Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 53 (2007) 209–215
Protein Expression & Purification
www.elsevier.com/locate/yprep

Production of polyclonal antibodies against the human respiratory syncytial virus nucleoprotein and phosphoprotein expressed in *Escherichia coli*

Fernando M. Simabuco, Cassiano Carromeu, Luis E. Farinha-Arcieri, Rodrigo E. Tamura, Armando M. Ventura *

Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenida Professor Lineu Prestes, 1374, São Paulo-SP 05508-900, Brazil

Received 13 November 2006, and in revised form 20 December 2006
Available online 27 December 2006

Abstract

The nucleoprotein (N) and the phosphoprotein (P) of the human respiratory syncytial virus (HRSV), A2 strain, were cloned into pMAL-c2e vector. The proteins were expressed fused with the maltose-binding protein (MBP) and were preferentially found in the soluble fraction of the bacterial lysate. After their purification using amylose resin, almost no other protein was detected in SDS–PAGE. The fused proteins were cleaved by digestion with enterokinase and then used as antigens for BALB/c mice immunization. The obtained polyclonal antibodies were tested against HRSV infected cells in immunofluorescence assays. The results indicate that the antibodies generated against the recombinant proteins were able to recognize the virus proteins. We now intend to purify the cleaved N and P proteins and use them in structural studies. The recombinant proteins will also be tested as potential inducers of a protective immunity against the HRSV.

Keywords: Human respiratory syncytial virus; Nucleoprotein; Phosphoprotein; Maltose-binding protein; Antibody production; Immunofluorescence

Human respiratory syncytial virus (HRSV)[1], a member of Paramyxoviridae family, is an enveloped virus, containing a single stranded RNA molecule with negative polarity [1]. HRSV infects the upper tract of the respiratory system, and is responsible for the majority of hospitalizations by viral respiratory disease in infants and neonates worldwide, causing serious problems such as pneumonia and bronchiolitis [2]. HRSV is also an important pathogen for elders [3] and immunocompromised patients, such as bone marrow transplant patients [4].

HRSV genome has about 15,000 nucleotides and contains 10 genes encoding 11 proteins [1]. Studies with other viruses of the same family revealed some important functions of the nucleoprotein (N) and the phosphoprotein (P). The nucleoprotein is responsible for forming the nucleocapsid structure, associating with the RNA molecule in a helical form resistant to RNases [5]. The phosphoprotein interacts with the N protein and acts like a chaperone, allowing specificity to viral RNA for encapsidation [6,7]. Other function of P protein is the interaction with L protein (the major unit of the virus replication complex), conferring stability and the correct positioning in the ribonucleo-complex for RNA synthesis [8,9]. The P protein has some residues that are phosphorilated, but they are not essential for viral transcription and replication [10].

The first clinical trials of a vaccine against HRSV, which consisted of formalin inactivated viral particles, were able

* Corresponding author. Fax: +55 11 3091 7354.
E-mail address: amventur@icb.usp.br (A.M. Ventura).

[1] *Abbreviations used:* N, nucleoprotein; P, phosphoprotein, HRSV, human respiratory syncytial virus; MBP, maltose-binding protein; BSA, bovine serum albumin; PEG, polyethylene glycol; RT-PCR, reverse transcription and polymerase chain reaction.

1046-5928/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.12.016

to generate specific neutralizing antibodies, but failed to protect the patients against the virus [11,12]. Moreover, an exacerbated disease was observed, and since then there is a search for an effective vaccine [13].

No structural studies of HRSV N protein have been reported. For HRSV P protein, a trypsin-resistant fragment has been characterized as containing an oligomerization domain that ranges from amino acids 104 to 163 [14]. New structural studies should provide a better characterization of these proteins.

In this report we describe the cloning and expression of the N and P proteins of HRSV into pMAL-c2e, a bacterial expression vector. Both MBP fused proteins were found in the soluble fraction of the bacterial lysate, allowing their direct purification using amylose resin. The results showed a considerable level of purity of the fused proteins, which were digested with enterokinase to cleave the recombinant proteins from MBP. The digestion of MBP-N revealed two sharp bands. However, the digestion of MBP-P showed some degradation of the P protein that increased with the time of digestion. The possible causes and implications of this degradation to our study will be discussed. Finally, BALB/c mice were immunized with the digested proteins and their sera were tested against HRSV infected cells. An immunofluorescence assay revealed that the produced polyclonal antibodies specifically recognized HRSV antigens in infected cells.

