Glycoproteins of Human Parainfluenza Virus Type 3: Affinity Purification, Antigenic Characterization and Reconstitution into Lipid Vesicles

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SUMMARY

Monoclonal antibodies to the envelope glycoproteins, HN and F, of human parainfluenza virus type 3 were coupled to a Sepharose 4B matrix and used for affinity purification of the viral glycoproteins. The purity of the glycoproteins was demonstrated by SDS-PAGE followed by fluorography or silver staining. The antigenicity of the glycoproteins was determined by immunization of rabbits; polyclonal rabbit antisera demonstrated inhibition of functional activities of the virus glycoproteins. The F glycoprotein, when reconstituted into lipid vesicles, showed distinct spike-like projections similar to those of intact virions.

INTRODUCTION

Human parainfluenza viruses are responsible for serious lower respiratory tract infections in infants (Chanock et al., 1961; Chanock & Parrot, 1965; Mufson et al., 1970; Glezen et al., 1971, 1984; Tyeryar, 1978, 1983). Parainfluenza viruses type 1 and type 2 have similar epidemiological patterns; they tend to cause epidemics in the autumn and early winter, usually appearing every other year and causing croup in children between 1 and 4 years of age. Infections usually do not occur in the first 6 months of life. On the other hand, human parainfluenza virus type 3 (PI3 virus) behaves epidemiologically more like respiratory syncytial virus. Parainfluenza virus type 4 is considered to be less important and less is known about this virus.

The haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins of paramyxoviruses form spikes on the external surface of the envelope and are responsible for initiation and progress of the infection processes (Scheid et al., 1972; Homma & Ohuchi, 1973; Scheid & Choppin, 1974). The importance of antibodies to parainfluenza virus HN and F glycoproteins for prevention of infection has been documented previously (Merz et al., 1980; Choppin & Scheid, 1980). The virion polypeptides of PI3 virus have been identified in several laboratories (Guskey & Bergtrom, 1981; Goswami & Russell, 1983; Storey et al., 1984; Sanchez & Banerjee, 1985; Jambou et al., 1985; Ray et al., 1985). Purified envelope glycoproteins of PI3 virus have been shown to be effective in eliciting an antibody response and confer protection from challenge infection in hamsters (Ray et al., 1985). On the other hand, immunization with formalin-inactivated whole virus induced an antibody response to the envelope glycoproteins, but provided only partial protection from challenge infection. Loss of important antigenic sites has been suggested to occur with formalin-inactivated mumps virus vaccine (Norby & Penttinen, 1978). In a serological study with naturally infected children, the antibody response to the HN glycoprotein has been reported to rise progressively during infection and reach a maximum at 8 to 10 months of age, after which antibody rises to the F glycoprotein (Kasel et al., 1984). Development of antibodies to both the glycoproteins has been correlated with protection from virus infection. The limited antibody response to the F glycoprotein, either after immunization of animals with envelope glycoproteins or in naturally infected children, appears to be due to the lower abundance or immunogenicity of the glycoprotein. Based on the previous experience with measles virus, inclusion of immunologically active fusion protein in vaccine...
preparations has been recommended by the Committee on Issues for New Vaccine Development (1985). The present study was aimed at isolation and purification of the two potentially important envelope glycoprotein antigen of PI3 virus, evaluation of the antigenicity of the purified molecules, and reconstitution of the glycoproteins into lipid vesicles for subsequent cell biological and immunological studies.

METHODS

Cells and virus. PI3 virus strain 47885 was obtained from the Division of Research Resources, National Institutes of Health (Bethesda, Md., U.S.A.). The virus was plaque-purified several times in African green monkey kidney (Vero) cells. Plaque-purified virus was subsequently passaged in rhesus monkey kidney cells (LLC-MK2) and the same cell line was used for virus growth. Inhibition of virus-induced cell fusion was done in baby hamster kidney (BHK) cells. Cells were grown in Dulbecco's medium containing 10% heat-inactivated newborn calf serum at 37 °C in a 5% CO2 incubator.

Preparation of detergent-soluble virus envelope glycoproteins and infected cell lysate. The envelope glycoproteins were solubilized by treatment of purified virions with n-octyl-β-D-glucopyranoside following procedures described previously (Ray et al., 1985). For preparation of cell lysates, virus-infected cells were lysed at about 36 h post-infection with 10 mM-Tris-HCl pH 7.8, 150 mM-NaCl, 600 mM-KCl, 0.5 mM-MgCl2, 2% Triton X-100, 1 mM-phenylmethylsulphonyl fluoride and 1% aprotonin as the lysis buffer (Varsanyi et al., 1984). The soluble supernatant of the virus-infected cell lysate was collected by centrifugation at 300000 g for 30 min at 4 °C.

