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# Glycosylation is not necessary for recognition of the fusion glycoprotein domain of the Human respiratory syncytial virus by a polyclonal antibody

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**Summary. –** Human respiratory syncytial virus (HRSV) is a leading pathogen causing lower respiratory tract infections in infants and young children worldwide. In line with the development of an effective vaccine against HRSV, a domain of the fusion (F) glycoprotein of HRSV was produced and its immunogenicity and antigenic properties, namely the effect of deficient glycosylation was examined. A His-tagged recombinant F (rF) protein was expressed in *Escherichia coli*, solubilized with 8 mol/l urea, purified by the Ni-NTA affinity chromatography and used for the raising of a polyclonal antibody in rabbits. The non-glycosylated rF protein proved to be a strong immunogen that induced a polyclonal antibody that was able to recognize also the glycosylated $F_1$ subunit of native HRSV. The other way around, a polyclonal antibody prepared against the native HRSV was able to react with the rF protein. These results indicated that glycosylation was not necessary for the F domain aa 212–574 in order to be recognized by the specific polyclonal antibody.

**Keywords:** Human respiratory syncytial virus; fusion protein; $F_1$ subunit; recombinant protein; glycosylation; polyclonal antibody

## Introduction

HRSV (the genus *Pneumovirus*, the family *Paramyxoviridae*) is an enveloped, negative-strand RNA virus (Collins *et al.*, 2007). The HRSV genome is transcribed into 10 transcripts encoding 11 proteins. Three of them are F (fusion), G (attachment), and SH (small hydrophobic) protein that are glycosylated and expressed on the surface of virion and infected cells. The SH and G proteins are not required for virus replication *in vitro*, but the recombinant viruses lacking these genes are attenuated *in vivo* (Day *et al.,* 2006). An estimated 64 million HRSV infections occur annually resulting in 160,000 deaths around the world (Girard *et al.*, 2005). In the USA, HRSV also causes 73,400 to 126,300 hospitalizations per year in children under the age of 1 year (Shay *et al.*, 2001).

Many attempts to develop a safe and effective HRSV vaccine have failed (Olmsted *et al.,* 1986). Palivizumab is currently the only approved monoclonal antibody (MAb) for prophylaxis against HRSV, but it does not protect a small percentage of patients nor does it inhibit HRSV replication effectively in the upper respiratory tract (Wu *et al.*, 2007).

The HRSV F glycoprotein is an important target antigen for the virus-specific cytotoxic T lymphocytes (Johnstone *et al.*, 2004). Neutralizing and protective responses against the F glycoprotein are broadly cross-reactive with different virus strains of the two antigenic subgroups (A and B) (Arbiza *et al.*, 1992). In addition, the F glycoprotein shares 89% homology among the strains of the different subtypes indicating that this glycoprotein is highly conserved among

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**Abbreviations:** HRSV = Human respiratory syncytial virus; MAb = monoclonal antibody; Ni-NTA = nickel nitrilotriacetic; rF protein = recombinant F protein

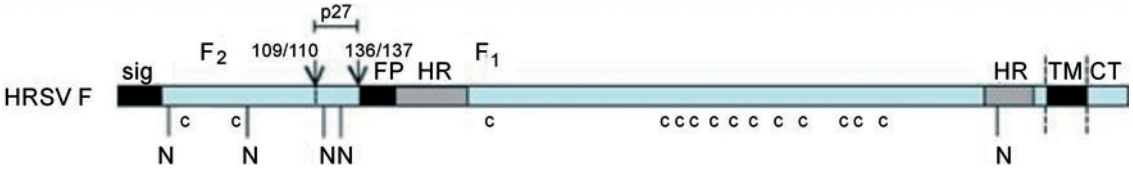


**Fig. 1**

**Primary structure of the HRSV F glycoprotein of strain A2**

Hydrophobic domains (black bars), signal peptide (sig.), fusion peptide (FP), transmembrane anchor (TM), and cytoplasmic tail (CT). Heptad repeat (HR, gray bars), cysteine residues conserved between the HRSV F proteins (c). Cleavage at the two sites at $F_0$ releases p27. Downward-facing arrows indicate the cleavage activation sites in F protein. Adapted from Collins *et al.* (2007).

the antigenic groups (Garcia-Barreno *et al.*, 1989). Thus, the F glycoprotein makes a good subunit vaccine candidate as it is more relevant for development of a vaccine that can confer adequate protection.

