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Journal of Virological Methods 76 (1998) 1–8

Journal of Virological Methods

Purification of the glycoprotein G from viral haemorrhagic septicaemia virus, a fish rhabdovirus, by lectin affinity chromatography

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Received 5 November 1997; received in revised form 12 January 1998; accepted 12 January 1998

Abstract

A new method for the isolation of glycoprotein G from viral haemorrhagic septicaemia virus (VHSV), a fish rhabdovirus, was developed by using affinity chromatography with immobilized Concanavalin A (ConA). The glycoprotein G was isolated from detergent solubilized concentrated virions and from large-volume virion-free supernatants from VHSV infected cells (soluble form). The purity achieved was higher than 85%. The stimated recovery of the initial glycoprotein G present in the virions was between 20 and 50%. These glycoprotein G preparations showed the presence of about 30% of trimers by ultracentrifugation, reacted with antibodies to the phosphatidylserine binding domain (p2) in a pH-dependent manner by ELISA and bound phosphatidylserine in a pH-dependent manner by solid-phase binding assays. These data suggest that ConA purified glycoprotein G conserved most of its native properties and conformation. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Chromatography; Glycoprotein; Haemorrhagic; Purification

1. Introduction

Enveloped viruses expose proteins that contain carbohydrates at their surface (glycoproteins). Viral glycoproteins have a native polymeric and conformation-dependent structure that plays a crucial role in virus attachment and entry into the cells. Moreover, induction of specific antibody responses to the viral glycoproteins normally leads to protection against infection. Because of the relevance of viral glycoproteins in viral infection, a considerable effort has been spent on their structural and functional analysis, making the availability of purification methods that preserve its native structures a demanding issue (Jones et al., 1994, 1995).

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0166-0934/98/$ - see front matter
PII S0166-0934(98)00028-7

Rhabdoviruses have a lipid bilayer containing conformation-dependent trimers of non-covalently associated monomers of its glycoprotein (glycoprotein G) (Coll, 1995a). The rhabdoviral glycoprotein G has about 500 amino acids (aa), 2–6 glycosylation sites, 12–16 cysteines, two regions of hydrophobic heptad-repeats (Coll, 1995b), a signal peptide absent from the mature protein, and a carboxy transmembrane sequence followed by a short cytoplasmic domain. The glycoprotein G of rhabdoviruses contains about 10% of N-linked carbohydrates (Reading et al., 1978), producing two molecular forms separable by gel electrophoresis in rabies virus (Wunner et al., 1985). Similarly, in the viral haemorrhagic septicaemia virus (VHSV), a fish rhabdovirus, the glycoprotein G of 507 aa (50 kDa of molecular weight of the monomer according to its aa composition) contains carbohydrates (about 5000 kDa) and several glycosylation-dependent forms separable by gel electrophoresis with different apparent molecular weights (Lorenzen et al., 1990). Because Concanavalin A (ConA) (fructose and mannose), wheat germ (*N*-acetyl-glucosamine) or garden pea (glucose and mannose) lectins agglutinated VHSV (Bernard et al., 1983), we attempted the affinity purification of the glycoprotein G of VHSV by using one of those lectins (ConA) as immobilized ligand with the purpose of obtaining a purification method that will help to preserve its native structure as much as possible.

