Isolation and Purification of a Polymeric Form of the Glycoprotein of Rabies Virus

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SUMMARY

Of the three major proteins associated with the rabies virus membrane, only the glycoprotein was found to be located on the external surface of the virus membrane. Glycoprotein prepared by treatment of rabies virus with Triton X-100 and purified by isoelectric focusing was found to be homogeneous with respect to size and isoelectric point. This material, which is free of phospholipids, is able to protect in vaccination experiments against a lethal challenge infection with rabies virus. The apparent mol. wt. of this component isolated under non-denaturing conditions is approx. 400000. The same material analysed by SDS polyacrylamide gel electrophoresis (PAGE) was found to consist solely of polypeptide chains of the G protein (mol. wt. 80000). A minor glycoprotein (gp 50), detected by PAGE of the Triton X-100 released material, appeared to be a breakdown product of the G-protein. Therefore the detergent released material represents homopolymers of the G-protein. Whether the antigenic determinants reside on the monomeric subunit or are a property of the polymeric form of the G-protein is discussed.

INTRODUCTION

The membrane of rabies virus is composed of a lipid bilayer and one glycoprotein (Sokol et al. 1971). Whether the two non-glycosylated proteins M1 and M2 are essential constituents of the virus membrane remains to be clarified. The G-protein contains three oligosaccharide side chains that account for about 11% of its total mass (Dietzschold 1977). Recently Atanasiu et al. (1976) and Cox et al. (1977) were able to solubilize rabies glycoprotein using the non-ionic detergent Triton X-100. This glycoprotein preparation not only reacts with and induces the production of rabies neutralizing antibodies but also protects mice in vaccination experiments from a challenge infection with rabies virus (Atanasiu et al. 1976; Cox et al. 1977). The glycoprotein vaccine had similar or better protective activity than a vaccine made from inactivated virus (Cox et al. 1977). To understand the immunological properties of the glycoprotein preparation released by non-ionic detergent, it is necessary to investigate the chemical purity and complexity of this material.

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The present report describes the structural organization of the envelope of rabies virus and the isolation and purification of the rabies virus G-protein. The data obtained here indicate that the glycoprotein of rabies virions is located on the surface of the virus membrane. The glycoprotein fraction released by Triton X-100 is homogeneous, as determined by isoelectric focusing and Sephadex G200 chromatography, and consists of homopolymers of the G-protein.

METHODS

Preparation of virus. The ERA strain of rabies virus was grown in roller cultures of BHK-21 S13 cells (Stoker & McPherson, 1964). Culture conditions, virus assays and purification techniques have been described (Wiktor et al. 1977). To prepare amino acid labelled virus, 48 h after infection the culture medium was replaced by minimal essential medium supplemented with 0.3% bovine serum albumin and 5 µCi/ml 3H-leucine or 0.5 µCi/ml 14C-amino acid mixture. For labelling of virus lipids with 32P the culture medium was replaced 48 h after infection by minimal essential medium lacking unlabelled phosphate and supplemented with 20 µCi/ml 32P-orthophosphate. The cells were then incubated at 37 °C for 24 h.

External labelling of rabies virus proteins. For labelling of the proteins located on the external surface of the virus envelope the method of Rifkin et al. (1972) was used.

Isolation and purification of the rabies virus glycoprotein. Treatment of rabies virus with Triton X-100 was done principally as described by Helenius & Söderlund (1973). Triton X-100 was added to a suspension of purified rabies virus to a final concentration of 2%. The mixture was kept at room temperature for 20 min and then chilled in an ice bath and centrifuged in a Beckman SW50.1 rotor at 45000 rev/min for 60 min at 4 °C. The supernatant containing the glycoprotein was extensively dialysed against 1% (w/v) glycine and 1% (w/v) glycerol in distilled water. After addition of 1% ampholine (pH 3.5 to 10), a 5 ml sample was layered on a 5 to 40% (w/v) sucrose gradient containing 1% ampholine (pH 3.5 to 10) and 0.1% Triton X-100. The preparation was electrofocused for 72 h in an LKB 110 ml column. Fractions (1 ml) were collected and each fraction was tested for pH and radioactivity.

SDS polyacrylamide gel electrophoresis. Electrophoresis of virus polypeptides was carried out in a discontinuous polyacrylamide system having a 4% (w/v) stacking gel on top of a 10% (w/v) resolving gel as described by Laemmli (1970). Purified virus or virus components were precipitated with 5 vol. of ethanol and solubilized with a small vol. of 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol. Prior to application to the gels, samples were incubated for 1 min in a boiling water bath. After electrophoresis the gels were sliced into 1 mm sections and individual slices were heated with Protosol (New England Nuclear) at 37 °C before adding scintillation fluid Omnifluor (New England Nuclear) toluene cocktail.