The F glycoprotein mediates the fusion of virus and cell membrane through syncytium formation during the infection process (Wu *et al.*, 2007). Synthesis of the F glycoprotein begins when an inactive precursor $F_0$ (574 aa) undergoes processing at the cleavage sites (aa 109 and 136), generating two biologically active, disulfide-linked polypeptide subunits, $F_1$ and $F_2$ (48 and 20 K, respectively) (Olmsted *et al.*, 1986; Garcia-Barreno *et al.*, 1989). The highly conserved hydrophobic N-terminus or fusion peptide of the $F_0$ protein acts as a cleaved signal peptide for the virus fusion (Fig. 1) (Feldman *et al.*, 2000). The $F_2$ subunit begins from the end of the signal peptide sequence up until a hydrophilic region consisting of six consecutive basic amino acids, which is the cleavage (aa 131–136) activation site (Fig. 1). The N-terminus of $F_1$ that begins at aa 137 contains a long stretch of hydrophobic amino acids (FP) and represents the domain responsible for the membrane fusion activity of F glycoprotein similar to the other paramyxoviruses. The transmembrane anchor region consists of 26 hydrophobic aa found near the C-terminus of $F_1$ (TM) (Anderson *et al.*, 1992). There are 4 potential N-linked glycosylation sites (N) on the $F_2$ subunit and one on the $F_1$ subunit (Fig. 1).

Several epitopes on the primary structure of the F glycoprotein has been identified (Gallardo *et al.,* 1991) especially in the $F_1$ subunit. This was confirmed when one of the neutralization epitopes on the $F_1$ subunit (aa 221 to 236) was localized (Trudel *et al.,* 1987). On the other hand, aa 190–289 have been reported to be involved in the neutralization process and provide partial protection in lungs of HRSV-infected mice (Werle *et al.,* 1998). Work has also been done on the truncated form of the F glycoprotein lacking the C-terminal 50 aa, by probing it with an antiserum raised against a synthetic peptide aa 255–275 bound to keyhole limpet hemocyanin (KLH) (anti-F255–275) (González-Reyes *et al.*, 2001). Finally, a new epitope positioned at aa 249–258 was identified (Johnstone *et al.*, 2004).

In addition, some studies identified synthetic peptides 10–20 aa long to be suitable for immunoprophylaxis (Barbas *et al.*, 1992). However, the passive immune response required longer-length molecules for lower degradability, because of the need of immunoglobulins that would circulate in the host as long as possible. Besides being more effective in therapy, a significant decrease in the protein dosage could be achieved to realize antibody-mediated administration (Barbas *et al.*, 1992). The HRSV F gene with $CTA_2B$ (a detoxified subunit of cholera toxin) has been proven to be successfully expressed in a bacterial system (Singh *et al.*, 2007). When the chimeric antigen was used to immunize mice *via* the intranasal route, it induced a strong mucosal as well as systemic immune response. The N-terminal signal sequence of the expressed F protein was found to be toxic in what is due to its interaction with bacterial membranes (Martin-Gallardo *et al.*, 1991). Deletion of this region resulted in the restoration of normal growth, which explains the beginning of the chosen domain at aa 212.