2. Materials and methods

2.1. Purification of VHSV

The VHSV 07.71 isolated in France (LeBerre et al., 1977) from rainbow trout *Onchorynchus mykiss* (Walbaum) was used throughout the experiments. The virus was grown and assayed for infectivity in epithelioma papillosum cyprini (EPC) cells as described previously (Basurco et al., 1991). To prepare purified VHSV, confluent cultures of EPC cells were grown in flasks of 150 cm^2 or roller bottles of 1500 cm^2 (Costar) with RPMI medium supplemented with 10% fetal calf serum as described before (Basurco and Coll, 1989) and inoculated with 10 plaque forming units (PFU) of VHSV 07.71 per cell. After 24 h post-infection, at 14°C the cell monolayer was washed with methionine/cysteine-free medium and incubated in the presence of 33 μCi/ml ^{35}S-trans label (1142 mCi/mol, ICN). The culture was harvested 4 days later and centrifuged at 12000 × *g* for 30 min to remove cell debris. VHSV was precipitated with 7% polyethyleneglycol 6000 (PEG) in 2.3% NaCl at pH 7.8. After overnight agitation at 4°C, the virus was pelleted by centrifuging at 20000 × *g* for 40 min and the supernatants kept at −20°C. The PEG-supernatants contained soluble glycoprotein G as shown after concentration by lyophilization, gel electrophoresis and autoradiography. The pellet was dissolved in 10 mM Tris, 1 mM EDTA and 100 mM NaCl, pH 7.6 (TEN) and frozen at −20°C until used.

2.2. Affinity chromatography

The concentrated VHSV samples were resuspended in 10 ml buffer I (0.1 M sodium acetate, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.5). The samples were sonicated three times, 1 min each in the presence of 1–2% Nomidet NP40, Triton X100 or CHAPS (Sigma). The samples were then passed twice through a Concanavalin-A (ConA) Sepharose (Pharmacia) column preeluted with buffer II (1 M methyl-D-glucopyranoside, 1 M methyl-D-mannopyranoside in buffer I) and then equilibrated with buffer I. Other elution buffers tested contained 20 mM citrate, or 20 mM citrate and 10 mM EDTA, in the absence of the metal salts mentioned above.

To prepare glycoprotein G from PEG supernatants from VHSV infected cells, about 2 l of PEG supernatants were supplemented with the components of the buffer I and then passed through a ConA column (1.5 × 1 cm). The supernatant was passed more than eight times through the column with the aid of a peristaltic pump at room temperature. After washing the column with 80–100 ml of buffer I, the bound glycoprotein G or soluble glycoprotein G, respectively, were eluted with elution buffer II. The fractions containing the glycoprotein G were pooled, dialyzed against 10 mM ammonium bicarbonate and concentrated by

lyophilization. Analysis of the bound glycoprotein G by polyacrylamide gel electrophoresis (PAGE), immunoblotting or autoradiography were performed as described (Estepa, 1992; Estepa et al., 1994). For autoradiography the gels were fixed in isopropanol/H_2O/acetic acid (25/65/10) for 15 min and incubated in fluorographic liquid (Amplify, Amersham, UK) for 30 min before drying and exposure to X-ray films (Agfa, Madrid, Spain).

Lectin purified glycoprotein G (10 μg) was loaded in a 15–30% sucrose continuous gradient in 10 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 7.5 (TEN) and ultracentrifuged at $124000 \times g$ during 41 h to differentiate between trimers (~165 kDa) and monomers (~55 kDa). Bovine serum, albumin (BSA 68 kDa), aldolase (ALD, 158 kDa), catalase (CAT, 240 kDa) and ferritin (450 kDA) were used as molecular weight markers run in parallel and in separated tubes.