In order to isolate proteins from SDS polyacrylamide gels, the gels were sliced and the proteins were eluted from the individual slices with 0.05 M-NH4HCO3, pH 8.1, and 0.1% SDS. The peak fractions were pooled and lyophilized.

Preparation of tryptic peptides of glycoproteins. The 3H-leucine labelled gp 50 and 14C-leucine labelled G-protein were purified by PAGE. The lyophilized proteins were suspended in NTE buffer (0.01 M-tris-HCl, pH 7.5, 0.1 M-NaCl, 0.001 M-EDTA). Bovine serum albumin (0.5 mg) was added and the suspension was heated at 100 °C for 1 min with 1/1000 vol. of a mixture of 2-hydroxyethylidisulphide and 2-mercaptoethanol (50:1). The sample was then precipitated with 5 vol. ethanol, dissolved in 8M-urea-0.05 M-tris-HCl (pH 7.5) and re-precipitated with ethanol. This precipitate was suspended in 1 ml 0.05 M-
NH₄HCO₃. Freshly prepared 1% TPCK-trypsin in 0.001 N HCl was added. After incubation at 37 °C for 2.5 h another 50 μl of 1% TPCK-trypsin was added and the incubation was continued for 2.5 h. The sample was then lyophilized.

Analysis of tryptic peptides by cation exchange chromatography. Tryptic peptides of the glycoproteins were dissolved in pyridine acetate buffer, pH 2.5, and applied to a chromobead-column (Chromobeads, Type P, Technicon Chemical, S.A.). The tryptic peptides were eluted with a pH gradient from pH 2.5 to pH 4.5 as described by Vogt et al. (1975). The eluted samples were dried at 100 °C, re-dissolved in 0.01 N HCl and counted directly in Formula 963 (NEN).

Gel filtration chromatography. The glycoprotein was released with Triton X-100 from purified ³H-leucine-labelled virus and purified by isoelectric focusing. The purified glycoprotein (pH 7.0-material) was dissolved in 0.05 M tris buffer, pH 7.8, and 0.5 M NaCl and 0.1% Triton X-100. A 0.5 ml sample was layered on a Sephadex G200 column (0.9 by 80 cm) equilibrated with 0.05 M tris-HCl, pH 7.8, 0.5 M NaCl and 0.1% Triton X-100. Fractions (1 ml) were collected and counted directly in Formula 963 (NEN). Blue dextran 2000 was used as a marker for void volume. The Sephadex G 200 column was calibrated by determining the elution volume (Ve) of the following proteins of known molecular weights: ferritin, catalase, aldolase and bovine serum albumin. The marker proteins were detected by the method described by Bramhall et al. (1969).

RESULTS

Electrophoretic analysis of the rabies virus polypeptides

Fig. 1 shows SDS gel electropherogram of purified rabies virus grown in the presence of either ¹⁴C-amino acids or ³H-glucosamine. A single virus G-protein and a second smaller glycoprotein, designated gp50, were detected.
Fig. 2. Polyacrylamide gel electrophoresis of rabies virus polypeptides after pyridoxylation and reduction with NaB\(_3\)H\(_4\). (a) A suspension of purified rabies virus was treated with pyridoxal phosphate and reduced with NaB\(_3\)H\(_4\) (---) or labelled with \(^{14}\)C-amino acid (-----) (b). Rabies virus was treated with 2% Triton X-100, then pyridoxylated and reduced with NaB\(_3\)H\(_4\). ---, NaB\(_3\)H\(_4\)-labelled virus. ----, \(^{14}\)C-amino acid-labelled rabies virus.

**Organization of the proteins of rabies virions**

To study the localization of the two glycoproteins, purified rabies virus was incubated with pyridoxal phosphate and then reduced with \(^3\)H-sodium borohydride. Since phosphate esters cannot penetrate biological membranes, the \(^3\)H incorporation is limited to proteins on the external surface of the membrane that possess exposed amino groups. Fig. 2(a) shows that after pyridoxylation and NaB\(_3\)H\(_4\) reduction of rabies virus, G protein, gp50 and each of the two membrane proteins incorporated, respectively, 73\%, 10\% and 8\% of the radioactivity. The small amount of \(^3\)H-incorporation into the M proteins is probably due to a non-specific reaction, since a similar amount of radioactivity was found in the M proteins when rabies virus was reduced with NaB\(_3\)H\(_4\) without prior pyridoxylation (data not shown). When purified rabies virions were first treated with 1\% Triton X-100, incubated with pyridoxal phosphate, then reduced with NaB\(_3\)H\(_4\) (Fig. 2b), 37\% of the \(^3\)H was incorporated.
in the M1 and M2 proteins, indicating that the low incorporation of 3H in the M proteins of intact virions is not due to a lack of available NH₂-groups. From the observation that in intact rabies virions it is predominantly the glycoproteins which have amino groups that can form Schiff bases with pyridoxal phosphate, we suggest that only the glycoproteins are exposed on the surface of rabies virus particles.