Polyclonal antisera raised against the purified F protein, or immune sera raised against vaccinia virus recombinants expressing F protein neutralized HRSV in tissue culture (Olmsted *et al.*, 1986; Stott *et al.*, 1987; Walsh *et al.*, 1987). In addition, the immunized animals were protected against a challenge with live HRSV, because the neutralizing antibody responses eliminated HRSV-induced infection from the respiratory tract (Olmsted *et al.*, 1986). Previously, a formalin-inactivated HRSV vaccine stimulated an imbalanced immune response. A large proportion of the induced antibodies were directed against non-protective epitopes rather than functional antibodies (Murphy and Walsh, 1988). The uncleaved F protein (F313) was not recognized by a panel of F protein-specific MAbs in ELISA or indirect immunofluorescence assays (Anderson *et al.*, 1992). Therefore, to ensure that a large variety of antibodies are available, polyclonal antibodies have been chosen to be produced for this study.

The aim of this work was to express domain 212–574 aa of the F glycoprotein of HRSV in *E. coli* in a non-glycosylated form and to examine its immunogenic and antigenic proper-

ties. For this purpose an expression plasmid harboring the domain 212–574 aa was constructed and used for the production of the corresponding recombinant protein, named rF protein. The protein was solubilized, purified and used as immunogen in rabbits. The obtained polyclonal antibody was examined for its reactivity with the native $F_1$ subunit. In addition, the rF protein was examined for its reactivity against a polyclonal antibody to the native virus.

## Materials and Methods

*Virus and cells.* The HRSV strain A2 was purchased from American Type Culture Collection (ATCC No. VR-1540D). Stock virus was grown on 75% confluent Vero cells in RPMI 1640 medium, containing 10% heat-inactivated FCS (both PAA). Cells and media were harvested when 75% CPE was observed.

*Cultivation of E. coli in LB broth. E. coli* was grown in 3 ml of LB broth supplemented with 50 µg/ml ampicilin (when necessary) at 37°C and 250 rpm agitation for 16 hrs. Induction of the culture with IPTG was carried out at the log phase at $OD_{600}$ ~0.6–0.8. The volumes of the cultures were scaled up to 1000 ml proportionately when larger amounts of the recombinant proteins were needed.

*Cloning and transformation.* Viral RNA was isolated using the ZR Viral RNA Kit (ZymoResearch). The F domain forward and reverse primers were 5'-acaaagatctctgcagcatatcaaatatagaaactgtg-3' and 5'-ctatccatggttagttactaaatgcaatattatttataccactcag-3', respectively. A combined reverse transcription and PCR was performed with the Access RT-PCR System (Promega) to synthesize and amplify the cDNA of the F domain (NC_001781.1) according to the manufacturer's instructions with the $T_A$ at 60°C. Five µl of the RT-PCR product was mixed with 1 µl of 6x loading dye (Fermentas) and resolved by electrophoresis in 1% (w/v) agarose gel using TAE buffer (40 mmol/l Tris-acetate, 1 mmol/l EDTA). The gels were stained with ethidium bromide (1 µg/ml) for 30 mins, analyzed and photographed with the Quantity One gel imaging system (Biorad). The PCR product and pRSetC plasmid (Invitrogen Life Technologies) were double digested with *Bgl*II and *Nco*I prior to the ligation. *E. coli* Top 10 cells (Invitrogen Life Technologies) were transformed with the ligation mixture. Then, the confirmed positive plasmids harboring the insert were again transformed into competent *E. coli* BL21 (DE3) cells (Invitrogen Life Technologies). The transformed *E. coli* BL21 (DE3) cells harboring the F domain in the pRSetC plasmid (Invitrogen Life Technologies) were designated as pRSetC(F).