2.3. Solid-phase phospholipid binding assays

Just prior to the assay, the labelled L-3-phosphatidyl-(L-C3-^{14}C) Serine, 1,2-dioleoyl, phosphatidylserine (PS) of 55 mCi/mmol (Amersham, UK) dissolved in organic solvents was dried in vacuo in glass tubes. A phosphate–citrate buffer was prepared from 0.1 M citric acid and 0.2 M dibasic sodium phosphate stock solutions at different pHs. After the addition of the phosphate–citrate buffer to the dried phospholipids, the mixture was sonicated for three 1 min periods in the cold. The aqueous suspension was immediately used for the subsequent binding assays (Coll, 1995c). To prepare the solid-phases, the purified glycoprotein G was diluted in distilled water to 2–40 μg/ml. Then, 100 μl/well were dried overnight at 37°C in 96-well plates (polystyrene from Costar/Nunc). Plates were kept at -20°C in a dried atmosphere until used. Just prior to use, the coated plates were washed with distilled water. Then, sonicated labelled phospholipids in phosphate–citrate buffer were pipetted in 100 μl of volume per well. After 4 h of incubation at 4°C the plates were washed three times with distilled water, and incubated with 100 μl/well of 2% SDS at 60°C during 30 min. The supernatants (100 μl/well) were then transferred into 96-well polyethylene terephthalate counting plates (Wallac-Pharmacia), the counting plates pipetted with Hiload-scintillation liquid (100 μl/well) (LKB, Loughborough, UK), mixed and counted on a 1450-Microbeta scintillation counter (Wallac, Oy, Turku, Finland and Pharmacia Iberica).

2.4. Enzyme linked immunosorbent assays (ELISA)

Balb-C mice polyclonal antibodies against the phospholipid-binding peptide p2 (aa 82–109) from VHSV (Coll, 1995c), were obtained as myeloma X63/Ag8653 induced ascites after following classical mice immunization protocols (Freund's adjuvant) as described previously (Estepa and Coll, 1996a). The p2 region was shown to be the major PS-binding domain of the glycoprotein G, to be related to fusion and to bind to VHSV in solution in a pH dependent manner (Estepa and Coll, 1996a,b). Mice ascites containing anti-p2 were then purified by affinity chromatography over p2-activated CH-Sepharose-4B column (Sigma).

The ELISA assays were carried out as described before (Sanz and Coll, 1992). Briefly, serial dilutions of ConA purified glycoprotein G were dried onto polystyrene solid-phases and allowed to react with 0.3 μg of p2 affinity purified anti-p2 per well at pH 5.5 or at pH 7.5. After a washing step, development was with rabbit anti-mouse immunoglobulins conjugated with peroxidase and *o*-phenylenediamine as described previously (Sanz and Coll, 1992).

As a control for denatured glycoprotein G, inclusion bodies of recombinant G4 (devoid of signal peptide and transmembrane region) were obtained in yeast as described previously (Estepa et al., 1994; Coll, 1995c; Lorenzo et al., 1995). This recombinant G control was chosen because of the larger amounts available compared with the lectin purified glycoprotein G that could be isolated by ConA. Large amounts (~20 mg of initial glycoprotein G were needed to obtain about 1 mg of pure completely denatured form of G. The G4 inclusion bodies were treated with 4 M guanidinium isothiocianate, 0.1 M β-mercaptoethanol at 100°C with constant agitation during 4 h. The

resulting clear supernatant was further purified by preparative gel electrophoresis (PAGE). The band corresponding to the glycoprotein G was cut and the protein electroeluted in 0.1 M ammonium bicarbonate. After lyophilization, the purified recombinant glycoprotein G4 was completely soluble in TEN and it was kept frozen until use.

3. Results

To isolate the glycoprotein G from concentrated VHSV the first step was the removal of the lipid membrane by detergents, a step which could change the glycoprotein G conformational structure. In a series of preliminary binding experiments using labelled purified VHSV, it was found out that treatment of the virions with different detergents increased the amount of radioactivity bound to ConA from 49% (non-treated) to 75% (2% CHAPS), 79% (2% Nonidet NP40) or 91% (2% Triton X100) (data not shown).