Monoclonal antibodies. Monoclonal antibodies to the envelope glycoproteins of the virus were prepared as described previously (Ray et al., 1985; Ray & Compans, 1986) and used for immobilization on a solid matrix for subsequent affinity purification of the envelope glycoproteins.

Immobilization of antibodies to Sepharose 4B matrix. Two monoclonal antibodies designated 13.5.9.6.2 (anti-HN) and 9.4.3 (anti-F) were used in the affinity chromatography procedure to purify the HN and F glycoproteins, respectively. Immunoglobulin portions from ascites fluids were prepared by 50% (w/v, final concentration) ammonium sulphate precipitation. The precipitate was dissolved and dialysed against 0.1 M-NaHCO3, 0.5 M-NaCl, pH 8.3, with frequent changes. Finally, the immunoglobulin portions were separately coupled to CNBr-activated Sepharose 4B (Pharmacia) following the manufacturer's procedure. The remaining active sites, if any, on the Sepharose 4B-Ig matrix were blocked with 0.1 M-Tris-HCl pH 8.0 and the gel was washed three times successively with 0.1 M-Tris-HCl, 0.5 M-NaCl, pH 8.9 and 0.1 M-sodium acetate, 0.1 M-NaCl, pH 4.0 to remove uncoupled protein. The ligand concentration in the Sepharose 4B matrix was determined spectrophotometrically at 280 nm by suspending the coupled gel in ethylene glycol or solubilizing in 0.1 M-NaOH, 0.1% (w/v) NaBH4 at 75 °C (Dean et al., 1985).

Affinity purification of virus envelope glycoproteins. Detergent-soluble envelope glycoproteins or virus-infected cell lysate were used for adsorption to the antibody-coupled Sepharose 4B matrix. The Sepharose 4B-Ig beads and the lysate of crude virus pellets or infected cells were allowed to mix end-over-end for 4 h at 4 °C. Alternatively, the beads were packed in a column and the antigen preparation was passed slowly over the column at a flow rate of 10 ml/h and recycled at least five times. The unadsorbed material was used for purification of the other glycoprotein through a corresponding antibody-coupled Sepharose 4B matrix. The column was washed with 20 vol. washing buffer (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 0.1% octyl glucoside; Varsanyi et al., 1984). Finally, HN or F was eluted with 3 M-sodium thiocyanate, and dissolved in washing buffer. The eluted material was collected and directly concentrated in a collodion bag (Schleicher & Schuell) against several changes of 10 mM-Tris-HCl, 150 mM-NaCl, 0.01% Na2SO4, pH 7.6.

Reconstitution of purified glycoproteins into lipid vesicles. Phosphatidyl choline (Sigma) along with a trace amount of the 14C-labelled lipid (New England Nuclear) was dried to a thin film by gentle evaporation of the organic solvents through a stream of nitrogen. The dried film was solubilized in chloroform and again evaporated to dryness. Traces of organic solvents were finally removed under vacuum and dissolved in 2% cholic acid. The [3H]leucine-labelled purified glycoprotein, solubilized in 10 mM-Tris-HCl, 150 mM-NaCl, 0.01% NaN3, 2% cholic acid, pH 7.6 was mixed with lipid for 1 h by vortexing. Finally, the mixture was dialysed extensively to remove detergent and concentrated by surrounding the dialysis tubing with Biogel P 200 (Bio-Rad).

Purification of reconstituted vesicles. The glycoprotein–lipid mixture was applied over a 10 to 60% linear sucrose gradient and centrifuged at 300000 g for 24 h at 4 °C. Fractions were collected from the bottom and aliquots analysed for radioactivity. The fractions containing 3H and 14C activity were pooled, dialysed overnight against 10 mM-Tris-HCl, 150 mM-NaCl, 0.01% NaN3 and examined in an electron microscope.

Electron microscopy. The purified glycoproteins or reconstituted vesicles were examined by negative staining. Samples were applied to copper grids (400 mesh) coated with 1% paraformaldehyde for 15 min. The excess fluid was absorbed with filter paper and the material was stained with 1% potassium phosphotungstic acid pH 6.0 for 15 s. After removal of excess stain the grids were examined in a Philips 300 electron microscope.

