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Purification of respiratory syncytial virus F and G proteins

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Abstract

Respiratory syncytial virus (RSV) is the most important cause of severe lower respiratory tract infections of infants in industrial nations. In addition, the participation of RSV in the genesis of asthma is under discussion. The RSV glycoproteins F and G have key positions in the viral pathogenesis. At present no satisfactory protein purification protocols are available for these proteins. The methods published for the G protein using preparative SDS-PAGE or immunoaffinity chromatography yield only small amounts of purified G protein that has partially lost its antigenicity. We describe a three-step purification protocol for these glycoproteins. RSV-infected HEp-2 cells were lysed by a Triton X-100 containing buffer. The viral proteins were captured by QAE-Sephadex A-50 material in a batch procedure. A first elution with 100 m*M* NaCl led to a crude F protein fraction, and a second elution with 300 m*M* NaCl led to a crude G protein fraction. The F protein was further purified on a Lentil-lectin Sepharose 4B column and finally polished using a Resource Isopropyl column. Lentil-lectin Sepharose 4B was also used to purify the G protein from the crude fraction, but polishing of the G protein was carried out on a Resource Q column. Homogenous RSV-F and RSV-G proteins were obtained by this protein purification protocol. No loss of antigenicity could be observed during this procedure as the highly purified viral proteins remain detectable by a set of monoclonal antibodies and specific antisera. The G protein was isolated as a 90 000 monomer, whereas the purified F protein was recovered as a functional homodimer of 140 000.

Keywords: Purification; Glycoproteins; Fusion protein F; Attachment protein G

1. Introduction

Human respiratory syncytial virus (RSV) is an enveloped, RNA-containing virus and the most important cause of infections of the lower respiratory tract in newborns and infants [1]. In addition, the involvement of RSV in the genesis of asthma has been discussed [2]. As a member of the family Paramyxoviridae, RSV is classified in the genus Pneumovirus. It lacks hemaglutinin and neuraminidase and the diameter of its nucleocapsid is 12–15 nm, smaller than that of other paramyxoviruses [3]. The RSV genome is a single strand of negative-sense RNA encoding at least 10 proteins [4]. Three proteins, namely the attachment glycoprotein G, the fusion protein F and the small hydrophobic protein SH, are expressed at the surface of infected cells and virions [1].

G protein mediates attachment to the cell by binding to heparin-like motifs [5]. The predicted molecular mass of this type II membrane protein is about 32 000, but heavy glycosylation, both with

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N-linked and O-linked sugars, leads to an apparent molecular mass of about 90 000 [6–8]. The fusion protein F is involved in cell fusion and consists of a dimer connected by disulfide bonds. The predicted molecular mass is about 70 000, but under non-reducing conditions it seems to form homodimers resulting in a molecular mass of 140 000 [9,10]. F protein shows in contrast to G protein only N-linked glycosylation, which seems to be involved in virus infectivity as well as in the transport of F protein to the cell surface [11,12].

Two subgroups of the virus, A and B, have been identified based on antigenic analysis with monoclonal antibodies [13–15]. The antigenic differences between the RSV subgroups A and B are much greater within the G protein than the F protein. Antibody response to the G protein is subgroup specific and extensive glycosylation affects its recognition by the immune system [16]. It has been shown that RSV-G protein favours the development of a Th_2-cytokine pattern [17–19].

Many neutralizing antibodies have been raised towards the fusion protein and recent studies with a humanized antibody showed its possible role as a prophylactic reagent for premature babies during the RSV season [20].

Therefore, it is interesting to study the role and influence of the RSV surface proteins on the immune system separately and in combination. Some methods for purification of the RSV surface proteins have been published based on immunoprecipitation or preparative SDS-PAGE [11,21,22]. The use of such techniques for the preparation of the RSV-G protein resulted in the partial loss of its antigenicity [21]. In this regard, we developed a new method based on a one-buffer system for easy scale-up. We present a combined chromatographic method for the purification of both the attachment and the fusion protein from infected cells.

2. Experimental

2.1. Chemicals

All chemicals were of the highest quality grade available and were purchased from Biomol (Hamburg, Germany), Boehringer (Mannheim, Germany), Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany) if not stated otherwise.

2.2. Virus and cells

Monolayer cultures of HEp-2 cells were grown in 25 m*M* *N*-2-hydroxyethylpiperazine-*N′*-2-ethane sulfonic acid (HEPES) buffered Dulbecco's modified Eagle Medium (DMEM) (Life Technologies, Karlsruhe, Germany) containing 5% v/v heat-inactivated foetal calf serum (Biochrom, Berlin, Germany) and a mixture of penicillin, streptomycin and neomycin as usually used (Sigma, Deisenhofen, Germany). Stocks of RSV long strain were propagated in HEp-2 cells, which were maintained in 25 m*M* HEPES-buffered DMEM containing 0.5% v/v foetal calf serum and the above-mentioned mixture of antibiotics. Viral titers were determined 48 h post-infection by immunostaining using RSV-antigen-specific monoclonal antibodies (mAb).

