Biochemical Characterisation of an Epitope on the Surface Membrane Antigen (Cs-gp200) of the Pathogenic Piscine Haemoflagellate Cryptobia salmositica Katz 1951

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INTRODUCTION

Monoclonal antibodies have been used to characterize specific epitopes on antigens of parasites. These include rhoptry proteins of Plasmodium falciparum (see Burns et al. 1989; Ahlborg et al. 1993; Sam-Yellow and Ndengelf 1993) and the major surface antigen of Toxoplasma gondii (Velge-Roussel et al. 1994). A few monoclonal antibodies are protective and the antigens that they recognize can be used to develop vaccines (Boyle et al. 1982; Oikawa et al. 1992; Saul et al. 1984).

Cryptobia salmositica is a haemoflagellate which causes disease and mortality in salmonids. It has been found in all species of Pacific salmon on the west coast of North America (Woo 1994). The clinical signs of cryptobiosis include exophthalmia, general oedema, splenomegaly, abdominal distention with ascites, and anorexia (Woo 1979; Li and Woo 1991; Woo and Thomas 1992). Cryptobiosis may result in high mortality in experimentally and naturally infected salmonids (Woo 1987). The parasite has also been identified as a lethal pathogen in seminatural and intensive salmon culture facilities on the Pacific coast of North America (Bower and Thompson 1987).

Little is known about the antigenic composition of C. salmositica. Using polyclonal antibodies, Woo and Thomas (1991) showed that C. salmositica has 21 polypeptide bands (21–200 kDa). The identification and characterization of individual antigenic bands could not be done using polyclonal antibodies. We have developed a monoclonal antibody (mAb-001) which recognizes a glycoprotein (Cs-gp200) on the surface of C. salmositica (see Feng and Woo 1996).

The present study was designed to characterize an epitope on Cs-gp200 on C. salmositica.
MATERIALS AND METHODS

Preparation of antigen. The crude antigen was prepared as described earlier (Feng and Woo 1996). Briefly, *C. salmositica* from the blood of an infected rainbow trout was cultured for about 10 weeks at 10°C in minimum essential medium (MEM) supplemented with 25% foetal bovine serum (Woo and Li 1990). Parasites were harvested by washing three times at 4°C (centrifugation at 10,000 g for 15 min each time) in cold-blooded vertebrate Ringer's solution (CBVR) and resuspended in cold CBVR. Parasite numbers were determined using a haemocytometer (Archer 1965). After the final wash, parasites were lysed in Ringer’s solution containing 0.5% Triton X-100, 2 mM ethylendiaminetetraacetic acid (EDTA), 2 mM phenylmethylsulphonyl fluoride (PMSF), and 100 μg/ml leupeptin, vortexed for 10 sec, and then centrifuged at 7000g for 10 min at 4°C after the last washing. The supernatant containing soluble protein was saved and total protein concentration was determined according to Bradford (1976). The sample was used immediately for immunoblot and lectin blots or stored at −100°C.

Electroelution was used to isolate the antigen from SDS gels. Briefly, SDS gel slices which contained protein (200 kDa) were loaded into an Electro-elutor (Model 422, Bio-Rad) and the protein was eluted at 8–10 mA/glass tube constant current for 5–8 hr using Tris-glycine buffer (Yu and Greenwood 1994). After elution, the electroelutor was removed from the buffer tank, and the buffer in the tube was carefully removed. The silicone adaptor, together with the membrane cap, was removed from the bottom of the glass tube and the liquid which contained the eluted protein in the membrane cap was pipetted into a microfuge tube. The membrane cap was rinsed with another 200 μl of fresh elution buffer and added to the microfuge tube. The eluted protein was run on SDS gel again to check for contamination with other proteins. The eluted protein without contamination was used for amino acid sequence and characterization using ELISA. The protein concentration was determined according to Bradford (1976) and then the sample was used for ELISA or stored at −100°C.

Monoclonal antibody. The monoclonal antibody was produced in an earlier study (Feng and Woo 1996). Briefly, BALB/c mice were immunized with 1 × 10⁷ live *C. salmositica*, and hybridomas were produced as described by Os and Herzenberg (1979) with some modifications (Lam et al. 1987). Hybridomas were selected with HAT medium and incubated at 37°C in a 5% CO₂ incubator, and screened for production of antibodies against *C. salmositica* using enzyme-linked immunosorbent assay (ELISA). The positive hybridomas were then cloned by the limited dilution and using the “single cell pick” method of Harlow and Lane (1988). One positive hybridoma was selected and the antibody it produced was designated mAb-001 and used in this and other studies (Feng and Woo 1996). mAb-001 was isotyped as an IgG1 and it has an ELISA optical density (OD) value of 0.49 before concentration.