To carry out the rest of the experiments, CHAPS was chosen since it has been reported to preserve best the trimeric structure of glycoprotein G from rabies virus (Gaudin et al., 1992). However, about 30% of the glycoprotein G still remained with the VHSV nucleocapsids after a 30-min treatment with 2% CHAPS, as estimated by autoradiography of the material pelleted by ultracentrifugation. Attempts to release more glycoprotein G by increasing the CHAPS treatment to 60 min or the CHAPS concentration to 4% resulted in the failure of the ConA column to retain the glycoprotein G protein (not shown). Therefore a 2% CHAPS was used for the rest of the experiments. Fig. 1 shows the relative absorbance at 280 nm (A280 nm) of the material which passed through the column (VHSV nucleocapsids and contaminating bovine serum albumin as shown by electrophoresis of the pooled fraction) and that which was retained and subsequently eluted from the column by 20 mM citrate (VHSV G glycoprotein). By densitometric scan analysis of the bands obtained by gel electrophoresis and autoradiography of the material retained and eluted from the column, a 3% final recovery of the initial ^{35}S-labelled glycoprotein G was estimated. However, proteins N/Nx contaminated the preparation of glycoprotein G and three more non-labelled bands appeared at 40, 17 and 15 kDa. These non-labelled bands were due to ConA leaking from the column as shown by parallel gel electrophoresis of ConA (not shown).

Better elution conditions were obtained by using 1 M glucopyranoside, 1 M mannopyranoside rather than by using buffers containing 20 mM citrate or 20 mM citrate and 10 mM EDTA. Maximal yields were obtained when a volume of the elution buffer was recirculated through the column during 16 h. Further improvement of the method led to the finding that pre-elution of the ConA column with the sugar containing buffer (buffer II) almost completely prevented both binding of N/Nx proteins to the lectin and leaking of ConA (Fig. 2). Fig. 2 also shows the purity of the glycoprotein G obtained and the decrease of intensity in the glycoprotein band of the material not retained by the ConA column respect to the initial material. By using all the conditions men-

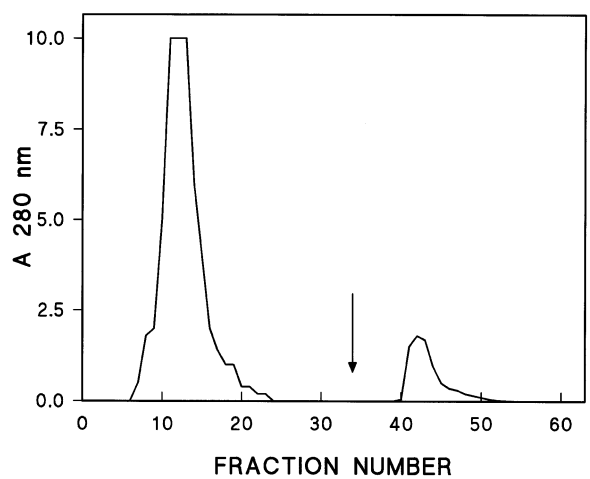

Fig. 1. Affinity chromatography over ConA-Sepharose of purified VHSV. 10 ml of concentrated VHSV were incubated with 1% Triton X100 for 30 min. After adding 0.1 M sodium acetate, 1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and adjusting the pH to 7.5 they were chromatographed. After washing with 85 ml of buffer I, the glycoprotein G was eluted with the same buffer but containing no metal salts (Ca, Mg and Mn) and 0.2 M sodium citrate. Fractions of 2.5 ml were collected. The arrow means the point at which the wash buffer I was changed for elution buffer II. Similar results were obtained by treatment of purified VHSV with 2% CHAPS.

