EXPRESSION AND CHARACTERIZATION OF VP2 PROTEIN OF HUMAN ROTAVIRUS A IN A MAMMALIAN LUNG CELL LINE

F. POURASGARI[1], S. AHMADIAN[1], A.H. SALMANIAN[2]

[1]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran; [2]National Institute for Genetic Engineering and Biotechnology, Tehran, Iran

Received April 4, 2007; accepted September 28, 2007

Summary. – A system for the expression and characterization of VP2 protein of Human rotavirus A, strain G3 was established in the mammalian lung cell line (A549). Expression of the recombinant VP2 (rVP2) protein was detected 48–72 hrs after transfection by Western blotting. The rVP2 protein expressed in A549 cells formed intracellular core-like particles (CLPs) 30 nm in diameter detected by electron microscopy (EM). These results showed that the A549 cells are suitable as efficient eukaryotic host for production of rVP2 protein.

Keywords: A549 cell line; core-like particles; expression; Human rotavirus A; VP2 gene

Introduction

Rotavirus A belonging to the genus *Rotavirus,* family *Reoviridae* is the most important pathogen causing severe diarrhea in infants and young children (Kapikian *et al*., 2001; Wilhelmi *et al*., 2003). More than 70% of serious diarrhea diseases in children worldwide are caused by the rotavirus A infection due to a lack of specific immunity (Adler *et al*., 2005; Simpson *et al*., 2003). The rotavirus A causes an estimated 111 million episodes of diarrhea annually requiring only home care, 25 million clinic visits, 2 million hospitalizations, and 352,000–592,000 deaths of children under 5 years of age (Parashar *et al*., 2003).

Morphologically, the triple-layered rotavirus particles are composed of (i) an outer protein layer consisting of VP4 and VP7 proteins, (ii) a middle protein layer containing VP6 protein, and (iii) an inner layer composed of VP1, VP2, VP3 proteins that encloses 11 segments of double-stranded genomic RNA (Estes and Cohen, 1989). It has been shown that the innermost capsid layer is composed of 120 molecules of the VP2 protein of 102 K (Labbe *et al*., 1991). The VP2 protein varies in size from 880 residues for Bovine rotavirus A/RF (Kumar *et al*., 1989), 881 residues for both Bovine rotavirus A/UK and Simian rotavirus A/SA11 (Mitchell and Both, 1990), to the 890 residues for Human rotavirus A/Wa (Ernst and Duhl, 1989). The VP2 protein binds to viral RNA and the corresponding binding domain is located between the amino acids 1 and 132 (Varghese *et al*., 2006). However, further analyses indicated that the N-terminus of VP2 protein is necessary also for the encapsidation of VP1 and VP3 proteins (Zeng *et al*., 1998). VP2 as a scaffolding protein permits the formation of virus-like particles (VLPs) of various protein composition that include VP2, VP4, VP6, and VP7 proteins (Crawford *et al*., 1994). VLPs can be triple-, double- or single-layered. Single-layered particles are called as core-like particles (CLPs).

High-level expression of recombinant proteins in eukaryotic cells provides a good opportunity to investigate the functions and molecular interactions of the proteins. The aim of the present study was to prepare plasmid containing the gene encoding VP2 protein of Human rotavirus A, transfect the cells and characterize the expression and production of VP2 protein in A549 cells. We also described the formation of CLPs in cells transfected with plasmid expressing the rVP2 protein.

Email: pourasgari@ibb.ut.ac.ir; fax: +9821-66404680.

Abbreviations: CLPs = core-like particles; EM = electron microscopy; rVP2 protein = recombinant VP2 protein; TEM = transmission EM; VLP(s) = virus-like particle(s)

Materials and Methods

Virus. Human rotavirus A, strain G3 was obtained from the stool of children suffering of diarrhea. The presence of rotavirus in stool specimens was confirmed by the standard assays (Department of Virology, Pasteur Institute of Iran).

