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Original Research Article

Glycosylation studies of G protein of group B human respiratory syncytial virus (hRSV) in eukaryotic system

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Keywords

hRSV, cloning, G protein, eukaryotic expression, purification, glycosylation study

A B S T R A C T

Human respiratory syncytial virus (hRSV) is the most common viral agent of acute respiratory tract infection (ARI). The G protein (glycoprotein) of hRSV helps in attachment of virion to the host cell. The G protein (90-120 kDa) has a protein backbone of about 32.6 kDa which is extensively modified by the addition of N- and O-linked oligosaccharides. The full length sequence of prototype group B strain and BA laboratory strain was optimized and synthesized by commercial means and inserted in pUC 57 vector. The ectodomain of the synthetic G protein gene was cloned in pGEMT-easy vector and subcloned into pcDNA 3.1 eukaryotic expression vector. The protein was expressed in Hep-2 cells and purified from the media of transfected cells by Ni-NTA column. The protein was further subjected to enzyme (tunicamycin and glycosidase F) based deglycosylation to investigate the N- and O-linked glycosylation sites. Our result indicates that the ectodomain G protein of hRSV has both N-linked and O-linked glycosylation with O-linked sugars contributing to the major part of the glycosylation. Further studies with metabolic inhibitors of glycosylation process are needed to elucidate the detailed mechanism and sites of glycosylation of G protein of hRSV.

Introduction

Human respiratory syncytial virus (hRSV) is a leading cause of broncholitis and pneumonia in infants and immunocompromised patients in both developed and developing countries. The G protein (glycoprotein) helps in attachment of virion to the host cell. The G protein (90-120 kDa) has a protein backbone of about 32.6 kDa (923 bp) for A2 strain (Satake et al., 1985, Wertz et al., 1985). The G protein of long strain of hRSV is a transmembrane glycoprotein with three functional domains: cytoplasmic domain (amino acid residue 1-37), transmembrane region (amino acid

residue 38-66) and ectodomain region (amino acid residue 67-298. The G protein contains both N- and O-linked oligosaccharides. With the addition of N-linked carbohydrates during synthesis, O-linked sugars are added in the golgi apparatus, and G protein is then transported to the cell surface (Wertz et al., 1989). There are two forms of the G protein: Gs (soluble G protein) and Gm (membrane anchored G protein), Gs represent the ectodomain of Gm, which is heavily glycosylated by the addition of N- and O-linked oligosaccharides in both form of the G protein. The G glycoprotein is highly glycosylated but still the role of sugars in immunogenicity and antigenicity is not defined. Studies have suggested that sugars are essential for infectivity and antigenicity of the G protein. A new group B genotype (BA viruses) was identified in Argentina in 1999 (Trento et al., 2003). The BA viruses illustrate a drastic change of 60 bp duplication in the second hypervariable region of G protein gene as compared to sequence of G protein gene of hRSV group B prototype strain (18537). Later this genotype was reported from different parts of the world, including Japan (Kuroiwa et al., 2005, Nagai et al., 2004, Sato et al., 2005), Kenya (Scott et al., 2006, Scott et al., 2004); Belgium; (Zlateva et al., 2005) , Canada, Brazil, the United Kingdom, the United States (Trento et al., 2010, Trento et al., 2006) and India (Parveen et al., 2006).

Limited information is available on cloning and expression of G protein gene of group B hRSV. However, Ryder and colleagues expressed the ectodomain of hMPV G protein using the codon optimized synthetic DNA in HEK-293 suspension cells (Ryder et al., 2010). There is no data on cloning, expression and glycosylation study of G protein of group B hRSV. Therefore, this study was planned to clone, express and purify the G protein of hRSV from eukaryotic system. The purified G protein was further subjected to the glycosylation studies.

Materials and Methods

The pcDNA 3.1 vector was used as expression vector and Hep-2 cells were used as expression host. Two G gene sequences for the prototype group B hRSV (accession number M17213) and BA laboratory strain (accession number KJ690610) were optimized and synthesized by commercial means (Bio Basic Inc., U.S.A). The accession numbers of the optimized synthetic sequence of the prototype group B hRSV (18537) and BA laboratory strain are KM373206 and KJ690590 respectively.