2.3. Enzyme-linked immunosorbent assay (ELISA) and protein determination

BCA assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as standard was applied to determine protein concentrations [23]. ELISA was carried out according to Clegg and Evans [24]. Briefly, samples were diluted 1:100 in 200 m*M* sodium carbonate buffer pH 9.6, and 100 μl of each was coated on the surface of the test plate (Nunc, Wiesbaden, Germany) and incubated overnight at 4°C. After removal of the supernatant the wells were washed three times with PBS-T (phosphate-buffered saline: 10 m*M* sodium phosphate, 150 m*M* NaCl, 0.05% v/v Tween 20, pH 7.2) and blocked for 30 min with blocking solution using 2% w/v non-fat dried milk powder (De-Vau-Ge GmbH, Lüneburg, Germany) in PBS-T. After washing three times wells were incubated with a dilution (0.1 μg/well) of a RSV-antigen-specific mAb in PBS-T containing 1% w/v non-fat dried milk powder for 1 h. Secondary antibody incubation was carried out by a 1:2000 dilution of anti-mouse-HRP conjugate (DAKO, Hamburg, Germany) for 1 h after washing three

times. Unbound antibodies were removed and the test was developed by addition of 50 μl of 0.67 mg/ml *ortho*-phenyl-diamine-dihydrochloride (DAKO, Hamburg, Germany). Development was stopped by adding 50 μl of 1 *M* H_2SO_4 to each well and absorbance was measured at 492 nm with an Anthos EIA reader (Köln, Germany).

2.4. SDS-PAGE, Western blot and silver staining

SDS-PAGE was performed in 10% w/v gels according to Laemmli [24,25] on Biometra equipment (Biometra, Göttingen, Germany). After electrophoreses the proteins were transferred to BA 85 nitrocellulose membranes (Schleicher und Schuell, Dassel, Germany) by Semi-Dry-Blot [26] using a Biometra Fast Blot system.

After blocking of the nitrocellulose membranes using 2% w/v non-fat dried milk powder in PBS-T, they were incubated in a 1:2000 dilution of goat RSV-specific biotinylated antiserum (Dunn, Asbach, Germany) and 1:500 dilutions of RSV-antigen-specific mAbs in PBS-T containing 1% w/v non-fat dried milk powder for 1 h. A second incubation followed in a 1:5000 dilution of an extravidin-alkaline-phosphatase conjugate (Sigma, Deisenhofen, Germany) and a 1:1000 dilution of an anti-mouse-alkaline-phosphatase conjugate (DAKO, Hamburg, Germany) for 1 h after washing three times. The tests were developed by adding 0.1 mg/ml Nitro-Tetrazolium-Blue (Sigma, Deisenhofen, Germany) and 0.05 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Sigma, Deisenhofen, Germany) dissolved in 150 m*M* Tris–HCl pH 9.2 containing 40 m*M* $MgCl_2$.

Silver staining of the polyacrylamide gels was carried out according to a method first described by Heukeshoven and Dernick [27].

2.5. Samples

Ten tissue culture flasks of 75 cm^2 growth surface (Becton Dickinson, Franklin Lakes, NJ, USA) covered with subconfluent grown HEp-2 cells were infected by RSV long strain at a multiplicity of infection (m.o.i.) of 0.1. Seventy-two hours post-infection the cells were scrapped into the medium, and pelleted by centrifugation (900 *g*, 5 min, 4°C) in a Beckman centrifuge GPR (Beckman Instruments Inc., Fullerton, CA, USA). The supernatant was removed, the cells were washed twice with ice-cold PBS containing 2 m*M* EDTA and again pelleted. The cells were then solubilized in 10 ml lysis buffer (250 m*M* saccharose, 25 m*M* MES-NaOH pH 5.7, 10 m*M* NaCl, 0.1% v/v Triton X-100, 2 m*M* EDTA, 1 m*M* PMSF) for 15 min at 4°C. The resulting lysate was clarified by centrifugation for 10 min at 2000 *g* at 4°C

2.6. Chromatography

2.6.1. Ion-exchange chromatography

The first purification step of the proteins was carried out by a stepwise gradient elution in batch at room temperature. The clarified lysate was added to 10 ml QAE-Sephadex A-50 equilibrated with 25 m*M* MES-NaOH pH 5.7, 0.1% v/v Triton X-100, 10 m*M* NaCl (MT-10) buffer. The proteins were allowed to bind to the material for 30 min, followed by washing five times with MT-10 buffer using the same volume as the batch volume. The RSV fusion protein was then eluted by increasing the NaCl concentration to 100 m*M*. Five fractions of 10 ml each were collected. Finally, the RSV attachment protein was eluted by increasing the NaCl concentration to 300 m*M* and again five fractions each of 10 ml were collected. Fractions were screened by ELISA using mAbs against the fusion and attachment protein.