Electrophoresis and immunoblotting. Crude parasite proteins were separated according to the discontinuous gel system of Laemmli (1970). The antigen (90 μg/ml protein) was mixed with an equal volume of a reducing buffer and heated at 95°C for 10 min. Samples were electrophoresed at 20°C in 10% running gel at 100 V for 1 hr. Gels were stained with Coomassie brilliant blue or used for immunoblotting. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Towbin et al. 1979). Electrophoretic transfer was carried out for 2 hr at 100 V. The membrane was blocked with 5% skim milk in Tris buffered saline (TBS), washed in TBS, and incubated in unconcentrated hybridoma supernatant overnight at room temperature. A nitrocellulose membrane incubated in culture medium was used as negative control. The membranes were washed in Tween 20-Tris-buffered saline (TTBS) followed by TBS (two washes each, 5 min/wash) and then incubated for 1 hr in goat anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad) diluted 1:3000 in 3% skim milk/TTBS. Membranes were washed as before and incubated in enzyme substrate for 20 min.

Reduction and carboxymethylation (Talbot et al. 1985). The antigen was coated on ELISA plates. Sample 1 was not denatured, reduced, or carboxymethylated; Sample 2 was denatured with 8 M urea in 0.2 M Tris-HCl with 1 mM EDTA (pH 8.2) for 60 min; Sample 3 was denatured 8 M urea for 60 min and then reduced with 1.5% DTT for 60 min at room temperature. Sample 4 was denatured with 8 M urea and then reduced with 1.5% DTT and carboxymethylated with 4% iodoacetic acid in the dark for 60 min to prevent reassociation of the SH groups during the subsequent reactions. All reactions were carried out at room temperature (22°C) and excess reagents were removed by washing with TBS. The wells were then blocked with 5% skim milk in TBS and screened with the monoclonal antibody. Hybridoma culture medium was used as a negative control.

Limited proteolysis. The plate with the coated protein was treated with proteinase K (EC 3.4.21.14) in 50 mM Tris-HCl, pH 8.0, or trypsin (EC 3.4.21.4) in 50 mM Tris-HCl, pH 7.5, α-chymotrypsin (Type 1-S; from bovine pancreas, Sigma, EC 3.4.21.1) in 50 mM Tris-HCl, pH 7.8, or protease V8 (EC 3.4.21.9) in 50 mM phosphate buffer, pH 7.8, respectively, at different concentrations for 60 min at 37°C. After incubation, the enzymes were inactivated with 15% TCA for 30 min at room temperature. Negative control wells for enzyme digestion received the same amount of buffer in place of protease. All treatments were done in triplicate.

Periodate oxidation. Periodate oxidation of the antigen was carried out as described by Woodward (1985). The 96 wells of a microplate were coated with antigen (100 μl, 5 μg/ml) and then rinsed with 50 mM sodium acetate buffer (pH 4.5). They were then exposed to varying concentrations of periodate (0–20 mM) in 50 mM sodium acetate buffer, pH 4.5, for 1 hr at room temperature in the dark. Following a rinse with 50 mM sodium acetate, the wells were then incubated with 50 mM sodium borohydride in TBS for 30 min at room temperature. Both the periodate and the borohydride solutions were prepared just prior to use. After five washes with TTBS, the plate was sequentially blocked with 3% skim milk and incubated with mAb-001, goat anti-mouse immunoglobulin conjugated with alkaline phosphatase and the substrate.

Deglycosylation (glycosidases digestion). The parasite-protein-coated ELISA plate was treated with O-glycosidase (from *Diplococcus pneumoniae* in a buffer containing 0.1 M sodium phosphate, 10 mM EDTA, 0.5% Triton X-100, 0.05% SDS, and 1% 2-mercaptoethanol, EC 3.2.1.97), or N-glycosidase F (from *Flavobacterium meningosepticum* in the buffer similar to that for O-glycosidase, EC 3.2.2.18), or neuraminidase (from *Arthrobacter ureafaciens* in 0.1 M sodium acetate buffer, pH 5.0, EC 3.2.1.18), or α-mannosidase (from jack bean, *Canavalia ensiformis* in ammonium sulphate solution, pH 6.0, EC 3.2.1.24) for 60 min at 37°C. The enzymes were inactivated with 15% TCA for 30 min at room temperature. Negative control wells received the same amount of buffer in place of glycosidase. All treatments were done in triplicate.

Deglycosylation was also performed using Western blot. The polypeptides transferred onto nitrocellulose were digested with endoglycosidase H (gene cloned from *Streptomyces plecatus* and expressed in
Escherichia coli, EC 3.2.1.96) and endoglycosidase F (from F. meningoseptium, EC 3.2.1.96). The blotted protein was also treated with neuraminidase, α-galactosidase (from green coffee beans in 100 mM potassium phosphate buffer at pH 6.0, EC 3.2.1.22) and α-mannosidase sequentially to determine the type of N-glycan. The strips of nitrocellulose then were subsequently blocked with 3% skim milk, incubated with either mAb-001 or lectin from Galanthus nivalis and then with either goat anti-mouse IgG or anti-digoxigenin enzyme conjugate and substrate.

