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Baculovirus-mediated Expression of *p35* Confers Resistance to Apoptosis in Human Embryo Kidney 293 cells*

Jian-hua SONG, Chang-yong LIANG and Xin-wen CHEN**

(State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China)

Abstract: Baculovirus has many advantages as vectors for gene transfer. We demonstrated that recombinant baculovirus vectors expressing *p35* (Ac-CMV-p35) and eGFP (Ac-CMV-GFP) could be transduced into human kidney 293 cells efficiently. The level of transgene expression was viral dose dependent and high-level expression of the target gene could be achieved under the heterogonous promoter. MTT assay suggested that both Ac-CMV-p35 and Ac-CMV-GFP did not have cytotoxic effect on human embryo kidney 293 cells. Cell growth curve showed the Ac-CMV-p35 and Ac- CMV-GFP transduced and non-transduced cells had similar proliferation rate, so baculovirus-mediated *p35* expression had no adverse effect on cell proliferation. In addition, baculovirus-mediated *p35* gene expression protected human embryo kidney 293 cells against apoptosis induced by various apoptosis inducers such as Actinomycin D, UV or serum-free media. These results suggested that the baculovirus vector mediated *p35* gene expression was functional and it could be widely used in molecular research and even gene therapy.

Key words: Baculovirus; Gene therapy vectors; *p35* gene; Expression

Baculovirus has been widely used for production of recombinant proteins in insect cells. Previous studies found when a reporter gene under the control of a mammalian promoter was cloned into the baculovirus genome, it was expressed in hepatoma cells (Huh7 and HepG2), primary rat hepatocytes, epithelioid cell lines and rabbit intervertebral disc cells (Hela and Cos7) *in vitro* (2, 7, 14). Since these findings demonstrated that baculovirus can efficiently transduce mammalian cells, the applications of baculovirus have been greatly expanded (23). Interestingly, although baculovirus can transduce mammalian cells very efficiently it only replicates in insect cells (10). Therefore, it has been suggested that baculovirus could be used as a gene therapy vector (11, 12).

Baculovirus *p35* gene is from the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and is known to inhibit cell apoptosis in

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** Corresponding author. Tel: + 86-027-87199106, Fax: +86-027-87199106, Email: chenxw@pentium.whiov.ac.cn

different organisms as diverse as *Caenorhabditts elegans*, *Drosophila melanogaster* and mammals, including humans (1, 4). Expression of *p35* prevents apoptosis induced by various stimuli in carcinoma cells, neurons, oligdendrocytes, vascular smooth muscle cell and pancreatic beta-cell (8, 17, 19). Compared with cardiomyocytos from wild-type mice, cardiomyocytes from *p35* expressing transgenic mice had longer lifetime under hypoxia conditions, and markedly reduced cytochrome C release, and caspase 3 activation. Additionally, purified P35 has been found to inhibit all the mammalian caspases except caspases 5 and 9 (20). Based on previous studies, the molecular mechanism of inhibiting apoptosis is most likely the direct inactivation of caspase by P35 protein. Because of these characteristics, P35 is also commonly referred to as a pan-caspase inhibitor (21). In this study, we investigated whether the expression of *p35* gene mediated by baculovirus vector can protect human embryo kidney 293 cells against apoptosis. The results demonstrated that baculovirus- mediated expression of *p35* effectively protected human embryo kidney 293 cells against apoptosis induced by various apoptosis inducers such as Actinomycin D, UV and serum-free media, and it could be widely used in molecular research and even gene therapy.

MATERIALS AND METHODS

Cell culture

The *Spodoptera frugiperda* (Sf9) cells were maintained at 28℃ in Grace's media supplemented with 10% fetal bovine serum (GiBco). Mammalian cell line human embryo kidney 293 was obtained from the China Center for Type Culture Collection, Wuhan (CCTCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (GiBco), at 37℃.

Construction of recombinant baculovirus vector

The baculovirus vectors were constructed as described previously. The *p35* gene was cloned by PCR from baculovirus genomic DNA. A ～1.0 kb *Eco*R I *p35* fragment was inserted into the *Eco*R I site of pFBDeGFPCMV (which contains the human cytomegalovirus enhancer/promoter, polyadenylation signal from SV40, eGFP gene was driven by *P10* promoter) (15). The correct construct was confirmed by PCR. With the donor plasmid pFBDGC*p35*, recombinant virus Ac-CMV-p35 was generated using the Bac-to-Bac system according to the manufacturer's protocols (Life Technologies). Viral titers were determined by the end-point dilution using Sf9 cells. Ac-CMV-GFP was used as a control (12).