2.6.2. Lentil-lectin chromatography

Lentil-lectin Sepharose 4B (4 ml) (Pharmacia, Freiburg, Germany) was filled into 15 mm diameter columns (Pharmacia) and chromatography was run under gravitational buffer flow at room temperature. Two millilitre fractions were collected and estimated by ELISA testing.

2.6.2.1. Purification of the RSV fusion protein. The fusion protein positive fractions (Section 2.6.1) were pooled and layered on a 25 m*M* MES-NaOH, pH 5.7, 0.1% v/v Triton X-100, 100 m*M* NaCl (MT-100) equilibrated Lentil-lectin Sepharose 4B column. Unbound proteins were removed by extensive wash-

ing with MT-100 buffer using 10 times the column volume. The fusion protein was eluted with 4% w/v α-methyl-d-mannoside in MT-100 buffer.

2.6.2.2. Purification of the RSV attachment protein. The attachment protein positive fractions (Section 2.6.1) were pooled and layered on a MT-100 equilibrated Lentil-lectin Sepharose 4B column. Unbound proteins were removed by extensive washing with MT-100 buffer using 10 times the column volume. The attachment protein was eluted with 8% w/v α-methyl-d-mannoside in MT-100-buffer after a second washing step using 2% w/v α-methyl-d-mannoside in MT-100-buffer.

2.6.3. Ion-exchange and hydrophobic-interaction chromatography

Source Q anion-exchange chromatography and hydrophobic-interaction chromatography (HIC) were performed on a Pharmacia FPLC system using a prepacked Resource Q column and a Resource HIC-Isopropyl column (Pharmacia). All buffers used for FPLC were degassed and filtered through 0.2 μm membrane filters (Millipore, Bedford, MA, USA). All steps were carried out at room temperature.

2.6.3.1. Purification of the RSV fusion protein. The positive fractions (Section 2.6.2.1) were pooled, raised to 1.5 *M* ammonium sulfate and applied to a 1 ml Resource Isopropyl column. Recommended buffers: A, 25 m*M* MES-NaOH pH 5.7, 0.04% v/v Triton X-100; B, 25 m*M* MES-NaOH pH 5.7, 1.5 *M* ammonium sulfate. Starting conditions were 100% v/v B. A stepwise gradient was performed using a constant flow of 1 ml min^{-1}, resulting in a hold of 100% v/v B for 4 min, a linear gradient to 60% v/v B over a period of 4 min, a secondary hold of 60% v/v B over a period of 6 min and a final linear gradient towards 0% v/v B over 12 min. Fractions (2 ml) were collected, screened by ELISA testing, and the UV absorbance was measured at 280 nm on a Lambda 3 UV–VIS spectrophotometer (Perkin-Elmer, Norwalk, CT, USA).

2.6.3.2. Purification of the RSV attachment protein. The positive fractions (Section 2.6.2.2) were pooled, diluted with one volume of distilled water and applied to a 1 ml Resource Q column. Eluents comprised: A, 25 m*M* MES-NaOH pH 5.7, 0.04% v/v Triton X-100; B, 25 m*M* MES-NaOH pH 5.7, 0.04% v/v Triton X-100, 1 *M* NaCl. Starting conditions were 5% v/v B for 24 min followed by a linear gradient over 40 min to 40% v/v B with a flow-rate of 1 ml min^{-1}. Fractions (2 ml) were collected, screened by ELISA testing, and the UV absorbance was measured at 280 nm on a Lambda 3 UV–VIS spectrophotometer (Perkin-Elmer).

3. Results and discussion

The RSV transmembrane surface proteins are important mediators of viral pathogenity. The purification of the fusion protein F and the attachment glycoprotein G by immunoprecipitation or preparative SDS-PAGE provide only small amounts of protein with a reduced antigenicity in the case of the G protein [19]. Therefore, we developed a new method for the purification of high yields of F and G proteins in a three-step chromatographic procedure.