Rabbit antisera to purified envelope glycoproteins. Rabbits were immunized to determine the antibody response to the individual glycoproteins. Affinity-purified HN or F glycoproteins were mixed with Freund's complete
Human P13 virus envelope glycoproteins

Fig. 1. Analysis of affinity-purified HN and F glycoproteins by SDS-PAGE (15%) followed by fluorography. A [3H]leucine-labelled crude virus preparation was treated with lysis buffer and the detergent lysate (lane 1) was employed for purification of HN and F using the respective antibody coupled to Sepharose 4B column. Lanes 2 and 3 show single bands corresponding to HN and F1, respectively, under reducing conditions. The HN glycoprotein did not show any disulphide-linked subunit structure, as it appeared as a band with the same molecular weight under non-reducing conditions (lane 4). On the other hand, the fusion glycoprotein appeared as a higher molecular weight band (lane 5) under non-reducing conditions.

Fig. 2. Analysis of the purity of HN and F glycoproteins by SDS-PAGE (10%) followed by silver staining. Virus-infected cells were treated with lysis buffer and the detergent lysate (lane 1) was used for purification of the two glycoproteins using the respective antibody coupled to a Sepharose 4B column. Lanes 2 and 3 show bands corresponding to the HN and F glycoproteins under reducing conditions. The fusion glycoprotein was resolved as two major bands (F0, F1). Vesicular stomatitis virus proteins were used as molecular weight markers (lane 4).

adjuvant and injected intramuscularly into rabbits. Rabbits were boosted subcutaneously 2 weeks later. Each rabbit received a total of 45 μg (3 x 15 μg) of the purified glycoprotein. Sera were collected following the last week of immunization.

Other methods. SDS-PAGE, immunoprecipitation (IP), haemagglutination (HA), haemagglutination inhibition (HI), fusion inhibition (FI) of virus-infected BHK cells, plaque neutralization (NT), immunofluorescence (IF) and protein estimations were carried out following the procedures described previously (Ray et al., 1985). Cleveland peptide mapping was performed using Staphylococcus aureus V8 protease (Cleveland et al., 1977). Silver staining of polyacrylamide gels was done following the procedure of Oakley (1980).

RESULTS

Purification of P13 virus envelope glycoproteins

The efficiency of coupling of the monoclonal antibodies to the Sepharose 4B matrix was determined to be about 6 mg Ig per ml of swelled gel. The glycoproteins bound to the affinity
column showed a broad elution peak which was probably due to antibody excess in the matrix. A detergent-soluble crude virus preparation was used initially for affinity purification of \[^3\text{H}\]leucine-labelled envelope glycoproteins, which were analysed by SDS–PAGE followed by fluorography (Fig. 1). Both HN and F appeared as single bands with mol. wt. of about 72 000 (72K) and 54K, respectively, under reducing conditions. Further analysis of the glycoproteins under non-reducing conditions did not alter the position of migration of HN. However, F migrated as a diffuse band at a higher mol. wt. of about 70K, presumably representing the disulphide-linked F1–F2 complex of the fusion protein as has been reported for other paramyxoviruses. We could not usually resolve the lower molecular weight F2 subunit of the glycoprotein under reducing conditions even in a 15% polyacrylamide gel.

To investigate further the purity of the glycoprotein preparations, viral glycoproteins isolated from infected cell lysates were analysed by silver staining (Fig. 2). HN appeared as a major band of 72K and a minor band of about 74K. The minor 74K band may represent trace contamination by the F0 glycoprotein or a different glycosylated form of HN present in the virus-infected cell lysate. The fusion glycoprotein showed two major bands around 74K and 54K, probably representing F0 and F1 respectively. The presence of F0 under reducing conditions is probably due to the lack of cleavage of some of the fusion protein present in the virus-infected cell lysate.

An average yield of 30 \(\mu\text{g}\) HN and 25 \(\mu\text{g}\) F could be recovered from \(10^8\) virus-infected cells or virions purified from \(10^9\) cells following the affinity purification procedure. The purified HN was tested for HA activity with 0·3% chicken red blood cells and showed a titre of 30 HAU/\(\mu\text{g}\) HN.