Cells. The isolated virus was multiplied in confluent MA104 cells (fetal rhesus monkey kidney cells) maintained in DMEM without serum and with 5 μg/ml trypsin (Conner *et al*., 1988; Mohan *et al*., 2003). Human lung epithelial carcinoma (A549) cells were grown in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and used for the expression of *VP2* gene. Transfection experiments were carried out with the cells maintained in DMEM.

Plasmids construction. The virus was grown in MA104 cells as previously described (Ahmadian and Shahrabadi, 1999). The *VP2* cDNA was prepared by the RT-PCR procedure. The primers were designed according to the genomic 3' and 5' flanking sequences of the RNA segment 2 of Simian rotavirus A/SA11. Oligonucleotide sequences included the restriction endonucleases sites *Bam*HI and *Xba*I to facilitate the cloning procedure. The primer sequences were 5'-AAAGGATCCATGGCGTATCGAAAACGT-3' (forward) and 5'-GGTTCTAGATTACAGTTCGTTCATGAT-3' (reverse). Underlined areas indicate the *Bam*HI and *Xba*I sites, respectively. *VP2* cDNA was amplified by High Fidelity PCR System (Roche) and the 2,700 bp fragment was cloned into the polylinker of pTZ57R/T cloning vector using InsT/Aclone™ PCR Product Cloning Kit (Fermentas). The target cDNA was subcloned into the polylinker of pcDNA3.1(+) (Invitrogen Life Technologies), downstream of the CMV immediate/early promoter using the primers restriction sites. The *VP2* gene in TA cloning and in the eukaryotic expression vectors was sequenced in both directions by M13 forward/reverse and Sp6/T7 primers, respectively. *VP2* gene nucleotide sequence is registered in GenBank database, accession no. DQ480724.

Transfection of A549 cells. The A549 cell line was transfected with recombinant plasmid pcDNA3-VP2 and with empty plasmid pcDNA3.1(+) as a control using lipofectamine reagent (Invitrogen Life Technologies).

Western blotting analysis was used for the detection of expressed VP2. In brief, 60 hrs after transfection, the A549 cells were treated by the lysis buffer (PBS pH 7.2 containing 1 mmol/l DTT, 1 mmol/l EDTA, 10 μg/ml PMSF, and 1 mol/l sucrose). Proteins in the cell lysates and the purified rotavirus preparations were separated on SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane. The rabbit antiserum against the Human rotavirus A/G3 produced in our laboratory was used as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG was used as the secondary antibody (Sigma).

Transmission electron microscopy (TEM). The A549 cells were prepared for TEM according to the standard procedure (Ahmadian and Shahrabadi, 1999). In brief, 48–72 hrs after transfection, the cells were fixed in glutaraldehyde and osmium tetroxide. After embedding in the agarose, the preparates were dehydrated in ethanol**,** embedded in epoxy resin and cured at 60şC. Ultrathin sections were stained with uranyl acetate and lead citrate. The formation of CLPs was detected by the Zeiss 902A transmission electron microscope operating at 80 KV.

Results

Expression of rVP2 protein in A549 cells

Western blotting of the cell lysate of A549 cells transfected with a recombinant plasmid pcDNA3-VP2 showed VP2 protein band identical with VP2 band present in the purified rotavirus preparation and in the cell lysate prepared from rotavirus-infected cells used as positive controls. The bands were visualized with rabbit antiserum against rotavirus (Fig. 1). No bands were detected in the lysates of mock-transfected cells or cells transfected with empty pcDNA3 used as negative controls. Both the purified rotavirus and the cell lysate from the A549 cells infected with rotavirus showed a strong VP2 band.