*Expression in E.coli BL21 (DE3) cells.* A single colony of the recombinant *E. coli* BL21-DE3 cells carrying the pRSetC(F) plasmid was grown in 1 ml of LB broth containing 50 µg/ml ampicillin at 37°C with agitation at 250 pm overnight. The following day, 200 µl of the overnight culture was inoculated into 5 ml of fresh LB broth containing 50 µg/ml ampicillin. Time-course optimization for growth temperature, shaking speed and IPTG concentration were carried out. After determining the optimum conditions for protein expression, clarified *E. coli* lysates were prepared by harvesting the bacterial cells containing the rF protein by centrifugation at 2, 500 x g at 4°C for 10 mins. The pellet was washed in 1 volume of ice-cold $dH_2O$, resuspended in 0.04 volume of lysis buffer (50 mmol/l $NaH_2PO4/Na_2HPO4$, 0.1 mol/l NaCl, pH 7.6) and lysozyme (Sigma) was added to the final concentration of 0.2 mg/ml followed by gentle mixing at 4°C for 1 hr. The viscous content was sonicated at power output of 40 W for 10 cycles of 30 secs burst (10 x 30 secs) and clarified by centrifugation at 2, 500 g at 4°C for 5 mins. The clear lysate was spun at 20,000 g at 4°C for 30 mins resulting in a pellet (insoluble proteins) and supernatant containing soluble proteins. The pellet was resuspended in an equal volume to that of the supernatant in PBS (pH 7.4). The same volumes of soluble and insoluble proteins were loaded into adjacent wells of the gels for SDS-PAGE. Immunoblotting was performed at the appropriate dilutions for different antibodies and the nitrocellulose membrane developed using BCIP/NBT after washing with TBS-Tween.

*Purification of His-tagged rF protein under denaturing conditions.* The method of the isolation of the inclusion bodies was adapted from Georgiou and Valax (1999). The pellet fractions containing insoluble recombinant proteins in inclusion bodies were prepared as mentioned. One gram/pellet wet weight was used for each round of purification using the ProBond™ Purification System (Invitrogen) through denaturing conditions using the Ni-NTA column according to manufacturer's instructions. Briefly, the clarified pellet was resuspended in the binding buffer (8 mol/l urea, 20 mmol/l $NaH_2PO_4{\cdot}H_2O$ and 500 mmol/l NaCl, pH 7.8) and added to 2 ml of the 50% Ni-NTA slurry. The lysate-resin mixture was loaded into a column and binding carried out for 15–30 mins with gentle agitation. The first post-bound aspirated supernatant was designated PB1. The column was washed again with binding buffer and the aspirated supernatant designated PB2. The column was washed twice with 4 ml wash buffer (8 mol/l urea, 20 mmol/l $NaH_2PO_4{\cdot}H_2O$, and 500 mmol/l NaCl, pH 6.0). Each wash fraction, W1 and W2 were kept for SDS-PAGE analysis. The recombinant protein was eluted 5 times each with two 0.5 ml elution buffers (8 mol/l urea, 20 mmol/l $NaH_2PO_4{\cdot}H_2O$ and 500 mmol/l NaCl, pH 5.3) and (8 mol/l urea, 20 mmol/l $NaH_2PO_4{\cdot}H_2O$, and 500 mmol/l NaCl, pH 4.5), designated D1, D2, D3, D4, D5, and E1, E2, E3, E4, E5. The collected fractions were then analyzed by SDS-PAGE for the determination of the eluted 6× His-tagged recombinant protein.

*Dialysis and concentration of protein.* Dialysis tubing cellulose membrane (MW 10,000) was prepared using the methods provided by the manufacturer (Thermo Scientific). The dialyzed protein was concentrated using Vivaspin centrifugal concentrators with MWCO (molecular weight cut-off) of 30,000 (Sartorius). The Bradford method was used to determine protein concentration (Bradford, 1976).

*Immunization protocol.* Two female rabbits (New Zealand White, 2–2.5 kg) were used for immunization. 500 µl of PBS (pH 7.4) containing approximately 250 µg of the purified rF protein was emulsified with an equal volume of complete Freund's adjuvant and injected. Four weeks later, rabbits were immunized with a booster dose at the same concentration in incomplete Freund's adjuvant. The animals were bled two weeks later.