Materials and methods

Cloning of the N and P genes

The HRSV A2 strain N and P genes (GenBank Accession Nos. AAC14896 and AAC14897, respectively) were amplified by reverse transcription and polymerase chain reaction (RT-PCR). Briefly, HEp-2 cells were infected with HRSV strain A2 and RNA was extracted using Trizol (Invitrogen). cDNA was generated using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Primers were designed for in frame subcloning into pMAL-c2e. The primer sequences are shown below with the incorporated restriction sites (underlined and indicated in parenthesis).

- Ns: 5′ GG GTA CCG ATG GCT CTT AGC AAA GTC 3′ (*Kpn*I)
- Nas: 5′ GG ACG TCA TTA AAG CTC TAC ATC ATT 3′ (*Pst*I)
- Ps: 5′ GG GTA CCG ATG GAA AAG TTT GCT CCT 3′ (*Kpn*I)
- Pas: 5′ GT TCG AAA TTA GAA ATC TTC AAG TGA 3′ (*Hin*dIII)

Following polymerase chain reaction (PCR) amplification, the products were cloned into the pCR-4-TOPO (Invitrogen), using *Escherichia coli* (DH5α strain). DNA sequencing was made using Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Finally, N and P genes were excised from pCR-4-TOPO and subcloned into pMAL-c2e vector, generating pMAL-N and pMAL-P, respectively.

Expressing MBP-N and MBP-P proteins

Escherichia coli strain BL21 (DE3) pLysS cells were transformed with the pMAL-N and pMAL-P and grown in LB medium supplied with ampicillin (100 μg/mL). When the OD_{600} reached an approximated value of 0.5, IPTG (isopropyl-β-D-thiogalactopyranoside), purchased from Invitrogen, was added at a final concentration of 0.3 mM and the cultures incubated overnight at room temperature. Cells were then harvested by centrifugation at 4000*g* for 20 min and the pellets resuspended in 10 mL of Column Buffer (20 mM Tris–HCl, pH 7.4; 200 mM NaCl and 1 mM EDTA). Cells were lysed by one cycle of freezing and thawing and sonicated for 2 min with pulses of 15 s and pauses of 5 s. The samples were centrifuged at 9000*g* for 30 min and both the supernatant (soluble fraction) and the pellet (insoluble fraction) were analyzed by SDS–PAGE.

MBP-N and MBP-P purification

The amylose resin (NEB) columns were mounted in 10 mL syringes with the bottom plugged with glass wool and washed 8 times with Column Buffer. The soluble fractions previously obtained were diluted 1:5 in Column Buffer and loaded into the columns at a flow rate of 375 μL per min. The columns were washed 12 times with Column Buffer and the proteins eluted with Column Buffer containing 10 mM maltose in 13 fractions of 1 mL each.

The amounts of purified MBP-N and MBP-P were determined by Bradford assay (Bio-Rad) and the purities were estimated by bands intensity in SDS–PAGE using the software ImageJ (National Institutes of Health).

MBP-N and MBP-P enterokinase digestion

Enterokinase (NEB) was added at a final concentration of 0.001% (w/w) and reactions incubated at room temperature. Samples were collected at 2, 4, 6, and 20 h for analysis by SDS–PAGE.

Western blot

After SDS–PAGE, proteins were transferred to a Hybond-ECL Nitrocellulose membrane (Amersham Biosciences). The membrane was incubated with blocking buffer (0.1% Tween and 5% non-fat dry milk in phosphate-buffered saline, PBS) overnight at 4 °C and then with the anti-P monoclonal antibody C771, kindly provided by Dr. Erling Norrby [15], diluted 1:2000. The membrane was washed 3 times with washing solution (Tween 0.1% in PBS) and incubated with an anti-mouse peroxidase-conjugated antibody (KPL), diluted 1:1000. After 3 washes with washing solution the membrane was treated with SuperSignal West Pico Chemiluminescent