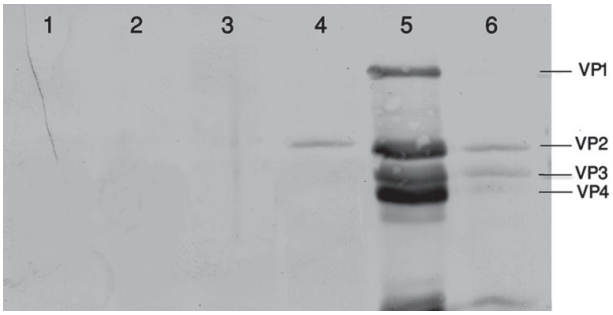

Fig. 1

Western blot analysis of the expression rVP2 in A549 cells

Lysates of cells non-transfected (1), mock-transfected (2), transfected with pcDNA3.1 (3), transfected with pcDNA3-VP2 (4), and infected with rotavirus (6). Purified rotavirus (5).

Formation of CLPs in A549 cells expressing rVP2 protein

Lipofectamine-mediated transfection of the A549 cells with pcDNA3-VP2 resulted in the formation of the particles structurally similar to the single shelled virions. These CLPs were detected by EM. The size of the particles was about 28–35 nm in positive staining (Fig. 2).

Discussion

Since the rotaviral disease results in a high rate of mortality in developing countries and is due in part to the absence of efficient antirotavirus drugs, various efforts have been made to design and develop a vaccine to prevent the disease (Coste *et al*., 2000). Among the various vaccine

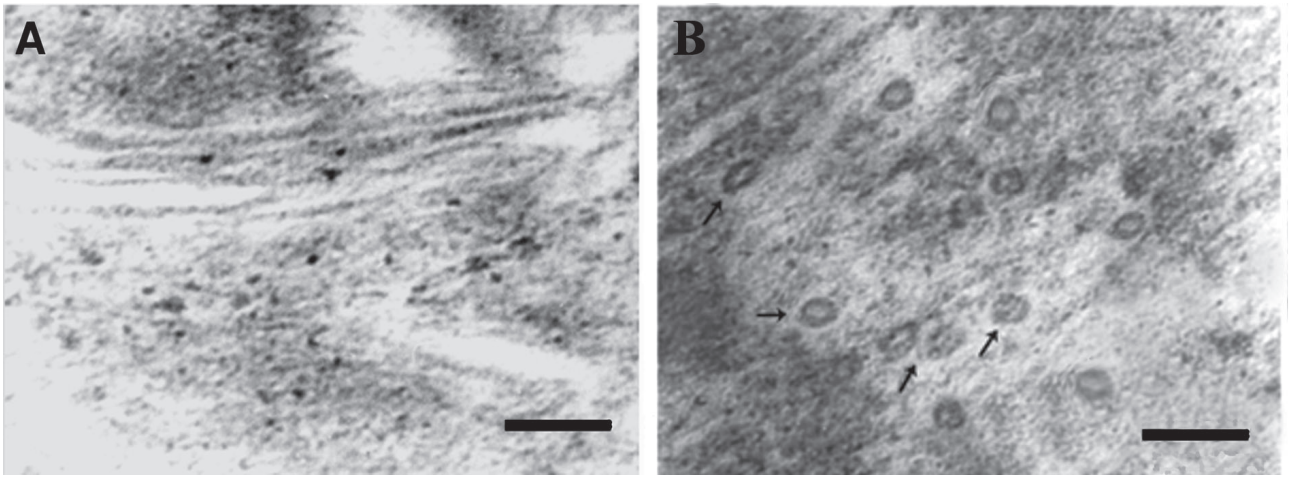

Fig. 2

EM of A549 cells transfected with pcDNA3.1 (A) and pcDNA3.1-VP2 (B) for 60 hrs

Arrows show CLPs. Bar = 100 nm.

candidates, the subunit vaccine is regarded as the most promising in vaccine development. Therefore, it is of value to characterize the expression of the recombinant proteins of rotaviruses in suitable host cells. The present study is aimed on the gene encoding the VP2 protein, an essential component of rotavirus inner capsid layer.