RSV-infected HEp-2 cells were solubilized to obtain the crude lysate. Testing of different detergents showed that the most effective lysis of the transmembranous proteins was achieved with Triton X-100. Increasing the pH or the concentration of Triton X-100 led to higher rates of solubilized proteins without changing the amount of surface proteins (data not shown). After clarifying the lysates by centrifugation they were tested in a Western blot analysis. The viral surface proteins G (90 000), F (70 000+140 000), and the nucleoproteins N (40 000) and P (36 000) could be detected with RSV-specific antiserum (see Figs. 2 and 6, lane 1).

The first grade of purification was achieved by a stepwise gradient elution using ion-exchange chromatography. Silver-stained SDS-PAGE (Fig. 1, lane 2) shows enrichment of the fusion protein after eluting a QAE-Sephadex A-50 with 100 m*M* NaCl. Western blot analysis with RSV-specific antibodies marked a 140 000 band corresponding to the homodimerized F protein and smaller traces of the 70 000 monomer F protein (Fig. 2, lane 2).

Further enrichment resulted from the following affinity chromatography. The Lentil-lectin Sepharose 4B columns led to an improved purity of the pooled

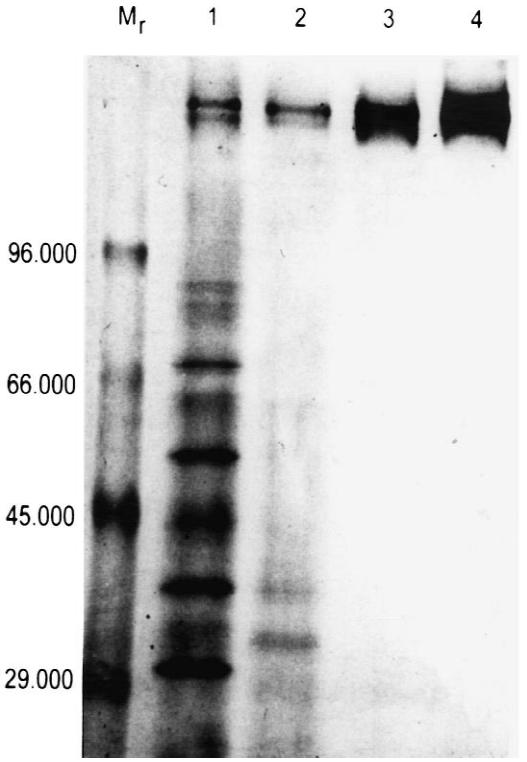

Fig. 1. Silver-stained SDS-PAGE showing the steps of the F protein purification protocol. Lanes: M_r marker proteins; 1=clarified lysate of RSV-infected HEp-2 cells (1.6 μg protein); 2=QAE-Sephadex A-50 eluate (0.45 μg protein); 3=Lentil-lectin Sepharose 4B pool (0.30 μg protein); 4=Resource Isopropyl column (0.28 μg protein).

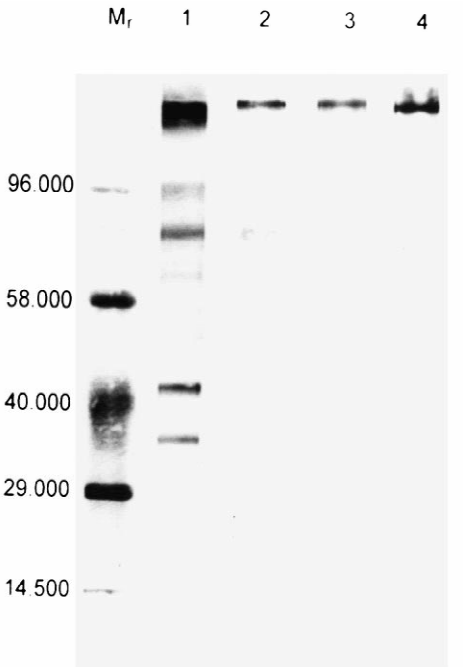

Fig. 2. Western blotted nitrocellulose membrane showing the steps of the F protein purification protocol treated with goat anti-RSV biotinylated serum followed by an extravidin-alkaline-phosphatase conjugate. Lanes: M_r=biotinylated marker proteins; 1=clarified lysate of RSV-infected HEp-2 cells (1.6 μg protein); 2=QAE-Sephadex A-50 eluate (0.45 μg protein); 3=Lentil-lectin Sepharose 4B pool (0.30 μg protein); 4=Resource Isopropyl column (0.28 μg protein).

Table 1
Protein content of each fraction determined by BCA assay and ELISA specific for RSV-F and RSV-G protein

	Preparation of the fusion protein		Preparation of the attachment protein	
	Total protein (μg)	Total yield[a] of F protein (μg)	Total protein (μg)	Total yield[a] of G protein (μg)
Clarified lysate	5800	824 (100%)	5800	87 (100%)
QAE-Sephadex A-50	1680	618 (75%)	900	83 (95%)
Lentin-lectin Sepharose	600	395 (48%)	100	44 (50%)
FPLC chromatography	280	280 (34%)	20	20 (23%)

[a] Estimated by specific F and G protein ELISA.

