A NOVEL DOUBLE-STABLE T-REX/GB CELL LINE EXPRESSING GLYCOPROTEIN B OF HERPES SIMPLEX VIRUS 1

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Received December 12, 2006; accepted February 20, 2007

Summary. – We established and characterized a new stable T-REx/gB cell line expressing gB of HSV-1 under tetracycline (Tet) control. The expression of complete gB (120 K) in T-REx/gB cells was detected by Western blot analysis with anti-HSV-1/ANG/gB monoclonal antibody as early as 2 days after Tet induction. Inducibility and tightness of Tet-regulated gB expression in T-REx/gB cell line was shown to be preserved after long-term culture (2 months) and after numerous freezing/thawing cycles as well. In this study, we described the conditions required for the generation of the T-REx/gB cell line, which can be useful as a host for the isolation and propagation of HSV-1 recombinant viruses defective in gB gene.

Key words: cell line expressing gB; Herpes simplex virus 1; inducible expression

Introduction

Herpes simplex virus 1 (HSV-1) is a neuroinvasive human pathogen that spreads from portal of entry (the epithelial cells of the skin or mucosa) along the nerves into regional ganglia, where the latency is established. The viral glycoproteins are the essential components controlling the virus infectivity and major determinants of virulence. The initial stage of virus-cell interaction is the attachment of virions to the surface receptor of susceptible cells. This event is mediated by the glycoproteins C and B (gC and gB), because they interact with the glycosoaminoglycans at the cell surface, especially with heparan sulphate (HS). An essential role in the virus-cell fusion has been attributed to gB (Herold *et al.*, 1994, 1995).

gB is a multifunctional protein. It plays an important role in membrane fusion, virion penetration, cell-to-cell spread, maturation and egress. gB is localized in the virion envelope and as a homooligomer in the membranes of infected cells. This property predicts gB as an important immunogen, which challenges both the humoral and cellular immune response (Potel *et al.*, 2002). gB is encoded by the UL27 gene and contains 904 aa. The first 29 or 30 aa constitutes the signal sequence (Claesson-Welsh and Spear, 1987). The ectodomain has 696 aa (Cai *et al.*, 1988), the transmembrane domain (TM) has 69 aa, and the endodomain consists of 109 aa (Pellett *et al.*, 1985). The TM domain of gB molecule three times traverses the membrane of infected cell.

To study the functional role of particular domains of gB in virus entry, maturation and pathogenicity, the construction of mutants with specific mutations or deletions is needed. For replication of a virus with defects in an essential viral gene, a complementing-cell line (helper cell line) must be available. We report here the establishment of a cell line for Tet-inducible gB expression. The evaluation of the double-stable cell line T-REx/gB revealed that it supports the growth of HSV-1/gB recombinant virus.

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Abbreviations: gB = glycoprotein B; HSV-1 = Herpes simplex virus 1; HS = heparan sulphate; IF = immunofluorescence; p.t. = post transfection; Tet = tetracycline; TetR = Tet repressor; TM = transmembrane domain

Materials and Methods

Virus and cells. HSV-1 strain ANG was propagated in Vero cells using DMEM supplemented with 10% (v/v) calf serum. Mammalian cell line T-REx-293 (Invitrogen) was grown in DMEM supplemented with 10% (v/v) fetal bovine serum and 5 µg/ml blasticidin (ICN).

Plasmids. To generate pcDNA3.1-*Kpn*I-nR recombinant vector, the *Kpn*I *n* fragment of HSV-1 strain ANG was cloned into pcDNA3.1 vector (Invitrogen) in reverse orientation. The 3.4 kbp *Nru*I-*Xba*I fragment with the gB coding region of HSV-1 strain ANG (UL27 gene) from pcDNA3.1-*Kpn*I-nR was inserted into the *Eco*RV and *Xba*I polycloning sites of pcDNA4/TO (Invitrogen). Recombinant pcD4gB/TO plasmid DNA used for transfection of T-REx-293 cells was purified using QIAfilter Plasmid Midi Kit (Qiagen). The gB coding region in the pcD4gB/TO was under control of Tet-inducible promoter of Human cytomegalovirus (HCMV) containing 2 copies of the *tet* operator type 2 ($tetO_2$) (Yao *et al.*, 1998).

Establishment of stable gB expressing cell line. The T-REx-293 cell line stably expressing the Tet repressor from the pcDNA6/TR plasmid (Invitrogen) in the presence of blasticidin (5 µg/ml) was used for transfection. Subconfluent mammalian T-REx-293 cells were transfected with 2 or 4 µg DNA of pcD4gB/TO using transfection reagent metafectene (Biontex). One day post transfection (p.t.), 5 µg/ml blasticidin and 10 µg/ml phleomycine (InvivoGen) was added. For selection of individual cell clones, the cell growth was maintained with the DMEM supplemented with 5 µg/ml of blasticidin and increased concentrations of phleomycine (from 15 µg/ml at 2 days p.t. to 25 µg/ml at 10 days p.t.).