Previous work has shown that the expression of rotaviral *VP2* and *VP6* genes in *Salmonella typhimurium* produced inclusion bodies, which aggregated in bacteria without the VLP assembly. This is probably due to either an inappropriate ratio of VP2 and VP6 proteins compared to those found in VLPs or due to the overexpression (Coste *et al*., 2001). In addition, it was shown that correct folding of VP2 proteins did not occur in *S. typhimurium*. In insect cells, the expression of rotavirus VP2 protein produced empty CLPs and the absence of the monomeric form of VP2 indicates that this protein was rapidly and efficiently assembled into the particles (Gallegos and Patton, 1989). These results indicated that VP2 alone provided the structural integrity of the core particles and therefore this protein was capable of protein-protein interactions. Consequently, the formation of core particles did not require interactions with other viral components.

In spite of the high level of expression in insect cells, there are some limitations in this system, such as the inappropriate post-translational modifications of the expressed proteins. There is only a simple N-linked glycosylation without any modification with sialic acid residues. However, mammalian cells, particularly lung cells do not belong to the expression systems producing high levels of proteins. In the present study, only a moderate level of CLPs was detected. The transfected lung cells that expressed rVP2 proteins showed a serological reactivity similar to that of the viral VP2, because they were able to react with rabbit antiserum to the rotavirus. On the other hand, the moderate level of VP2 expression in A549 cells might be due to transient transfection and expression. Probably, the stable transfection would show a high level of expression, what makes this mammalian cell line more suitable system for efficient production and purification of rVP2. It is noteworthy that we observed the CLPs directly in the intact cells, what was the finding not reported previously. Other researchers reported the presence of VP2 in the insect cells only after purification of the protein (Labbe *et al*., 1991).

It seemed that all types of post-translational modifications took place within the A549 cells. The EM studies showed that the synthesized rVP2 proteins were formed as CLPs that were morphologically similar to the rVP2 containing CLPs formed in the insect cells (Labbe *et al*., 1991). Moreover, they were similar in size to the authentic viral CLPs (about 30 nm).

It has been shown that lung epithelial cells can act as antigen-presenting cells (Eriksson and Holmgren, 2002; Ogra *et al*., 2001; Wu *et al*., 2001). Therefore, the expression of *VP2* in the lung cells creates a possibility for processing and presenting of VP2 epitopes in combination with MHC class I or class II. In addition, the moderate level of expression of *VP2* gene in the lung cells provided the sufficient material for functional analysis and especially for studying the interactions of VP2 protein with nucleic acids or with other rotavirus core proteins expressed in the same

system. In addition, it will be of interest to determine the function of CLPs as precursors for the encapsidation of viral RNA (Gottlieb *et al*., 1990).

Acknowledgements. This work was partially supported by the research grant 6401007/06/02 from the Institute of Biochemistry and Biophysics, University of Tehran. We feel indebted to the National Institute for Genetic Engineering and Biotechnology, Tehran, Iran for providing the facilities. We would like to thank Dr. S. Modarres for provision and testing of the stool samples and Ms. M. Shafiezadeh for help in EM studies. We also are very grateful to Dr. G. Sing for his critical review.

References

Adler M, Schulz S, Fischer R, Niemeyer CM (2005): Detection of rotavirus from stool samples using a standardized immuno-PCR ("Imperacer") method with end point and real-time detection. *Biochem. Biophys. Res. Commun*. **333**, 1289–1294.

Ahmadian S, Shahrabadi MS (1999): Morphological study of the role of calcium in the assembly of the rotavirus outer capsid protein VP7. *Biotech. Histochem*. **74**, 266–273.

Conner ME, Estes MK, Graham DY (1988): Rabbit model of rotavirus infection. *J. Virol.* **62**, 1625–1633.

Coste A, Cohen J, Reinhardt M, Kraehenbuhl JP, Sirard JC (2001): Nasal immunization with Sallmonella typhimurium producing rotavirus VP2 and VP6 antigens stimulates specific antibody response in serum and milk but fails to protect offspring. *Vaccine* **19**, 4167–4174.