Transduction of mammalian cells by baculovirus

Cells were seeded in 35 mm culture dishes at 5×10^5 cells per dish. Culture medium was removed, replaced with virus, and incubated for 2 h at 37℃. After removal of the virus, fresh medium was added and cultures were incubated at 37℃. Cultures were harvested, washed, and resuspended in phosphate-buffered saline. The GFP-expressing populations were analyzed by FACS (Beckman).

MTT assay

3-(4, 5-Dimethythiazal-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay was used to estimate cell survival by quantifying the total cell numbers. In this assay, 293 cells (2×10^4 cells/well) were seeded into a 96-well plate. Culture medium was removed, replaced with virus, and incubated for 2 h at 37℃. After removal of the virus, fresh medium was added and the

cultures were incubated. After 72 h treatment, the medium was replaced by 100μL/well normal medium containing 0.5 mg/mL MTT (sigma). Cells were incubated at 37℃ for 4 h. Then the medium containing MTT was removed and 100 μL/well 100% DMSO was added. After formazan was dissolved completed, the plate was placed on the microplate reader (Bio-Rad) to measure optical density at 570 nm.

Analysis of apoptotic cell death

Cell death was studied morphologically using a DNA dye, Hoechst 33258, which stains nuclei. Apoptotic cells have condensed chromatin and nuclear fragmentation that produces a characteristic, irregular staining of their nuclei, its chromosomes fluoresces brightly. 293 cells treated with different apoptosis-inducing stimuli such as Actinomycin D or UV radiation (w312 μmol/L, 1.8×10^{-6} watts/cm^2 at lamp to target distance). The percentage of apoptotic cell death was assessed by Hoechst 33258 staining according to the manufacturer's protocols (Bi Yuntian Company, Haimen, China). Starvation was induced by exposure to free serum cell culture and the survival rate was calculated by trypan blue, a vital dye, staining. Under a microscope, observe if non-viable are stained and viable cells excluded the stain.

Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde at 72 h post transduction and processed for immunofluorescence. P35 was visualized by indirect immunofluorescence using rabbit-anti-*p35* primary antibody (BD Biosciences) diluted 1:200. The bound antibody was visualized with FITC-conjugated goat- anti-rabbit IgG (San Ying Company, Wuhan, China) diluted 1:200 secondary antibody. Slides can be stored in the fridge or freezer until analyzed by confocal microscopy (Leica, Heidelberg, Germany).

RESULTS

Construction of recombinant baculovirus

Recombinant viruses Ac-CMV-p35 were generated using the Bac-to-Bac system (Fig. 1). After tansfection and infection, viral titers were determined by the end-point dilution with Sf9 cells. The titer of recombinant viruses Ac-CMV-p35 is 10^8 PFU/mL.

Recombinant baculovirus-mediated GFP gene expression

Expression of eGFP in mammalian 293 cells was investigated by FACS analysis. These cells were transduced with Ac-CMV-GFP (12) (with a series of MOI) following the addition of a deacetylase inhibitor, butyrate. The cells were harvested 48 h later. The percentage of cells expressing GFP was detected at different MOIs. With the addition of 10 mmol/L sodium butyrate, the percentage of eGFP-expressing cells expressing GFP increased as the MOI increased. The highest percentage was 86.6 ± 2.26% when using 200 MOI baculovirus (Fig. 2). This result indicated that recombinant baculovirus vectors are capable of transducing 293 cells efficiently.

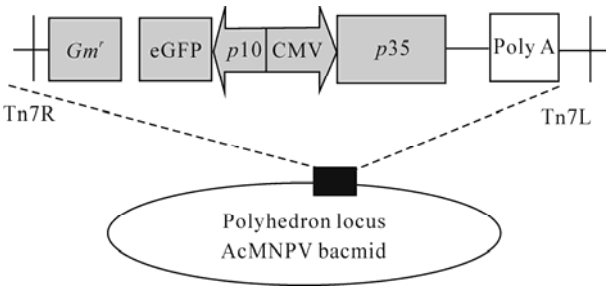

Fig. 1. Schematic diagram of recombinant baculovirus constructs. The strategy for insertion of the p35 protein gene constructs into the *polyhedrin* locus of the AcMNPV bacmid. Express the *p35* gene under a CMV promoter.