**Isolation and purification of rabies virus glycoproteins**

In order to characterize the two glycoproteins of rabies virus, we attempted to isolate and purify these proteins in a biologically active form. After treatment of rabies virus with Triton X-100 at a protein : detergent ratio of 1 : 10, on the basis of mass, 18% of the virion proteins and 93% of the virion phospholipids were solubilized. Fig 3(a) shows that
Fig. 4. Isoelectric focusing of proteins released by Triton X-100 treatment of rabies virus. Rabies virus labelled with $^3$H-leucine and $^{32}$P-orthophosphate was purified as described in Methods. Triton X-100 (2% w/v) was added to a suspension of purified virus in NTE buffer (0·1 M-NaCl, 0·05 M-tris-HCl, pH 7·5, 0·001 M-EDTA). The mixture was incubated for 20 min at room temperature then centrifuged in a Beckman SW50.1 rotor at 40000 rev/min for 60 min. The resulting supernatant was extensively dialysed against 1% glycine and 1% glycerol and a 5 ml sample was layered on a 5 to 40% sucrose gradient containing 0·1% Triton X-100 and 1% ampholine (pH 3·5 to 10). The preparation was electrofocused for 72 h in an LKB 110 ml column. Fractions were collected and assayed for pH (○○○○), $^3$H activity (●●●●) and $^{32}$P activity (---).

G-protein represents the major fraction of the solubilized virion protein which also contains gp50 and small amounts of M1 and M2 protein. To achieve a further purification of the glycoproteins, the material released with Triton X-100 was subjected to isoelectric focusing. Fig. 4 shows the isoelectric profiles of Triton X-100-released material from rabies virus labelled with either $^3$H-leucine or $^{32}$P-orthophosphate. The $^3$H-leucine-labelled material is focused in a major peak corresponding to a pH value of 7·0. A second smaller peak of $^3$H activity can be detected at pH 4·7. The main activity of $^{32}$P was focused on a region between pH 7·8 and 8·5. The $^{32}$P peak is almost completely separated from the $^3$H peak which indicates that the material focusing at pH 7·0 is essentially free of phospholipids.

Electrophoretic analysis and biological characterization of isolated rabies virus proteins

The materials focusing at pH 7·0 and 4·7 were analysed by polyacrylamide gel electrophoresis (PAGE). In Fig. 3(b), the protein pattern of the material focusing at pH 4·7 is shown. The pH 4·7 material was heterogeneous; it contained M1, M2 and gp50 as well as small amounts of G-protein. However, when the material focusing at pH 7·0 was analysed by PAGE only the glycoproteins (G-protein and gp50) could be detected (Fig. 3c). When the materials focusing at pH 7·0 and pH 4·7 were tested for their capacity to bind neutralizing antibodies and to protect mice from challenge infection with rabies virus, only the
Rabies virus glycoprotein

Fig. 5. Analysis by cation exchange chromatography of tryptic peptides of the rabies virus glycoproteins G (---) and gp50 (○○○). G-protein was prepared from 14C-leucine-labelled rabies virus by SDS polyacrylamide gel electrophoresis. To obtain gp50, 3H-leucine-labelled rabies virus was treated with Triton X-100 and the Triton-released material was subjected to isoelectric focusing as described in Fig. 4. The material focusing at pH 7 was then resolved on 10% SDS gels. The radioactivity associated with the 14C-leucine-labelled G-protein and the 3H-leucine-labelled gp50 was eluted from the SDS gels. The G-protein and the gp50 were treated with TPCK trypsin as described in Methods and the tryptic digest was applied to a chromo bead column. The tryptic peptides were eluted with a pyridine acetate gradient pH 2.5 to 4.5. The eluted samples were dried at 100 °C, re-dissolved in 0.01 N-HCl and counted directly in Formula 963 (NEN). ○—○, pH.

material focusing at pH 7.0 was able to perform both activities. Nine ng of purified G-protein (pH 7.0 material) protected 50% of the mice compared with 1630 ng of undisassociated virus.