Coste A, Sirard JC, Johansen K, Cohen J, Kraehenbuhl JP (2000): Nasal immunization of mice with virus-like particles protects offspring against rotavirus diarrhea. *J. Virol.* **74**, 8966–8971.

Crawford SE, Labbe M, Cohen J, Burroughs MH, Zhou YJ, Estes MK (1994): Characterization of virus-like particles produced by the expression of rotavirus capsid proteins in insect cells. *J. Virol*. **68**, 5945–5952.

Eriksson K, Holmgren J (2002): Recent advances in mucosal vaccines and adjuvants. *Curr. Opin. Immunol*. **14**, 666–672.

Ernst H, Duhl JA (1989): Nucleotide sequence of genomic segment 2 of the human rotavirus Wa. *Nucleic Acids Res*. **17**, 4382.

Estes MK, Cohen J (1989): Rotavirus gene structure and function. *Microbiol. Rev*. **53**, 410–449.

Gallegos CO, Patton JT (1989): Characterization of rotavirus replication intermediates: A model for the assembly of single-shelled particles. *Virology* **172**, 616–627.

Gottlieb P, Strassman J, Qiao X, Frucht A, Mindich L (1990): In vitro replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage phi 6: studies with procapsids assembeled from plasmid-encoded proteins. *J. Bacteriol*. **172**, 5774–5782.

Kapikian AZ, Hoshino Y, Chanock RM (2001): Rotaviruses. In Knipe D, Howley M (Eds): *Fields Virology*. Vol. 14, Lippincott. Williams & Wilkins, Philadelphia, PA, pp. 1787–1833.

Kumar A, Charpilienne A, Cohen J (1989): Nucleotide sequence of the gene encoding for the RNA binding protein (VP2) of RF bovine rotavirus. *Nucleic Acids Res*. **17**, 21–26.

Labbe M, Charpilienne A, Crawford SE, Estes MK, Cohen J (1991): Expression of rotavirus VP2 produces empty core-like particles. *J. Virol.* **65**, 2946–2952.

Mitchell DB, Both GW (1990): Completion of the genomic sequence of the simian rotavirus SA11: Nucleotide sequences of segment 1, 2, and 3. *Virology* **177**, 324–331.

Mohan KVK, Kulkarni S, Glass RI, ZhiSheng B, Atreya CD (2003): A human vaccine strain of Lamb rotavirus (Chinese) NSP4 gene: Complete nucleotide sequence and phylogenetic analyses. *Virus Genes* **26**, 185–192.

Ogra PL, Faden H, Welliver RC (2001): Vaccination strategies for mucosal immune responses. *Clin. Microbiol. Rev*. **14**, 430–445.

Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI (2003): Global illness and deaths caused by rotavirus disease in children. *Emerg. Infect. Dis*. **9**, 565–572.

Simpson R, Aliya S, Iturriza-Gomara M, Desselberger U, Gray J (2003): Infantile viral gastroenteritis: On the way to closing the diagnostic gap. *J. Med. Virol.* **70**, 258–262.

Varghese V, Ghosh S, Das S, Bhattacharya SK, Krishnan T, Karmakar P, Kobayashi N, Naik TN (2006): Characterization of *VP1*, *VP2* and *VP3* gene segments of a human rotavirus closely related to porcine strains. *Virus Genes* **32**, 241–247.

Wilhelm I, Roman E, Sánchez-Fauquier A (2003): Viruses causing gastroenteritis. *Clin. Microbiol. Infect.* **9**, 247–262.

Wu Y, Wang X, Csencsits KL, Haddad A, Walters N, Pascal DW (2001): M cell-targeted DNA vaccination. *Proc. Natl. Acad. Sci. USA* **98**, 9318–9323.

Zeng CQ, Estes MK, Chrpilienne A, Cohen J (1998): The N terminus of rotavirus VP2 is necessary for encapsidation of VP1 and VP3. *J. Virol.* **72**, 201–208.