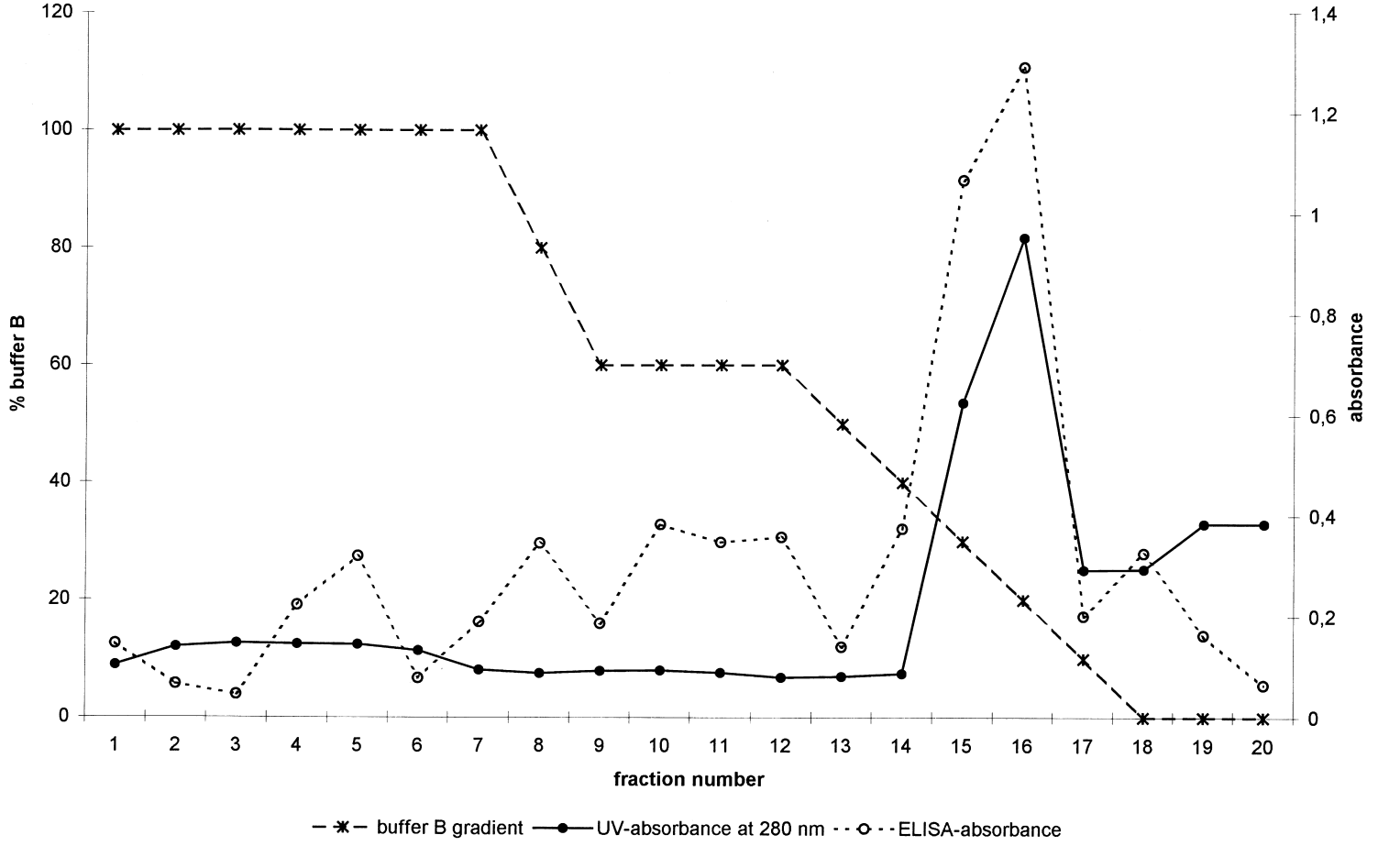

Fig. 3. HIC on the Resource Isopropyl column. Solid line (●): UV absorbance at 280 nm; dashed line (×): buffer B gradient; dotted line (○): F protein content estimated by ELISA (absorbance at 492 nm).

fractions as determined with an F-protein-specific ELISA (Table 1) and immunodetection of Western blotted F protein (Fig. 2, lane 3). Interestingly, the F monomers, which were still immunodetectable after ion-exchange chromatography, vanished, so only the homodimerized form of F could be detected (Figs. 1 and 2, lane 3). Hydrophobic-interaction chromatography on a Resource Isopropyl column resulted in fractions of homogenous F (Figs. 1 and 2, lane 4). It eluted at a concentration of about 0.5 *M* ammonium sulfate (Fig. 3) as determined with ELISA. The peak fraction in particular proved to contain highly purified F. Two hundred and eighty micrograms of fusion protein could be prepared from 5800 μg applied protein. RSV-F-specific ELISA showed that our protocol preserved 34% of the original F protein (Table 1).