**Morphology of purified glycoproteins**

Purified glycoproteins eluted from affinity columns were directly concentrated with simultaneous removal of detergent. Electron microscopic examination of the HN glycoprotein

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Fig. 3. Electron micrographs of negatively stained (a) HN glycoproteins and (b) F glycoproteins. Bar marker represents 100 nm for (a) and (b).
Human PI3 virus envelope glycoproteins

Fig. 4. Immunoprecipitation of radiolabelled viral polypeptides with rabbit antisera to affinity-purified HN and F glycoproteins. Radiolabelled polypeptides in immunoprecipitates of [35S]methionine-labelled virus-infected LLC-MK₂ cell lysate with rabbit antisera to HN (lane 2) and F (lane 4) glycoproteins were analysed by SDS-PAGE. Polypeptide profiles of the immunoprecipitates with preimmune rabbit sera to HN (lane 1) and F (lane 3) were similarly analysed to determine any pre-existing antibodies to these glycoproteins. Cleveland peptide maps of the immunoprecipitates of HN (lane 5) and F₀ (lane 6) are shown to demonstrate the differences in these two closely migrating polypeptides.

showed the appearance of small star-shaped aggregates with an amorphous appearance (Fig. 3a). In contrast, the purified F glycoprotein consisted of aggregates of spike-like projections (Fig. 3b).

Antibody response to purified glycoproteins

Rabbit antisera to the affinity-purified fractions were tested by IP to determine the antibody response to the individual glycoproteins. Sera from rabbits immunized with HN or F glycoproteins were each found to precipitate the respective polypeptide bands (Fig. 4). To demonstrate that the two closely migrating polypeptide bands, HN (lane 2) and F₀ (lane 4), were distinct polypeptides, the bands were cut out from the dried gel and analysed by peptide mapping (Cleveland et al., 1977). The S. aureus V8 protease-digested polypeptide cleavage products showed differences in their electrophoretic patterns (lanes 5 and 6), confirming each polypeptide to be unique. Rabbit antisera were also tested for inhibition of the biological activities of the virus by HI, NT and FI tests. The antiserum to the HN glycoprotein showed a reciprocal HI titre of 128 and NT titre of 200 but failed to show any inhibition of virus-induced cell fusion up to an Ig concentration of 1·6 mg/ml of culture medium. On the other hand, rabbit
Fig. 5. Test of rabbit antisera to purified HN or F glycoproteins in fusion inhibition of virus-infected BHK cells viewed by phase-contrast microscopy. (a) Untreated virus-infected cells; (b, c) virus-infected cells incubated with rabbit antisera to (b) HN at an Ig concentration of 1-6 mg/ml culture medium or (c) F at an Ig concentration of 0.8 mg/ml culture medium; (d) uninfected cells.

antiserum to the F glycoprotein did not show a detectable HI titre (8) but had a NT titre of 400 and inhibited fusion of virus-infected cells up to an Ig concentration of 0.8 mg/ml of culture medium (Fig. 5).

Characterization of glycoproteins reconstituted in lipid vesicles

Initially, we attempted to reconstitute the glycoproteins into lipid vesicles by solubilizing both the components in octyl glucoside with subsequent removal of the detergent by dialysis, which yielded aggregated glycoproteins and mostly naked vesicles. This was probably due to irreversible aggregation of the glycoproteins during concentration. We observed that such aggregation could be avoided by directly concentrating the eluted glycoproteins under vacuum in a collodion bag, immersed in buffer containing 2% cholic acid instead of octyl glucoside to remove sodium thiocyanate from the eluted glycoproteins. To prepare reconstituted vesicles, a dried film of phosphatidyl choline and a trace amount of 14C-labelled lipid were dissolved in sodium cholate, and mixed with [3H]leucine-labelled purified glycoproteins solubilized in the same detergent. The sample was concentrated following removal of detergent by dialysis and analysed by electron microscopy. A heterogeneous mixture of aggregated glycoproteins and mostly naked lipid vesicles was observed in the reconstitution experiment with HN (not shown). However, similar experiments with the F glycoprotein yielded lipid vesicles with spike-like projections. In order to purify further the lipid vesicles reconstituted with F, the material was applied on to a 10 to 60% linear sucrose gradient for centrifugation. Positions of lipid and protein were determined from radioactivity of aliquots collected from the gradient (Fig. 6). A parallel control of lipid vesicles without glycoprotein was also run to ascertain the differences in density of the reconstituted glycoprotein vesicles. The reconstituted vesicles appeared as a diffuse band around a density of 1.156 g/ml, as compared to a density of 1.08 g/ml of the naked
Fig. 6. Analysis of F glycoprotein reconstituted in phosphatidyl choline vesicles at a ratio of 1:1 (w/w) on a 10 to 60% linear sucrose gradient. The gradient, in 10 mM-Tris–HCl, 150 mM-NaCl, 0.001% NaN₃, pH 7.6, was centrifuged at 300 000 g for 24 h at 4 °C. Radioactive counts of [3H]leucine-labelled F glycoprotein (upper curve) and 14C-labelled lipid (lower curve) were determined for a 50 μl aliquot of each fraction using Budget-Solve (Research Products International, Mount Prospect, Ill., U.S.A.).