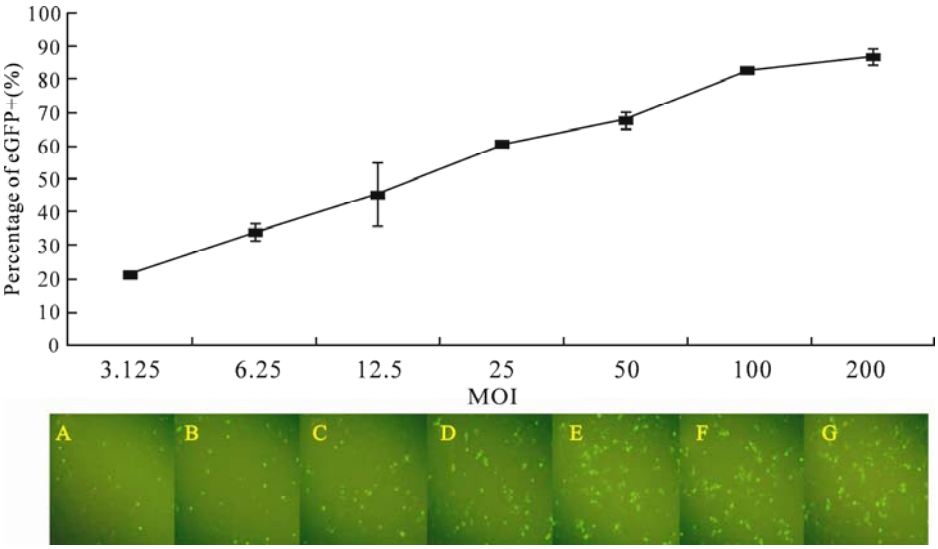

Fig. 2. Baculovirus-mediated GFP gene expression. 293 cells were transducted with Ac-CMV-GFP at 3.125 (A), 6.25 (B), 12.5 (C), 25 (D), 50 (E), 100 (F), 200 (G) MOI for 2 h, and percentages of GFP+ cells were detected at 48 h post-transduction. Results are shown as the mean ±standard error of 3 independent experiments.

Baculovirus-mediated *p35* gene transfer into 293 cells

The recombinant baculovirus Ac-CMV-*p35* (MOI= 100) was transduced into 293 cells. At 24 h, the transcription of *p35* gene was detected by reverse transcriptase polymerase chain reaction (RT-PCR). The 1 kb fragment was only found in the sample of transduced 293 cells indicating the successful transcription of the *p35* gene in 293 cells (Fig. 3). Further evidence of *p35* expression in transduced cells was found at the protein level by immunostaining 293 cells with an anti-*p35* antibody (Fig. 4). The transduced cells showed staining for *p35* in the intact cells in both the cytoplasm and the nucleus.

Cell viability assay and cell curve

Transduction of recombinant baculovirus vector is not toxic to mammalian cells. Transduction with Ac-CMV-p35 up to a MOI of 200 did not reduce cell viability, as measured by the MTT assy. MTT was also performed to estimate the effect of baculovirus-mediated expression of *p35* on mammalian cell

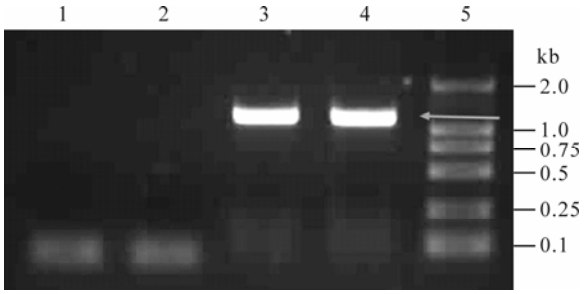

Fig. 3. Transcription analysis of *p*35 in 293 cells via RT-PCR. 1, Total RNA was isolated from 293 cells; 2, 293 cells transduced with Ac-CMV-p35 at oh; 3, 12 h; 4, Plasmid pT-*p35* as the positive control; 5, Marker DL2000.