## Results and Discussion

### *Expression and purification of the rF protein*

In this study, a domain of the F protein gene of HRSV from 212–574 aa (rF protein) was amplified, digested with *Bgl*II and

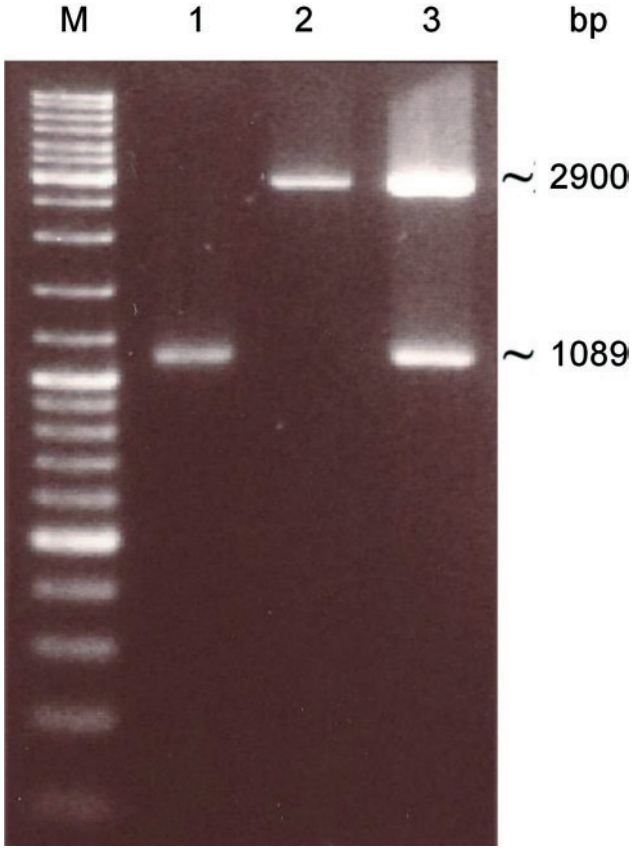


**Fig. 2**

**Confirmation of the F domain cloning into plasmid pRSetC**

Agarose gel electrophoresis. DNA ladder (lane M), double-digested RT-PCR product (lane 1), double-digested original plasmid pRSetC (lane 2), and double-digested recombinant plasmid pRSetC(F) (lane 3).

*Nco*I and cloned into the *E. coli* expression plasmid pRSetC. Restriction analysis (Fig. 2, lane 3) and DNA sequencing (data not shown) confirmed that the F glycoprotein domain was successfully cloned into pRSetC in the correct orientation.

*E. coli* BL21 (DE3) was transformed with the recombinant plasmid pRSetC(F) that is able to express genes under the regulation of T7 promoter. The presence of 6x His amino acids fused to the N-terminal of the rF proteins enables the purification of recombinant proteins using Ni-NTA column.

The optimized conditions for expression of the rF protein in pRSetC in *E. coli* BL21 (DE3) were: the temperature at 26°C, 9 hrs of induction with 1.5 mmol/l IPTG, and shaking at 200 rpm. These conditions were based on the band with highest intensity displayed after the time-course optimization (results not shown). Even under these optimized conditions, the expression of the rF protein was restricted. This outcome may be explained by the possibility that expression of the hydrophobic fusion-related domain of F subunit could be toxic, because of its fusogenic activity in mammalian cells as well as of its association with the bacterial membranes (Davis and Hsu, 1983).

The rF protein was found to be completely insoluble and produced as inclusion bodies due to the protein aggregation after prolonged incubation (Leinweber *et al.*, 2004). There are three hydrophobic sequences in the F polypeptide including its signal peptide located at the N-terminus of $F_2$ chain, fusion peptide at the N-terminus of $F_1$ chain, and transmembrane region located near the C-terminus of $F_1$ (González-Reyes *et al.*, 2001). These regions account not only for the hydrophobicity of the entire F glycoprotein of HRSV, but also for the hydrophobicity of the synthesized rF protein as well.