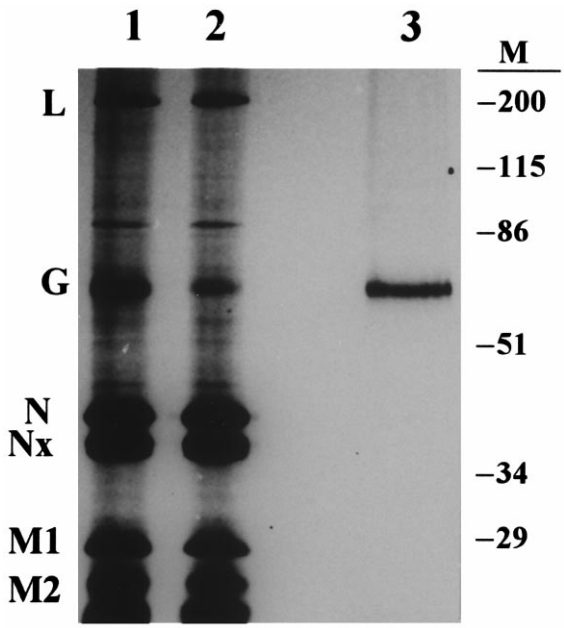

Fig. 2. Autoradiography of polyacrylamide gel electrophoresis of the ^{35}S-labelled VHSV glycoprotein G purified by affinity chromatography over ConA. The radioactive samples were subjected to electrophoresis on a 12% acrylamide gel. 1, purified VHSV; 2, viral proteins not retained by the ConA-Sepharose column; 3, material bound and eluted from the column. M, molecular weight protein standards co-electrophoresed are indicated on the right on kDa. The identification of the VHSV proteins is indicated on the left.

tioned above a final recovery of about 20% of the initial amount of glycoprotein G present in the purified VHSV was obtained as estimated by densitometric scan of the bands in the PAGE and quantification of the radioactivity in the samples.

That soluble glycoprotein G was present in the supernatants from labelled VHSV infected cells after PEG precipitation (PEG supernatants), was shown by concentrating the supernatants 20-fold followed by gel electrophoresis and subsequent autoradiography of the gel. A band at about 55–60 kDa was radioactive (not shown). Affinity chromatography of 2 l of PEG supernatants over a ConA column and subsequent elution of the retained material showed a peak of radioactivity in a band at 55–60 kDa when analyzed by PAGE (not shown). The peak of eluted radioactivity was coincident with the A280 nm peak with an estimated yield of about 50% of the total soluble glycoprotein G initially present in the PEG supernatant. About 1 mg of purified soluble glycoprotein G could be obtained by ConA chromatography of 2 l of the PEG supernatant.

To characterize preliminarily the lectin-purified glycoprotein G obtained after ultracentrifugation, anti-p2 binding and PS-binding assays were performed. Prolonged ultracentrifugation showed that at least about 30% of the purified glycoprotein G was in the form of trimers (peak 2 in the representative experiment in Fig. 3). The presence of bands at the molecular weight of glycoprotein G (peak 2) was confirmed by immunoblot of the fractions with polyclonal antibodies anti-VHSV (not shown). Anti-p2 binding and PS binding were estimated by solid-phase assays by coating plates with different amounts of lectin-purified soluble glycoprotein G and completely

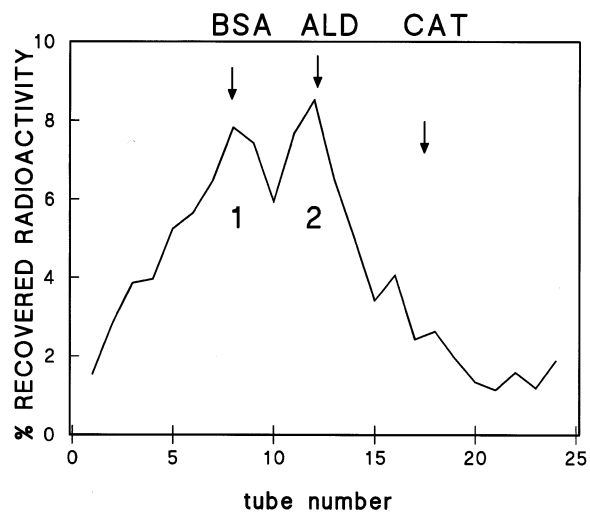

Fig. 3. Ultracentrifugation profiles of soluble G isolated from ConA-Sepharose chromatography. About 10 μg of soluble G isolated by ConA chromatography from labelled VHSV infected cell supernatants were loaded in a 15–30% sucrose continuous gradient in TEN and ultracentrifuged at 124000 × g for 41 h. BSA, bovine serum albumin (68 kDa). ALD, aldolase (158 kDa). CAT, catalase (240 kDa). Ferritin (450 kDa) was recovered at the bottom of the ultracentrifugate tube (fraction number 24). Peak numbers 1 (fractions 6–8) and 2 (fractions 11–14) correspond to the stimated molecular weights of monomers (~55 kDa) according to its aa composition (50 kDa) plus its carbohydrate content (5 kDa) and trimers (~165 kDa), respectively.