Cloning of ectodomain G protein gene of group B hRSV

The forward primer 5'*GCGGCCGC catggcaatgCACAAAGTGACCCTGACCAC*3' with Not 1 site and reverse primer 5'*cggtGGATCC*GAGTTGGGATT CAGGG TGGA3' with Bam H1 site were used for the amplification of the ectodomain G gene sequence of the prototype group B hRSV. The forward primer 5'*GCGGCCGCc atggcaatgCACAAAGTGACCCTGACCAC*3' with Not 1 and reverse primer 5'-*ttgagttgatt*GGATCCGAGGTGCTGTTGC T-3' with Bam H1 site were used for the amplification of the ectodomain G gene sequence of the BA strain of hRSV using synthetic sequences inserted in pUC 57 vector. The restriction sites are underlined in primer sequences and the extra sequences are in small italics. PCR was standardized using the following final reaction concentrations as suggested by manufacturer (Invitrogen Life Technologies, Carlsbad CA, USA Inc.), 1X pfx amplification buffer, 0.3 mM dNTPs, 1mM $MgSO_4$, 10 pmol of each forward and reverse primers, platinum Pfx

DNA polymerase 0.5 unit and template cDNA 2.0 µl in a 25 µl reaction. Optimized PCR cycling conditions were as follows: initial 5 min denaturation at 94°C, followed by 35 cycle of 1 min denaturation at 94°C, 1 min annealing at 59°C, 1 min extension at 72 °C and final extention was put up at 72 °C for 10 min. The amplified fragments of desired size were gel extracted and proceeded for TA cloning. A 10 µl reaction was put up using 5 U of Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad CA, USA Inc., USA); 1X PCR buffer; 1.5 mM $MgCl_2$; 0.2 mM dATP (Promega Corp., Madison, WI, USA); and 1-1.5 µg of the purified DNA insert. The reaction mixture was incubated at 72°C for 30 minutes. Ligation reaction was set up using the pGEM®T Easy vector kit (Promega Corp., Madison, WI, USA). A 10 µl reaction was put up using 50 ng (1 µl) of the pGEM®T Easy vector, A-tailed insert, 1X ligation buffer and 3 Weiss units (1 µl) of T4 DNA ligase. The reaction mixture was incubated at 4°C overnight. The ligation mixture was transformed into DH5α cells. The plasmid was isolated from the positive colonies and inserts were released by using their respective restriction enzymes. The gene was subcloned into pcDNA3.1, eukaryotic expression vector with C-terminal six histidine tag. The expression clones were confirmed by colony PCR, restriction digestion and sequencing method.

Expression and purification of ectodomain G protein of group B hRSV

Expression of GΔTM in mammalian cells was carried out in Hep-2 cells obtained from cell repository, National Centre for Cell Science (NCCS), Pune, India. Hep-2 cells were cultured in 75 cm² cell culture flask (Corning Inc., USA) in DMEM media (Gibco, life technologies, USA) with 10 % Fetal Calf Serum (FCS), 4mM glutamine, 1X penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and pH 7.2 in a humidified 5 % CO2 incubator at 37 °C. The GΔTM expression construct of the prototype and BA strain was transfected into adherent Hep-2 cells for protein production using lipofectamine 2000 (Invitrogen Inc., USA) according to manufacturer's protocol. Media was centrifuged for 5 min at 100×g at room temperature to remove debris. The supernatant was harvested and filtered through 0.2 µm filters. The his-tagged ectodomain G protein (GΔTM) was purified by immobilized metal ion affinity chromatography using pre-packed HisTrap Ni-NTA columns. The protein was loaded in a 5 ml His-Trap column and the binding buffer contained 20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole (pH 7.4). Unrelated proteins were washed out with four column volumes of 8 % elution buffer, and the his-tagged G protein was eluted with four column volumes of 25 % elution buffer containing 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole (pH 7.4). The purified protein was concentrated and dialyzed against PBS through amicon ultra centrifugal filters with 30,000 and 100,000 MWCO.

Expression of GΔTM of prototype group B hRSV and BA strain and cleavage through tunicamycin and O-glycosidase F

The pcDNA 3.1- GΔTM of group B prototype and BA strain recombinant plasmid was transfected to adherent Hep-2 cells. Briefly, $1x10^8$ number of cells and 32 µg plasmid were used for each transfection. Four days post-transfection, supernatant was taken and centrifuged for 5 min at 500 x g at room temperature. Supernatant containing the expressed, soluble proteins was removed for purification. To determine the N-glycan and O-glycan the purified proteins were treated with tunicamycin (Fluka, USA) and O-glycosidase F (calbiochem, USA). The 50

µg of purified, protein of prototype group B hRSV were heat denatured at the 95 ^{o}C for 10 min in denaturation buffer. After cooling, 2.5 µl of NP-40 and 5 µg of tunicamycin were added and in another fraction of the protein, amount of O-glycosidase F (10 µg and 15 µg) were added and the proteins were digested at 37 ^{o}C overnight. Similarly, seventy five microgram (75 µg) of purified concentrated protein of BA strain was heat denatured at 95 ^{o}C for 5 min in denaturation buffer. After cooling 2.5 µl of NP-40 and in different fractions of protein, 5 and 10 µg of O-glycosidase F and 5 µg of tunicamycin were added and the proteins were digested at 37 ^{o}C overnight. The digested protein was analyzed by the western blotting.

Polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze the digested protein. The 12 % resolving gel was allowed to polymerize. The samples were mixed by vortexing, boiled at 100 °C for 10 minutes, spun down at 9000 x g for 3 min and the supernatant was loaded in the wells. The gel intended for western blot was fixed in transfer buffer and transferred to nitrocellulose membrane (MDI**,** membrane technologies, India) at a constant voltage of 100 V for 1 hr. The membrane was blocked in 5 % BSA (prepared in 1X PBS) at 4 ºC for overnight.

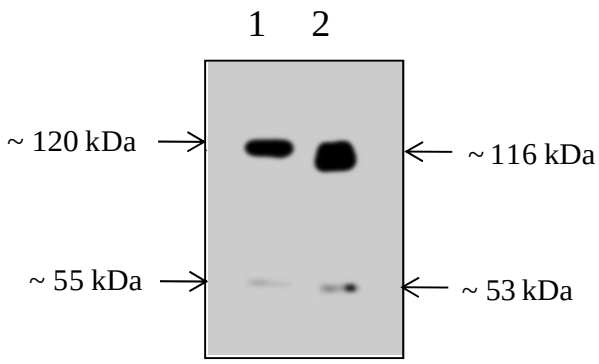
The membrane was then washed thrice with 1X PBS and incubated with 1:7000 dilution of the mouse anti-his monoclonal antibody (Invitrogen Inc., USA) for 2 hr at room temperature. Blots were then washed thrice with 1X PBS (pH 7.2) and incubated with 1:7000 dilution of the HRP-conjugated anti-mouse IgG antibody (Banglore Genei, India). The blot was developed with super signal west picochemiluminiscent substrate (Thermo Inc., USA).

Results and Discussion

Expression and purification of GΔTM of prototype group B hRSV and BA strain in eukaryotic expression system:

The GΔTM of prototype group B hRSV and BA strain was expressed in Hep-2 cells. The GΔTM was purified from the media of transfected cells using Ni-NTA column. In western blotting analysis two bands were observed (~116 kDa, ~53 kDa for the prototype strain and ~120 kDa, ~55 kDa for the BA strain) (Figure 1). The purified protein was used for the deglycosylation studies.

Figure.1 Expression of GΔTM in Hep-2 cells, Lane 1: GΔTM of BA strain of hRSV, Lane 2: GΔTM of prototype group B hRSV.

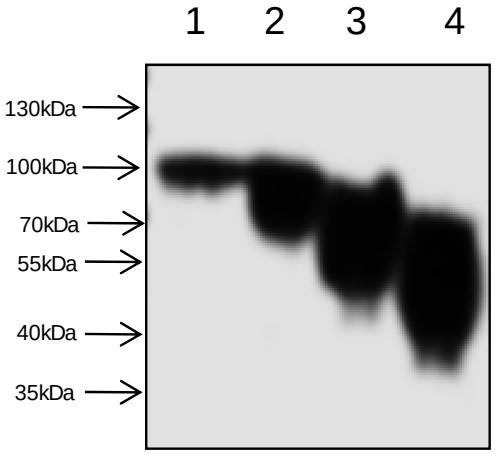


N and O-linked glycosylation pattern of GΔTM of prototype group B hRSV

The purified GΔTM of hRSV prototype strains expressed in mammalian cells was digested with tunicamycin and O-glycosidase F to study the glycosylation pattern of the N-linked and O-linked glycosylation sites respectively. The digested proteins were analyzed by western blotting. The ectodomain G protein of hRSV group B prototype showed a band around 100 KDa after western blotting. This protein

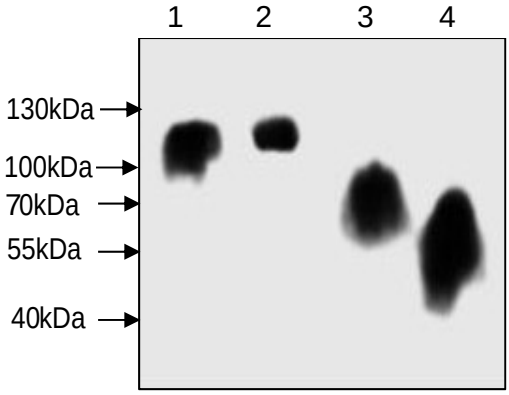
after tunicamycin treatment demonstrated a smear by western blotting with lower band of around 70 kDa. The protein on treatment with 5 and 10 µg of O-glycosidase F showed smear after western blotting with lower band of 45 kDa and 37 kDa respectively (Figure 2).