In order to acquire a sufficient amount of the rF protein, the bacterial cells were lysed and the rF protein was purified using the Ni-NTA purification system under denaturing conditions using 8 mol/l urea followed by dialysis (Fig. 3a, b). The cell lysate prior to the purification and proteins not bound to the column were showed in lanes 1 and 2, respectively (Fig. 3a). The washing fraction was examined in lane 3 and the concentration of the rF protein at 47 k was markedly decreased after its binding on the Ni-NTA slurry (Fig. 3a, lane 2). This decrease suggested that large amounts of the rF protein was bound to the column. No protein was eluted at pH 5.9 (Fig. 3a, lanes 4–9), whereas elution was completed in the last fractions at pH 4.5 (Fig. 3b, lanes 1–5). Next, the fractions containing purified rF protein were dialyzed to remove urea. SDS-PAGE showed that the protein remained intact and its concentration was determined after treatment with Vivaspin 6 centrifugal concentrator (Sartorius) with 30, 000 MWCO. In summary, the presence of the recombinant protein confirmed that it was successfully purified using the Ni-NTA affinity chromatography.

*Binding of polyclonal antibody against the rF protein to the native $F_1$ subunit*

In this work, the prepared antibodies were denoted as rabbit anti-rF polyclonal antibodies. Western blot analysis showed that these antibodies could bind to the rF protein (47 K) produced in *E. coli* BL21 (DE3) indicating that the raised antibodies were specific to this protein (Fig. 4, lane 4). Interestingly, this polyclonal antibody also recognized the $F_1$ subunit protein of HRSV at 48 k (Fig. 4, lane 5). Cell lysates from pRSetC (as the negative control) and pRSetC(F) were also probed with anti-His MAb (GE Healthcare) to prove the successful raising of polyclonal antibodies against the rF protein at the same molecular mass of 47 K (Fig. 4, lanes 1 and 2, respectively). $F_0$ protein (70 K) was not detected on the membranes of HRSV-infected cells (data not shown). It had been shown earlier that $F_0$ could not be detected on the surface of normal HRSV-infected cells even when the intracellular cleavage of F protein was partially inhibited (Collins