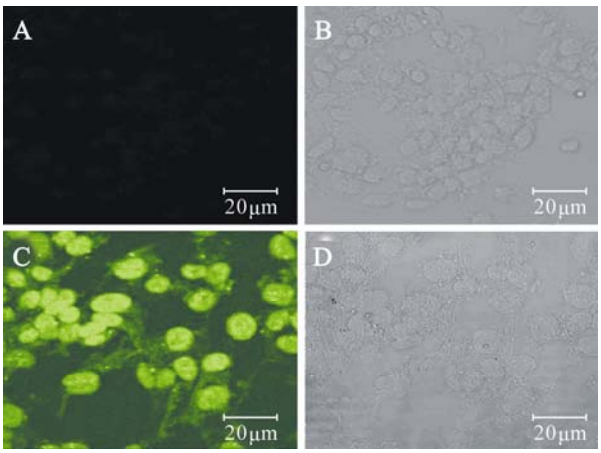

Fig. 4. Expression of P35 in transduced 293 cells by immunofluorescence. A/B, Negative control; C/D, 293 transduced with Ac-CMV-p35.

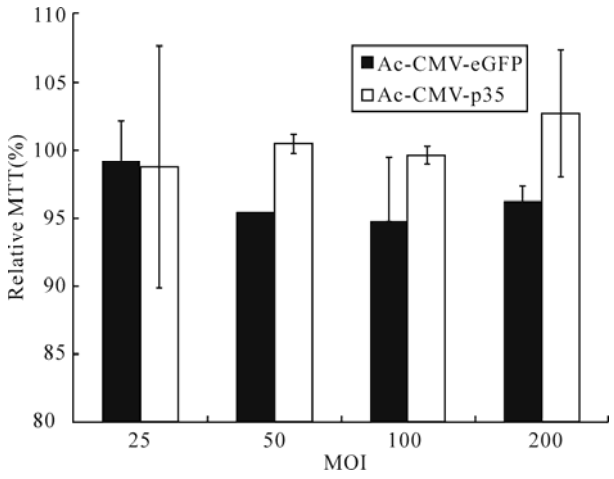

Fig. 5. Cytotoxic effects analysis by an MTT assay. 293 cells were seeded in 96-wells culture dish at 2.0×10^4 cells per well and transducted with Ac-CMV-GFP and Ac-CMV-p35 at 100 MOI. Cell survival was quantified by the MTT assay after 72 h post-transduction. Results are expressed as the mean ± standard error of 3 independent experiments.

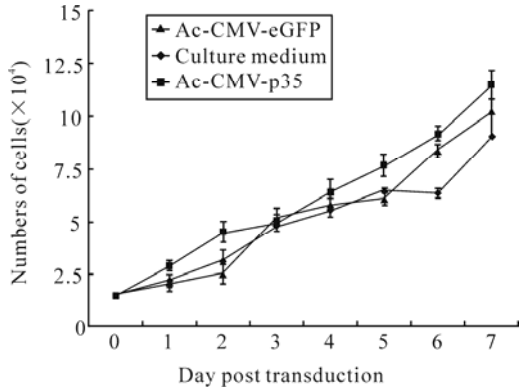

Fig. 6. Growth curve of 293 cells transducted by baculovirus. 293 cells were seeded in 96-wells culture dish at 2.0×10^4 cells per well, and transducted with Ac-CMV-GFP and Ac-CMV-p35 at 100 MOI for 2 h, Numbers of trypan blue negative cells were counted manually using a haemocytometer. Results are expressed as the mean ± standard error of 3 independent experiments.

proliferation. The viability of 293 cells transduced by Ac-CMV-GFP and Ac-CMV-p35 was not changed more than 5% (Fig. 5). In addition, compared to time-matched controls, there was no difference in the growth curve of cells transduced by Ac-CMV-GFP or Ac-CMV-p35 for 7 d (Fig. 6). Cell viability and cell growth curve assays demonstrated baculovirus-mediated expression of *p35* had no toxic effects on mammalian cell viability and growth.

Antiapoptosis induced by Actinomycin D

To assess the effect of baculovirus-mediated expression of *p35* on 293 cells resistance to apoptosis, the percent apoptosis cells induced by Actinomycin D was determined by Hoechst 33258 staining assay. The percentage of apoptotic cells decreased 34.14±7.05% and 64.51±4.86%, respectively, by using 12.5 MOI and 25 MOI Ac-CMV-p35 compared with the control baculovirus Ac-CMV-eGFP. The percentage of apoptotic cells with 50 MOI Ac-CMV-p35 decreased approximately 2-fold than that with 12.5 MOI Ac-CMV-p35 (Fig. 7). However, the efficiency of apoptosis inhibition could not increase correspondingly by using the higher MOI Ac-CMV-p35 once MOI was more than 50 MOI.

Antiapoptosis induced by UV

To verify that baculvirus-mediated expression of