Western blot analysis. Lysates of the cells prepared by lysis in 2x concentrated electrophoresis sample buffer (125 mmol/l Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 1.8% ß-mercaptoethanol) were analyzed on 7.5% SDS-PAGE gel and the proteins were transferred to PVDF membranes (Amersham). Membranes were blocked in 5% non-fat milk made in PBS with 0.01% Tween (T-PBS), incubated for 1 hr in T-PBS containing a mouse anti-HSV-1/ANG/gB monoclonal antibody diluted 1:1,000 and finally in a goat-anti-mouse/Px conjugate diluted 1:5,000. The blots were developed by chemiluminescence (ECL, Amersham).

Immunofluorescence (IF) analysis. To detect gB expression, T-REx/gB cell line grown on cover slips was induced with 2 µg/ml Tet for 2, 3 or 4 days and then cultivated without Tet for 2, 3, 4, 5, 6 or 8 days. To detect intracellular or membrane surface gB localization, the cells were fixed either in methanol or in 4% paraformaldehyde for 20 mins at 4°C. Then, the cells were incubated with the mouse anti-HSV-1/ANG/gB monoclonal antibody diluted 1:40 for 40 mins at 37°C, three times extensively washed with PBS, and incubated with goat anti-mouse/FITC conjugate diluted 1:40 for 30 mins at room temperature. After three washes with PBS, cover slips were mounted using Tris-glycerol mounting media, and viewed with an E400 Nikon fluorescence microscope.

Results and Discussion

The gene UL27 of HSV-1 encoding gB is essential for viral replication (Little *et al.*, 1981). To construct viruses with a mutated gB gene for functional studies as well as for the growth of viruses with deleterious gB mutations a helper cell line expressing gB is required.

Cai *et al.* (1987) used gB-transformed cells to isolate and propagate HSV-1 viruses with mutations in gB. This cell line, designated D6, was prepared via transformation of Vero cells with gB coding region of the KOS strain of HSV-1. By the help of this cell line, the mutant gB-del virus K082 derivated from KOS strain was isolated.

We used the T-REx™ System to generate double-stable inducible cell line expressing gB gene of the ANG strain of HSV-1. The major component of this system is inducible expression plasmid – pcDNA4/TO. It provides the expression of gene of interest under the control of the strong HCMV promoter with 2 inserted copies of the *tet* operator 2 ($tetO_2$) sequence (Yao *et al.*, 1998). Each $tetO_2$ element coming from the Tn*10*-encoded tetracycline resistance operon of *Escherichia coli* (Hillen and Berens, 1994; Hillen *et al.*, 1983) serves as the binding site for two molecules of the Tet repressor (TetR). The second major component of expression system used in this study was the T-REx-293 cell line that stably expressed TetR from the regulatory plasmid pcDNA6/TR (Postle *et al.*, 1984).

The tetracycline regulation in the T-REx™ System is based on a gene repression of interest in the absence of Tet (TetR binds to the $tetO_2$ sequence) and induction of its expression in the presence of Tet, when binding of Tet to the TetR homodimers leads to the release of TetR from the $tetO_2$ sequences (Yao *et al.*, 1998).

The stable gB expressing cell line T-REx/gB was established by transfection of T-REx-293 cell line with the recombinant inducible expression plasmid pcD4gB/TO. After transfection, the cell clones were selected in the presence of blasticidin and increasing concentration of phleomycin. Viable cell clones were expanded and after induction with 2 µg/ml Tet were tested for gB expression by Western blot analysis and IF. A single cell clone signed P3 was selected and characterized and the requirements for its stability and the optimal concentration of antibiotics were determined.

Presence of gB (120 K) in T-REx/gB cells was detected with anti-HSV-1/ANG/gB monoclonal antibody in Western blot analysis as early as 2 days after induction with Tet. Further experiments confirmed that gB protein expression in these cells was strictly Tet-dependent, showing no detectable protein in the absence of Tet (Fig. 1, line 2). Moreover, the inducibility of gB expression in the T-REx/gB cell line was preserved even after long-term culture (2 months) (Fig. 1, line 3, 4). Furthermore, even after repeating freezing/thawing the established double-stable cell line exhibited identical properties and an unchanged gB expression profile.

Indirect IF staining with anti-HSV-1/ANG/gB monoclonal antibody was used to detect the expression of gB protein in T-REx/gB cells from 2 to 7 days after Tet