Figure.2 Glycosylation pattern of GΔTM of prototype group B hRSV treated with tunicamycin and O-glycosidase F. lane 1: Untreated, lane 2: 5 µg tunicamycin , lane 3: 5 µg O-glycosisase F, lane 4: 10 µg O-glycosidase F



N and O-linked glycosylation pattern of GΔTM of BA strain of hRSV: The purified ectodomain G protein of hRSV BA strain expressed in mammalian cells was digested with tunicamycin and O-glycosidase F to study the glycosylation pattern of the N-linked and O-linked glycosylation sites respectively. The digested proteins were analyzed by western blotting. The ectodomain G protein of hRSV BA strain showed a band around 120 kDa after western blotting. This protein after tunicamycin treatment demonstrated a smear by western blotting with lower band of around 80 kDa. The protein on treatment with 5 and 10 µg of O-glycosidase F showed smear after western blotting with lower band of 60 kDa and 39 kDa respectively (Figure 3).

Figure.3 Glycosylation pattern of GΔTM of BA strain of hRSV treated with tunicamycin and O-glycosidase F. lane 1: 5 µg tunicamycin, lane 2: Untreated, lane 3: 5µg O-glycosisase F, lane 4: 10 µg O-glycosidase F.



hRSV is one of the most commonly identified viral pathogen of ARI. RSV G protein is a transmembrane glycoprotein that helps in attachment of virion to the host cells. One of the striking features of G protein is the large number of serine and threonine residues (33-35 %) that are potential O-linked sugar acceptors (Wertz et al., 1985). Putative O-glycosylation sites are mainly concentrated at I^{st} and II^{nd} hyper variable regions of the G protein. The attachment G protein precursor is synthesized as 32 kDa polypeptide which is extensively modified by the addition of both N-linked and O-linked oligosaccharides (Wertz et al., 1985). High mannose N-linked carbohydrates side chains are added co-translationally to aspargine residues to yield intermediate species of 45-50 kDa (Wertz et al., 1985). This step is followed by conversion of the N-linked sugars to complex type and addition of O-linked oligosaccharides to serine and threonine

residues in the golgi compartment yielding a mature molecule of around 90-120 kDa, as estimated by SDS-PAGE. Less glycosylated forms of 45-80 kDa can also accumulate in substantial quantity.

The ectodomin G protein of prototype group B hRSV and BA strain was purified from Hep-2 cells. The purified protein showed two bands of different sizes on western blotting. The upper bright bands (~116 and ~120) are probably the mature glycosylated form of the G protein of prototype group B hRSV and BA strain respectively. The lower bands (~53 and ~55) are probably the partially glycosylated form of the G protein of prototype group B hRSV and BA strain respectively. The 60 bp duplication in the second hypervariable region of the G protein gene resulted in 20 amino acid duplication in BA viruses. These additional amino acids probably resulted in slight increase in molecular weight of the G protein of BA strain as compared to the prototype strain.

The enzyme based deglycosylation was used to investigate the N- and O- linked glycosylation sites of G protein. In the present study, tunicamycin and O-glycosidase F enzyme based deglycosylation of ectodomain G protein resulted in partial degradation of the G proteins that appeared as smears in western blotting. The findings of deglycosylation experiment demonstrated that N-linked glycosylation contributed modestly to the apparent molecular weight of ectodomain G protein by digestion with tunicamycin which removes sugar moieties at N-linked glycosylation sites. On the other hand O-linked glycosylation did contribute to the apparent molecular weight of ectodomain G protein as O-glycosidase F based deglycosylation remove sugar residue at O-linked glycosylation sites. In addition, this O-glycosidase F based enzymatic digestion resulted in observation of band around 37 kDa and 39 kDa for the prototype strain and BA strain respectively which are more close to the unglycosylated precursor of G protein (32 kDa). Thus we concluded that the ectodomain G protein of hRSV has both N- and O-linked glycosylation sites with O-linked sugars contributing to major part of the glycosylation. hRSV G protein is similar to the human metapneumovirus (hMPV) G protein. The G protein of hMPV is also transmembrane glycoprotein and is involved in the attachment of virion to the host cells just like G protein of hRSV. Ryder and colleagues showed that G protein expressed in hMPV infected cells was both N and O-linked glycosylated and similarly the sugars contribute to the majority of the G protein apparent molecular weight (Ryder et al., 2010). In depth analysis of the glycosylation process with different inhibitors is needed to explain the detailed mechanism of N- and O-linked glycosylation of G protein of hRSV.

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