The purified F protein retained its antigenicity as it still reacted with a set of specific monoclonal antibodies and antisera (Fig. 4). Boiling of the F homodimers separated them into monomers preserving their antigenicity (Fig. 4, lanes 2, 4 and 6). Treatment of the samples with β-mercapto-ethanol, however, caused a total loss of antigenicity (data not shown).

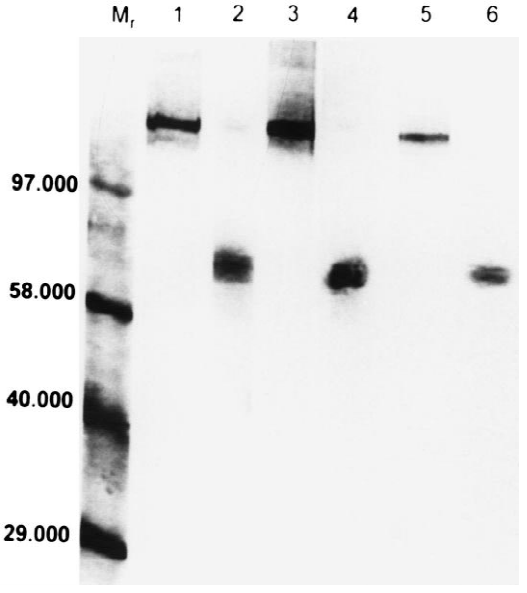

Fig. 4. Western blotted nitrocellulose membrane treated with goat anti-RSV biotinylated serum (lanes 1 and 2), mAb 18F12 (lanes 3 and 4) and subunit F1-specific mAb 621 (lanes 5 and 6), followed by an extravidin-alkaline-phosphatase conjugate (for mAbs with anti-mouse-alkaline-phosphatase conjugate). Even numbers contain the native HIC eluate, uneven numbers contain the boiled HIC eluate. M_r, biotinylated marker proteins.

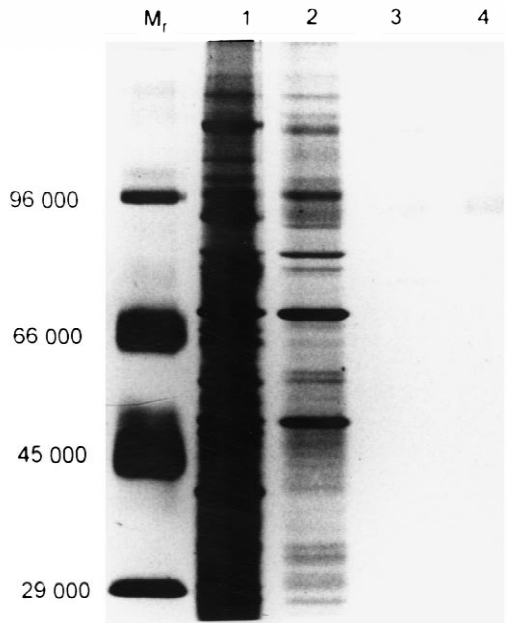

Fig. 5. Silver-stained SDS-PAGE showing the steps of the G protein purification protocol. Lanes: M_r=marker proteins; 1=clarified lysate of RSV-infected HEp-2 cells (5.8 μg protein); 2=QAE-Sephadex A-50 eluate (0.9 μg protein); 3=Lentil-lectin Sepharose 4B pool (0.1 μg protein); 4=Resource Q column (0.1 μg protein).

Our combined chromatography allows the preparation of both viral proteins F and G from the same cellular lysate. Increasing the NaCl concentration to 300 m*M* during the ion-exchange chromatography eluted glycoprotein G (Figs. 5 and 6, lane 2). As for the fusion protein the Lentil-lectin Sepharose 4B columns showed enrichment of the G protein in the pooled fractions (Figs. 5 and 6, lane 3). Nevertheless, Western blot analysis demonstrated that the fractions were not completely pure (Fig. 5, lane 3). FPLC was performed using a Resource Q column. RSV-G protein eluted at about 250 m*M* NaCl (Fig. 7). The peak fractions containing the purest degree of attachment protein were collected. The resulting homogenous G protein could be detected with a set of monoclonal antibodies and specific antisera (Fig.