Analysis by cation exchange chromatography of tryptic peptides of the rabies virus glycoproteins G and gp50

In order to determine the relationship between the two glycoproteins, the tryptic peptides of G-protein and gp50 were analysed by cation exchange chromatography. 14C-leucine-labelled G-protein was eluted from SDS gels and 3H-leucine-labelled gp50 was isolated by isoelectric focusing of Triton X-100-released material followed by SDS gel electrophoresis. After ethanol precipitation, the glycoproteins were digested with trypsin and the trypsin digestion products of G-protein and gp50 were applied to a column of chromo beads type P and eluted with a pH gradient of 2.5 to 4.5. Fig. 5 shows an analysis of 14C-labelled tryptic peptides of G-protein and 3H-labelled tryptic peptides of gp50. A comparison of the tryptic peptides obtained from G-protein and gp50 shows that all peptides of gp50 co-migrate with glycopeptides of G-protein. However, the observation that G-protein is resolved into a higher number of components than gp50 suggests that the gp50 is a breakdown product of G-protein.

Chromatography of isolated rabies glycoprotein on Sephadex G200

To investigate whether the glycoprotein released by Triton X-100 and purified by isoelectric focusing represents the monomeric form or a complex of the 80000 dalton G-protein detected by PAGE, this material was chromatographed on Sephadex G200 in the presence of
Fig. 6. Chromatography of isolated rabies glycoprotein on Sephadex G200. The glycoprotein was released with Triton X-100 from purified \(^2\)H-leucine-labelled virus and purified by isoelectric focusing as described in Methods. The purified glycoprotein (pH 7.0) was dissolved in 0.05 M-tris buffer, pH 7.8 and 0.5 M-NaCl and 1% Triton X-100. A 0.5 ml sample was layered on a Sephadex G200 column (0.9 by 80 cm) equilibrated with 0.05 M-tris-HCl, pH 7.8, 0.5 M-NaCl, and 1% Triton X-100. One ml fractions were collected and counted directly in Formula 963 (NEN). Blue dextran 2000 was used as a marker for void volume. The Sephadex G200 column was calibrated by determining the elution volume (Ve) of the following glycoproteins of known molecular weights: ferritin (■), catalase (□), aldolase (▲) and bovine serum albumin (△). The marker proteins were detected by the method described by Bramhall et al. (1969).

0.1% Triton X-100. As shown in Fig. 6, the glycoprotein migrates in a single symmetrical peak slightly behind the ferritin marker. We have estimated the mol. wt. of this material to be approx. 400,000. The elution volume of the Triton X-100 solubilized glycoprotein fraction was neither influenced by the concentration of Triton X-100 (0.1 to 1.0%) nor by using different mild ionic and non-ionic detergents such as sodium deoxycholate and Nonidet P40.

DISCUSSION

Since it is known that the nucleocapsid of rabies virus only induces the formation of complement binding and precipitating antibodies (Schneider et al. 1973) it was the purpose of our investigations to isolate and characterize components of the virus membrane which are responsible for eliciting the production of neutralizing antibodies and protecting in vaccination experiments against a challenge infection with rabies virus. The results presented here allow us to conclude that a polymeric form of the rabies virus glycoprotein represents the antigen which induces and reacts with rabies virus neutralizing antibodies and protects mice, in vaccination experiments, from challenge infection with rabies virus. We have estimated the mol. wt. of the glycoprotein-complex, released by Triton X-100 and purified by isoelectric focusing, to be 400,000.
Although the elutions of the marker proteins were not significantly changed in the presence of Triton X-100, the effect of this detergent on the elution characteristics of the rabies virus glycoprotein is unknown. Since conformational differences may also exist between the rabies virus glycoprotein and the marker proteins used in this procedure, it is difficult to determine precisely the mol. wt. of the glycoprotein preparation. However, the discrepancy between the mol. wt. measured by SDS gel electrophoresis (80,000) and that measured by Sephadex G-200 chromatography (400,000) appears to be too great to be due only to differences in conformation. A more reasonable interpretation would be that the high mol. wt. form resolved under non-denaturing conditions by Sephadex G-200 gel chromatography represents a homopolymer of the G-protein.

Various mild detergents of different chemical composition such as Nonidet P-40 and sodium deoxycholate in different concentrations had no influence on the size of the glycoprotein complex. Therefore it seems to be unlikely that these complexes represent artifactual aggregates of undenatured G-protein.

Using several crosslinking agents, Dubovi & Wagner (1977) demonstrated recently that the glycoprotein spike of vesicular stomatitis virus is composed of more than one G-protein. The present results suggest that the rabies virus glycoprotein has a polymeric subunit structure, but the exact number of subunits has not been determined.

The observation that the M proteins are not exposed on the surface of the virus is consistent with our immunological analysis that showed that the M proteins do not elicit the production of neutralizing antibodies (Cox et al. 1977). Since it is clear that rabies virus glycoprotein is solely responsible for inducing the production of neutralizing antibodies, it remains to be determined whether these antigenic determinants reside on the monomeric subunit or are a property of the polymeric form of the G-protein.

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REFERENCES


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