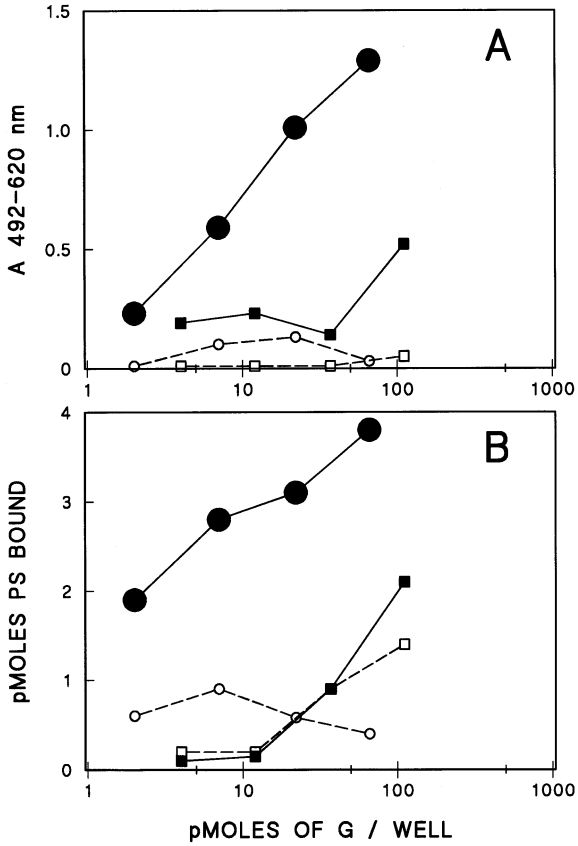

Fig. 4. PH-dependence of anti-p2 (A) and phosphatildylserine (B) binding to soluble glycoprotein G isolated by ConA chromatography and to denatured recombinant G4. Soluble glycoprotein G isolated by ConA-Sepharose chromatography (●, ■) or recombinant denatured G4 (○, □) were bound at different concentrations into solid-phase polystyrene wells. A unique concentration of anti-p2 antibodies (A) or radioactively labelled PS (B) were added to the solid-phase glycoproteins at a pH of 5.5 (●, ○) or 7.5 (■, □), respectively (Section 2). Recombinant G4 was denatured, purified and used as a conformationally altered glycoprotein G because of the limited amounts available of soluble glycoprotein G isolated by ConA chromatography. – • –, Binding at pH 5.5 to soluble glycoprotein G isolated by ConA chromatography. – ◦ –, Binding at pH 5.5 to recombinant denatured glycoprotein G4. – ■ –, binding at pH 7.5 to soluble glycoprotein G. – □ –, Binding at pH 7.5 to recombinant denatured glycoprotein G4.

denatured recombinant G4. The number of glycoprotein G molecules per VHSV virion were calculated from densitometry of the autoradiography of electrophoresed concentrated labelled VHSV and estimated to be about 300 pmol of glycoprotein G per 200 μg of concentrated VHSV. Anti-p2 antibodies obtained in mice against the major PS-binding domain of the glycoprotein G bound to the lectin-purified glycoprotein G in solid-phase and showed a low pH-requirement which was absent from completely denatured recombinant G4 (Fig. 4A). Binding of labelled PS to lectin-purified glycoprotein G in solid-phase also showed a low pH requirement which was absent from completely denatured recombinant G4 (Fig. 4B).