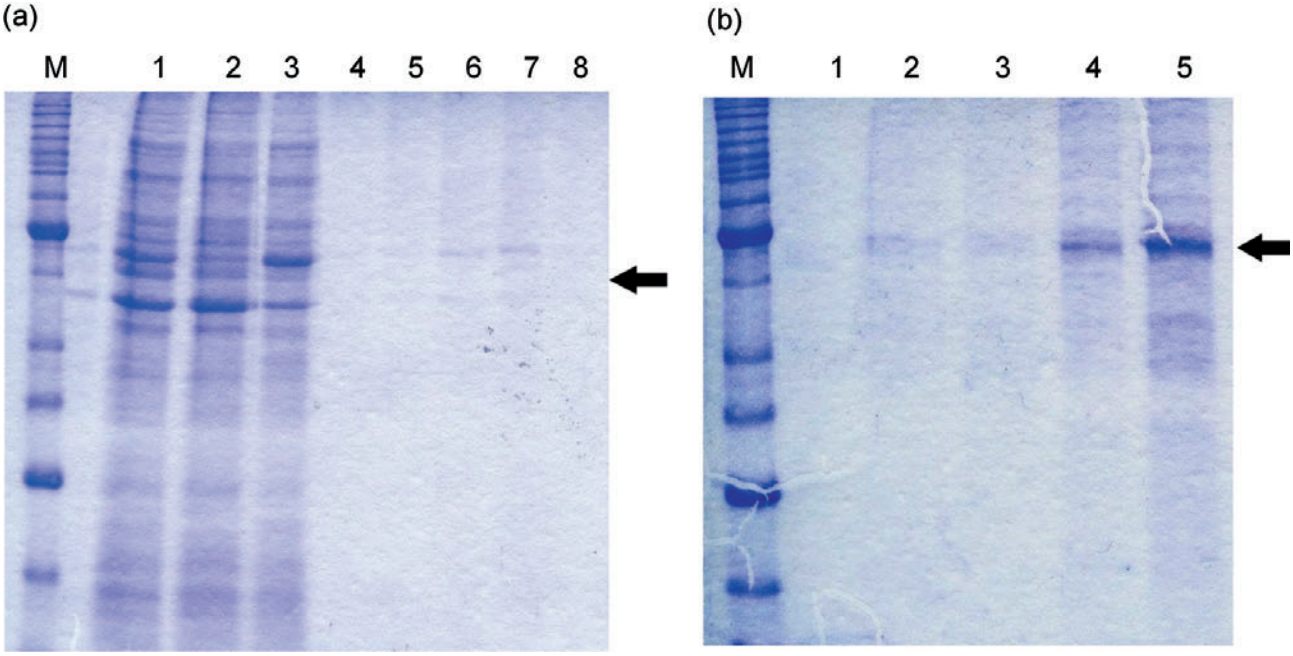


**Fig. 3**

**Purification profile of the rF protein**

Protein size marker (lanes M). (a) Solubilized cells (lane 1), post-binding fraction (lane 2), washing fraction (lane 3), elution fractions at pH 5.9 (lanes 4–8). (b) Elution fractions at pH 4.5 (lanes 1–5). The arrow indicates the rF protein at 47 K.

and Mottett, 1991). This outcome could be due to the rapid break-down of the uncleaved F glycoprotein or to its intrinsic property that prevents the complete migration of F glycoprotein to the cell membrane (Collins and Mottett, 1991; Bolt *et al.*, 2000). Polyclonal antisera against the reduced form of the F glycoprotein of HRSV were raised and the antisera could neutralize virus infectivity and cell-to-cell fusion, whereas antibodies against the non-reduced form were neutralizing, but could not inhibit the cell-to-cell fusion (Subbarao *et al.,* 1994). Furthermore, the polyclonal antisera that were raised against the purified F glycoprotein in cotton rats could successfully neutralize HRSV in tissue culture and protect immunized animals during challenge with live HRSV (Walsh *et al.*, 1987). A six-fold higher titer of serum-neutralizing antibodies was also elicited by the vaccinia F recombinant providing complete resistance to the HRSV-induced lower respiratory tract infection in cotton rats (Olmsted *et al.*, 1986; Wertz *et al.*, 1987). Epitopes in the $F_1$ subunit were found to be resistant to the high doses of trypsin (Arbiza *et al.,* 1992). This indicates that a certain three-dimensional conformation is required for the antibody recognition that might be preserved in Western blot analysis, but not in short peptides.

*Binding of polyclonal antibody against the native virus to the rF protein*

A nitrocellulose membrane was probed with a commercial polyclonal goat anti-HRSV IgG antibody (Chemicon) and


**Fig. 4**

**Western blot analysis of the rF protein**

Blot was probed with the antibodies to His-tag (lanes 1–2), to the rF protein (lanes 3–5), and to HRSV (lanes 6–8), respectively. Cell lysate from *E. coli* harboring plasmid pRSetC (negative control, lanes 1, 3, 6), cell lysate from *E. coli* harboring recombinant plasmid pRSetC(F) (lanes 2, 4, 7), and HRSV (lanes 5, 8). The values 47 K (red arrow) and 48 K (black arrow) correspond to rF and native $F_1$ protein, respectively.

the detected bands were similar to those detected with rabbit polyclonal anti-rF antibodies. The rF protein was observed at 47 K and the $F_1$ subunit protein from the whole virus at 48 K (Fig. 4). No bands at the corresponding $M_r$ could be detected in the negative controls (Fig. 4, lanes 1, 3, 6). These results indicated that although the anti-rF polyclonal antibodies have been raised only against a part aa 212–574 of the F protein and even though the glycosylation did not occur, the corresponding antibodies were capable of reaction with the native $F_1$ protein making it a potential vaccine candidate. So far, much research has been done on the entire full-length F (Wertz *et al.,* 1987; Martin *et al.*, 2006; Ternette *et al.*, 2007; Fu *et al.*, 2009) as well as short peptides (Trudel *et al.*, 1987; Scopes *et al.*, 1990; Arbiza *et al.*, 1992; Johnstone *et al.*, 2004; Marsh *et al.,* 2007; Singh *et al.*, 2007). However, further work is required to demonstrate the virus-neutralizing property of this rF protein and its potential as a vaccine candidate.

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