsackievirus in experimental murine viral myocarditis. Cardiovasc. Res. 71, 517–526. doi:10.1016/j.cardiores.2006.05.016

Martensen PM, Justesen J (2001): Specific inhibitors prevent proteolytic degradation of recombinant proteins expressed in High Five cells. Biotechniques 30, 782–784, 786, 788 passim.

Nemerow GR (2000): Cell receptors involved in adenovirus entry. Virology 274, 1–4. doi:10.1006/viro.2000.0468

Pham TT, Burchette JL, Jr, Hale LP (2003): Fatal disseminated adenovirus infections in immunocompromised patients. Am. J. Clin. Pathol. 120, 575–583. doi:10.1309/AWXDGNC5D70EN7YT

Savon C, Acosta B, Valdes O, Goyenechea A, Gonzalez G, Pinon A, Mas P, Rosario D, Capo V, Kouri V, Martinez PA, March-

ena JJ, Gonzalez G, Rodriguez H, Guzman MG (2008): A myocarditis outbreak with fatal cases associated with adenovirus subgenera C among children from Havana City in 2005. J. Clin. Virol. 43, 152–157. doi:10.1016/j.jcv.2008.05.012

Schneider-Schaulies J (2000): Cellular receptors for viruses: links to tropism and pathogenesis. J. Gen. Virol. 81, 1413–1429.

Tomko RP, Xu R, Philipson L (1997): HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. USA 94, 3352–3356. doi:10.1073/pnas.94.7.3352

Wang P, Wang H, Zhou M, Liu S, Lin Z, Xu Y, Li Z (2007): Expression, Purification of Ad-5 Knob Protein and Preparation of Its Monoclonal Antibody. Chin. J. Cell. Mol. Immunol. 23, 1038–1040.

Wickham TJ, Davis T, Granados RR, Shuler ML, Wood HA (1992): Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol. Prog. 8, 391–396. doi:10.1021/bp00017a003

Wu Y, Chen H, Huang X, Chen H, Liu J (2004): Cloning and expression of human coxsackie-adenovirus receptor and production of anti-car polyclonal antibody. Acad. J. Shanghai Second Med. Univ. 24, 726–730.

Yamaji H, Hirakawa D, Tagai S, Fukuda H (2003): Production of protein kinase C-delta by the baculovirus-insect cell system in serum-supplemented and serum-free media. J. Biosci. Bioeng. 95, 185–187.

Yamaji H, Tagai S, Fukuda H (1999): Optimal production of recombinant protein by the baculovirus-insect cell system in shake-flask culture with medium replacement. J. Biosci. Bioeng. 87, 636–641. doi:10.1016/S1389-1723(99)80127-3

Zhang K, Zhou X, Guang L, Zhou S, He X, Sheng H, Qian G (2009): Expression of human exCAR protein in E. coli and its purification and identification. Chongqing Med. J. 38, 2691–2694.

Zhang S, Jia HB, Gong BS, Zhang SJ, Li X, Yu B (2008): Role of coxsackievirus and adenovirus receptor in the pathogenesis of dilated cardiomyopathy and its influencing factor. Chin. Med. J. 121, 1445–1449 (in English).

Zor, T, and Selinger, Z (1996): Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. Anal. Biochem. 236, 302-308. doi:10.1006/abio.1996.0171