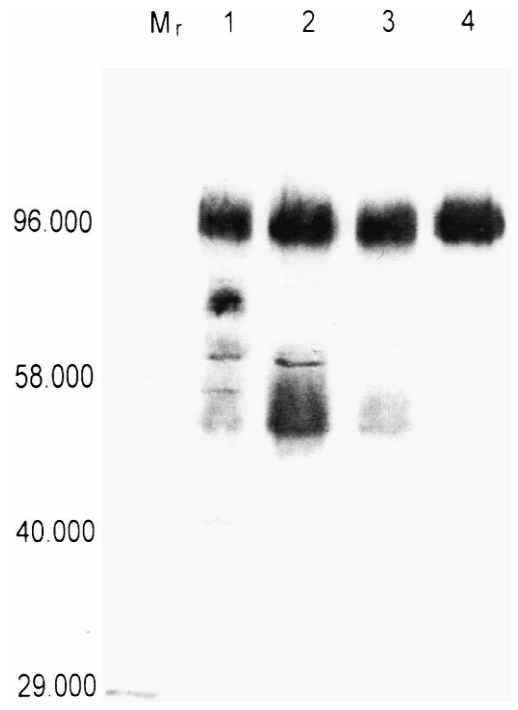

Fig. 6. Western blotted nitrocellulose membrane showing the steps of the G protein purification protocol treated with goat anti-RSV biotinylated serum, followed by an extravidin-alkaline-phosphatase conjugate. All samples were heated and reduced. Lanes: M_r=biotinylated marker proteins; 1=clarified lysate of RSV-infected HEp-2 cells (5.8 μg protein); 2=QAE-Sephadex A-50 eluate (0.9 μg protein); 3=Lentil-lectin Sepharose 4B pool (0.1 μg protein); 4=Resource Q column (0.1 μg protein).

8). The monoclonal antibodies were developed in our group by immunisation of BALB/c mice with infectious RSV. They are able to detect different carbohydrate–protein structures on the G protein, shown by a partial deglycosylation of the purified G protein (data not shown).

Table 1 compares the amount of G protein to total protein. The total protein content decreased from 5800 to 20 μg after the three purification steps, whereas the total yield of G protein decreased only from 87 to 20 μg, which is 23% of the original protein.

The successive employment of ion-exchange chromatography, affinity chromatography and FPLC using HIC and Resource Q successfully separated RSV attachment glycoprotein and fusion protein of a lysate of infected cells. The resulting homogenous eluates retained full antigenicity (Figs. 4 and 8) and verified biological activity (data not shown).

Chromatographic separations have already been used successfully for the isolation of viral proteins [28]. Our three-step purification of RSV membranous proteins might also be helpful to obtain a variety of viral surface proteins as it renders high yields of homogenous protein. Furthermore, the application of our protocol allows an easy scale-up for higher protein output simplified by the use of a one-buffer system.

Arumugham and coworkers [29] detected small quantities of disulfide-linked F and G protein in infected cells, raising the possibility that these two proteins form heterooligomers. Our data are, in contrast, consistent with the findings of Collins and Mottet [30], who could not show chemical cross-linking of these proteins. The separate purification of G and F under non-reducing conditions makes the existence of disulfide-linked heterooligomers unlikely.

Purified G and F should help to elucidate the function and structure of these viral proteins and to obtain further insight into their roles in immunopathogenity and in virus–host interactions.

4. Conclusions

We have developed a new method for the purification of the RSV membranous proteins G and F in a three-step chromatographic process. It renders high yields of homogenous proteins which were shown to maintain full antigenicity and biological activity. The uncomplicated procedure based on a one-buffer system allows simple and fast preparation with easy scale-up. Our protocol should be helpful in the search for answers to a variety of virological questions.