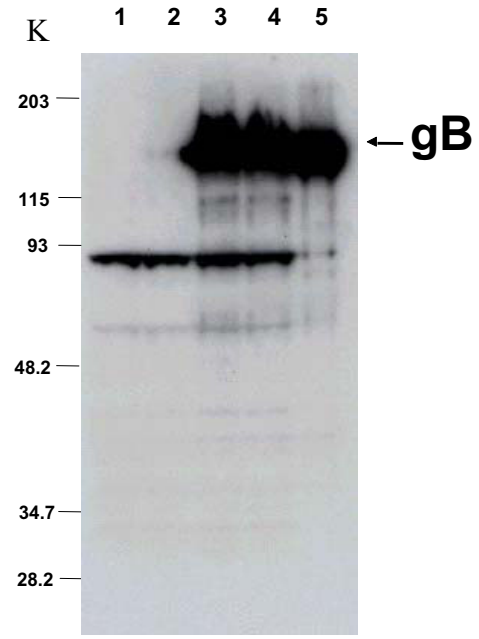

Fig. 1

Western blot analysis of T-REx/gB cells expressing HSV-1 gB

Line 1: negative control – T-REx-293 cells transfected with control plasmid pcDNA4/TO cultivated in the presence of Tet (2 μg/ml). Line 2: T-REx/gB cells cultivated in the absence of Tet. Line 3, 4: T-REx/gB cells cultivated in the presence of Tet (1 and 2 μg/ml, resp.). Line 5: positive control – T-REx-293 cells infected with HSV-1 strain ANG.

induction. The gB antigen was predominantly localized in the cytoplasm of the cells as well as around the rim of the nuclear membrane (data not shown).

A major goal of this study was to achieve the gB expression preferably on the cell membrane in order to enhance the maturation of gB mutant viruses. The expression of gB in T-REx/gB cells should be used mainly for the complementation of the deleterious gB mutant viruses. We observed that gB expression was abundantly present on the membranes of cells when induced with Tet for 3–4 days and then kept without Tet for 3–5 days. When induction with Tet proceeded for 3 days followed by 4 days cultivation in the absence of Tet, the peak of gB expression was detected (Fig. 2). The surface membrane localization of gB was also documented by indirect IF using permeabilized and non-permeabilized cells. Presence of gB in non-permeabilized cells was detected equally distributed on the cell membranes (Fig. 2A), whereas gB in permeabilized cells was found only in a few cells and cell clusters (Fig. 2B).

We established a novel stable T-REx/gB cell line expressing HSV-1 gB. Unique attribute of this cell line is the inducibility of gB expression. Efficient gB expression in T-REx/gB cell line could enable the successful growth of

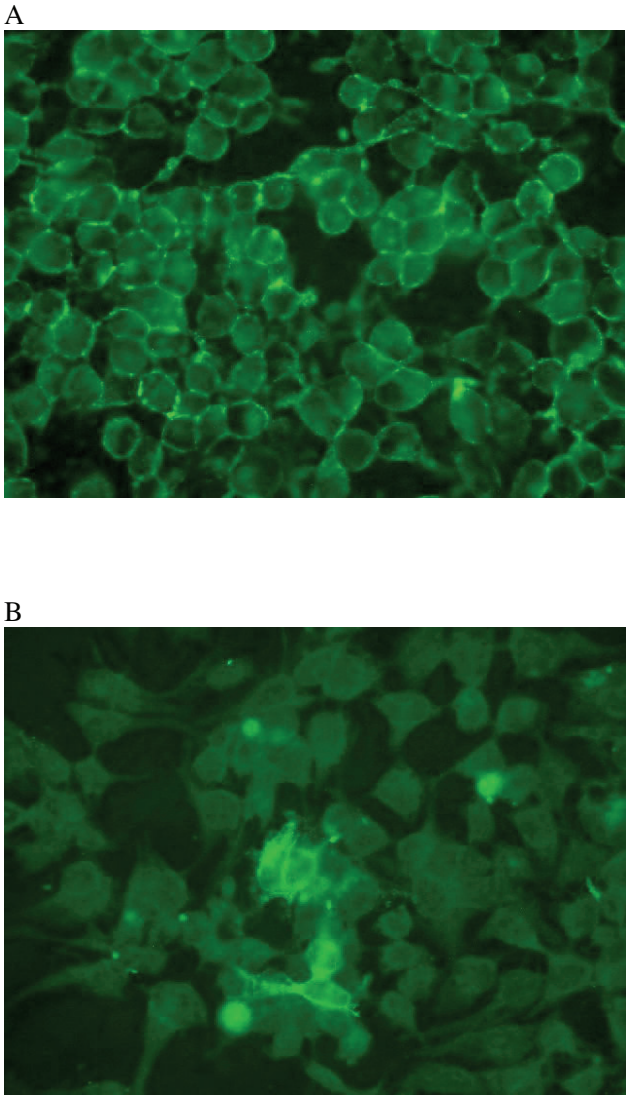

Fig. 2

IF analysis of T-REx/gB cells expressing HSV-1 gB

(A) non-permeabilized and (B) permeabilized cells (magnification 200x).

HSV-1/gB mutants. The stable T-REx/gB cell line will be very useful for studies of the functional domains of gB and its cellular interactions by the help of specifically designed HSV-1 gB mutant viruses.

Acknowledgement. This work was supported by the contract APVT-51-0347/02 from Science and Technology Assistance Agency and by the grants 2/4122/04 and 2/7095/7 from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences. We feel indebted to Jochen Kühn, Universität Münster, for providing the anti-HSV-1/ANG/gB monoclonal antibody.

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