4. Discussion

This report describes a one step technique to carry out rapid purification of conformationally-active glycoprotein G from purified VHSV by ConA affinity chromatography. The existence and purification of the soluble form of the glycoprotein G released to the cell culture medium by the VHSV infection is also preliminarily described.

In the purification method reported above mild detergent conditions (2% of CHAPS) were employed as described for the rabies virus glycoprotein G (Gaudin et al., 1992). Evidence for the presence of about a 30% of trimers in the lectin-purified glycoprotein G was obtained by ultracentrifugation analysis in sucrose gradients of labelled glycoprotein G (Fig. 3) and immunoblot of the fractions. A certain amount of glycoprotein G aggregates and/or whole virions at the bottom of the gradient were also detected (not shown). The major problem encountered was copurification of the VHSV N/Nx protein, but this was later overcome successfully by preeluting the column with elution buffer (Fig. 2). Although the protein N does not possess carbohydrates, because of the mild solubilization conditions used, the partially disrupted virions containing both G and N might bind to ConA. It is also possible that a certain degree of non-specific interaction with the ConA-sepharose bed column occurs.

An elegant reported alternative, affinity chromatography to isolate VHSV glycoprotein G made use of immobilized specific monoclonal antibodies (Lorenzen, 1992), however no characterization of the conformation of the glycoprotein G

obtained were reported. Both the binding of anti-p2 antibodies (antibodies against the major PS-binding region of the glycoprotein G of VHSV) and the binding of phosphatidylserine (PS) by the lectin-purified glycoprotein G were dependent on pH as shown before for purified VHSV (Estepa and Coll, 1996a,b), suggesting a high the degree of conformational conservation of the lectin purified glycoprotein G.

About 50% of the total soluble glycoprotein G present in virion-free supernatants obtained from PEG-concentrated VHSV could be removed from the supernatants by ConA affinity chromatography. A soluble form of the glycoprotein G of VSV (Chatis and Morrison, 1983; Cheng and Huang, 1986) and of rabies (Dietzschold et al., 1983) had been also described to accumulate in the extracellular medium of cells infected in vitro (for VSV, one every six glycoprotein G molecules made in infected cells is secreted to the medium). Similarly to the VSV and rabies soluble glycoprotein G (Dietzschold et al., 1983; Florkiewicz et al., 1983) purified from VHSV-depleted extracellular medium was about 5 kDa smaller than the virus associated glycoprotein G (not shown), due to the absence of the carboxy terminal region which contains the hydrophillic cytoplasmic and the membrane-anchoring hydrophobic domains (Browning et al., 1990). The results obtained here demonstrate that soluble glycoprotein G is also produced during VHSV infections suggesting that a similar phenomenon occurs in this fish rhabdovirus.

Throughout conformational changes, the rhabdoviral G trimers are responsible for virus attachment to cell receptors, for low-pH dependent membrane fusion (Gaudin et al., 1993; Konieczko et al., 1994; Lecocq-Xhonneux et al., 1994) and for reacting with host neutralizing antibodies (Kelley et al., 1972; Engelking and Leong, 1989; Lorenzen et al., 1990). However, trimer-specific MAbs have not yet been described for any rhabdoviruses, probably because the glycoprotein G isolated under a variety of conditions is often found to be monomeric (Lyles et al., 1990). Antigenic differences could exist between monomers and trimers, which may have implications for further vaccine developments. The availability of mild isolation methods like the one described here to isolate pure and as close as native glycoprotein G as possible, would help to the study of all the phenomena mentioned above.

Acknowledgements

We appreciated the help of J.P. Coll in typing. This work was supported by Research Grants CT94-1334 from the AIR2 Program of the European Economic Community, from the INIA project SC94-102 (Spain) and from CICYT projects AGF95-910 and AGF97-297 (Spain). L.P. was funded by the Spanish Ministerio de Educación y Cultura/INIA research contract.

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