Acknowledgements

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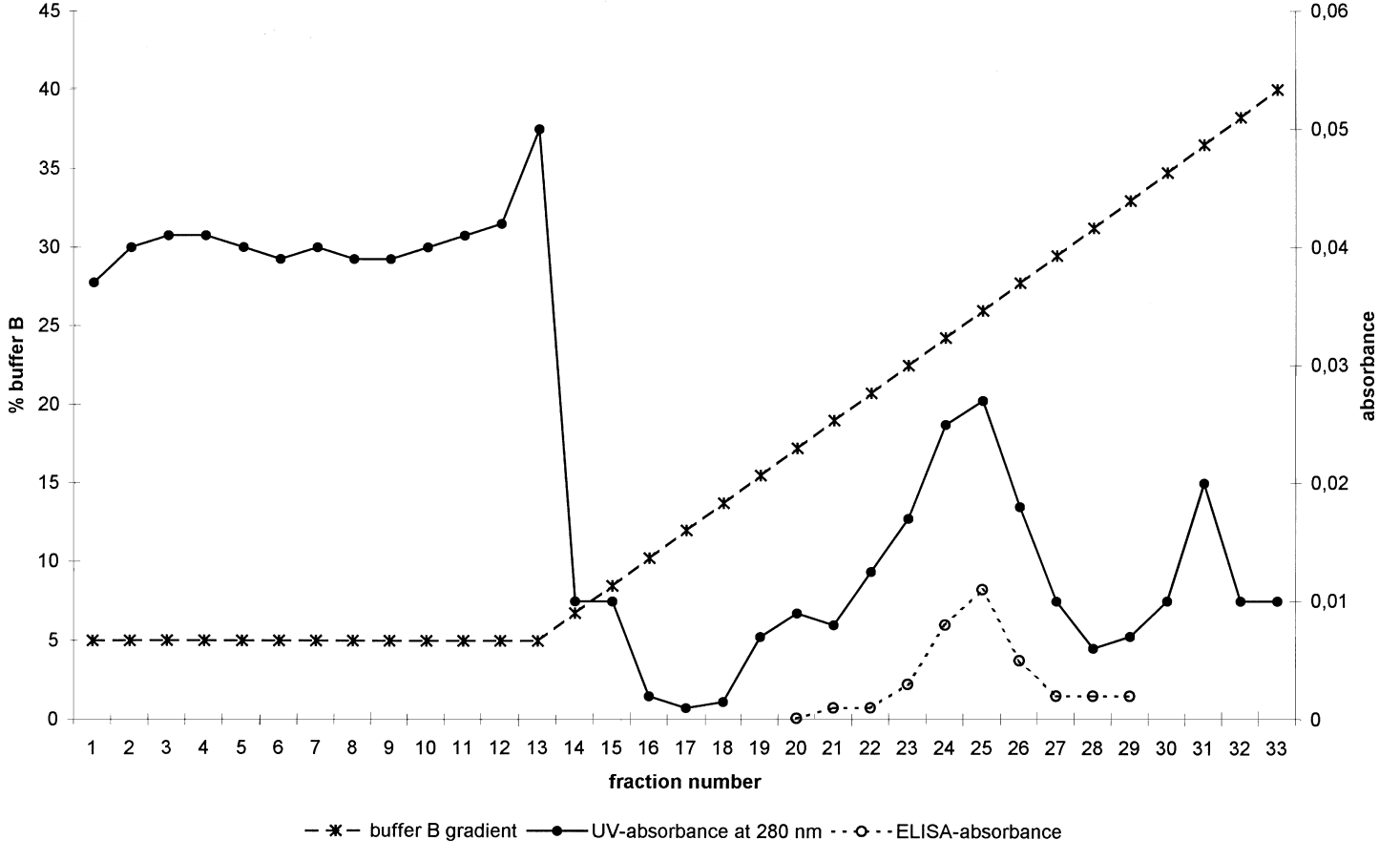

Fig. 7. Reversed-phase on the Resource Q column. Solid line (●): UV absorbance at 280 nm; dashed line (×): buffer B gradient; dotted line (○): G protein content estimated by ELISA (absorbance at 492 nm).

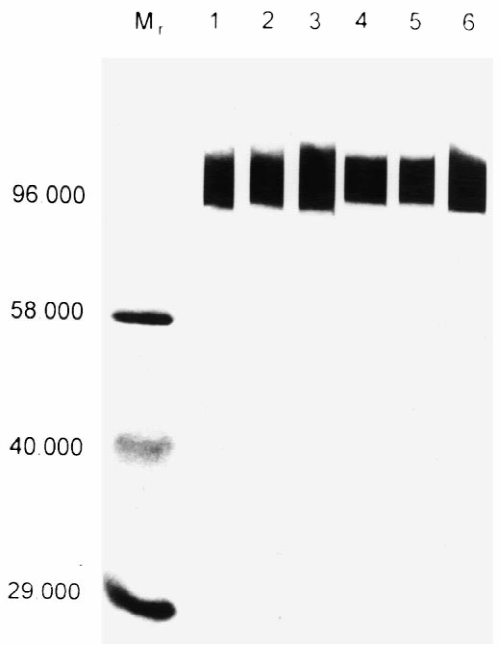

Fig. 8. FPLC purified G protein Western blotted on a nitrocellulose membrane treated with goat anti-RSV biotinylated serum and G-specific mAbs. Lanes: M_r=biotinylated marker proteins; 1=anti-RSV serum; 2=mAb 9D7; 3=mAb 11C9; 4=mAb 12E2; 5=mAb 14C10; 6=mAb 14E6.

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