Fig. 7. Electron micrograph of negatively stained reconstituted F glycoprotein on phospholipid vesicles. Bar marker represents 100 nm. Inset shows higher magnification of a single vesicle. Bar marker represents 50 nm.

lipid vesicles. Reconstituted glycoprotein fractions were pooled and dialysed overnight against 10 mM-Tris–HCl, 150 mM-NaCl, 0.01% NaN₃, pH 7.8. After dialysis, the preparation was examined in the electron microscope. The reconstituted vesicles containing the F glycoprotein showed distinct projections radiating from the surface of the lipid vesicles (Fig. 7). The average number of surface projections was of the order of 18 to 20 and their average length was 12 nm.
The diameter of the lipid vesicles usually varied from 40 to 45 nm, while a heterogeneous population with different diameters was observed with control lipid vesicles.

**DISCUSSION**

This report describes the successful purification of the two PI3 virus envelope glycoproteins, HN and F, using specific monoclonal antibodies coupled to Sepharose 4B as the matrix. The glycoproteins are present in the plasma membranes of infected cells and are probably the primary targets for immune recognition. Antibodies to these glycoproteins are expected to prevent initiation and spread of the infection process. In an earlier study, the mixed envelope glycoproteins were evaluated as a subunit vaccine and found to confer protection against challenge infection in hamsters (Ray et al., 1985). Purification of measles virus glycoproteins and the fusion protein of mumps virus has been reported using immunoaffinity chromatography with monoclonal antibodies (Bellini et al., 1981; Varsanyi et al., 1984, 1985; Server et al., 1985). The present work was undertaken to purify the two parainfluenza virus glycoproteins and to obtain them in sufficient quantity for eventual use as a subunit vaccine. Purified HN retained HA activity, although the titre, when compared with virus preparations, was observed to be lower. This probably resulted from aggregation of the purified HN as reported with purified measles virus glycoproteins (Bellini et al., 1981).

Rabbit antiserum raised against purified HN exhibited surface IF with virus-infected cells, neutralization of infectivity and HI. On the other hand, rabbit antiserum to the purified F glycoprotein demonstrated surface IF with infected cells, neutralization of virus infectivity and FI of virus-infected cells. These results indicate that antigenic determinants of the two glycoproteins are conserved during the purification procedures. The eluted glycoproteins showed extensive aggregation during concentration. Similar aggregation was encountered with envelope glycoproteins of SV5 (Scheid et al., 1972). The concentrated glycoproteins were successfully reconstituted into lipid vesicles by using dialysis for removal of cholate. The availability of reconstituted glycoproteins in lipid vesicles should facilitate studies of the biological interactions of F with mammalian cells. The reconstituted vesicles are also of interest for investigation of the cell-mediated immune response, particularly the generation of cytotoxic T cells in animal models, as has been studied with Sendai virus (Finberg et al., 1978), influenza virus (Koszinowski et al., 1980), Semliki Forest virus (Morein et al., 1978) and vesicular stomatitis virus (Loh et al., 1979). The individual subunits of viral glycoproteins have been reported to be less immunogenic when compared with their multimeric forms (Jennings et al., 1974; Morein et al., 1978, 1983). An increase in efficacy of subunit vaccines by reconstitution of the spike proteins into lipid vesicles (virosomes) was suggested to occur because of the formation of multimeric structures with a high surface density of spike proteins, similar to the surface of the virion (Morein et al., 1978). The availability of purified PI3 virus F glycoproteins in the form of reconstituted vesicles will facilitate studies of the immune response to this glycoprotein and formulation of the required proportions of HN and F necessary for an optimum immune response in a subunit vaccine preparation.

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