Terminal sugar determination. The terminal sugar on the epitope was determined using a carbohydrate differentiation kit [it contains five lectins: GNA (G. nivalis agglutinin), SNA (Sambucus nigra agglutinin), MAA (Maackia amurensis agglutinin), PNA (peanut agglutinin) and DSA (Datura stramonium agglutinin)] from Boehringer Mannheim Biochemica (Boehringer Mannheim Canada, Laval, Quebec). The Con A (concanavalin A from Canavalia ensiformis), ECA (Erythria cristagalli agglutinin), HPA (Helix pomatia agglutinin), LcH (Lens culinaris), PCA (Phaseolus coccineus agglutinin), PWM [Phytolacca americana agglutinin], LA (lotus agglutinin), WGA (Triticum vulgaris agglutinin), and PSA (Pisum sativum agglutinin) were from Sigma (Sigma-Aldrich Canada Ltd., Missisauga, Ontario). Briefly, antigens were transferred to nitrocellulose after electrophoresis. Strips were blocked with a blocking agent for 1 hr, washed with TBS, and incubated subsequently with digoxigenin-labelled or biotin-labelled lectins, anti-digoxigenin, or streptavidin (conjugated with alkaline phosphatase) for 1 hr. Finally, the strips were incubated with phosphatase substrate to develop a grey to almost black colour to visualize the terminal sugar.

Deacylation (hydrolysis with potassium hydroxide and phospholipase C). Hydrolysis of the acyl moiety on the epitope was carried out on the protein coated on the plate. Fifty microliters of 0.1 M KOH in 20% methanol was added and incubated for 90 min at room temperature and then neutralized with 1 M HCl. The plate was rinsed three times with TBS (pH 7.5) before it was blocked with 5% skim milk.

Phospholipase C (from Bacillus cereus, phosphatidylinositol-specific, EC 3.1.4.10) in 0.1 M sodium phosphate buffer (pH 7.7) at different concentrations (0.05, 0.1, 0.5, and 1 U) was added to the wells.

Negative control wells for enzyme digestion received the same amount of buffer in place of glycosidase, and the negative control wells for antigen and antibody binding had no mAb-001 before they were incubated with the second antibody.

RESULTS

Reduction and carboxymethylation. After denaturation with 8 M urea (Sample 2), the epitope was still recognized by mAb-001, i.e., the ELISA OD values were similar to those in Sample 1 without treatment. However, the ELISA OD values were significantly lowered after denaturation and reduction (Sample 3) and a similar result was obtained after denaturation, reduction, and carboxymethylation with 4% iodic acid (Sample 4). There were no reactions in the negative control (Fig. 1).

Proteinase hydrolysis of Cs-gp200. The ELISA OD values were significantly lowered after exposure to 5 μg/ml protease K (Fig. 2), and higher concentration of protease K did not further reduce the ELISA OD values. The treatment with 5 μg/ml of trypsin was the optimum to initiate hydrolysis by the enzyme and increased concentrations of trypsin continuously reduced the ELISA OD values. In addition, the ELISA OD values decreased after protease V8 digestion. There were no declines in ELISA OD values after α-chymotrypsin treatment. There was also no decline in ELISA OD values in the negative controls for all enzyme digestions.

Carbohydrate moieties of Cs-gp200. ELISA OD values were significantly reduced from 0.42 to 0.125 with increasing concentrations (0.0, 0.5, 1.0, 5.0 mM) of sodium periodate. Complete loss of antibody binding occurred in 10 mM periodate and greater than 90% inhibition occurred at 5 mM. No reactions were obtained with hybridoma culture medium (negative control).

Cs-gp200 was subjected to digestion with N-glycosidase F. ELISA OD values were drastically reduced, but there
FIG. 2. ELISA OD values after site-specific and nonspecific proteinase digestion of the epitope. Open square, α-chymotrypsin; solid square, trypsin; open circle, protease V8; solid circle, protease K; open diamond, negative controls screened with hybridoma culture medium.

FIG. 3. ELISA OD values after digestion of glycosidase. Solid square, digested with N-glycosidase F with concentration of 0.0, 0.5, 1.0, 2.0, and 4.0 U/1 ml; Solid circle, digested with O-glycosidase with concentration of 0.0, 0.005, 0.01, 0.02, and 0.04 U/1 ml; open circle, screened with mAb-001 without enzyme digestion. Solid triangle, screened with hybridoma culture medium.

were no reductions in ELISA OD values in positive and negative controls for N-glycosidase digestion. There were also no reductions in ELISA OD values after O-glycosidase digestion (Fig. 3). These results indicate that one or more N-linked glycans comprise the epitope in Cs-gp200 recognized by mAb-001.

Because N-linked glycan has at least three structure types (high mannose, hybrid, and complex), Cs-gp200 was treated with glycosidases specific for high-mannose and hybrid types of N-glycans and with sequential deglycosylation using glycosidases specific for terminal sialic acid, α-D-mannose, and β-D-galactose. After N-glycosidase F digestion, there were no reactions between lectin GNA and the antigen, however, mAb-001 still recognized the 200-kDa band. Similar results were seen after endoglycosidase H and endoglycosidase F digestions (Fig. 4a). After neuraminidase, α-mannosidase, or galactosidase digestion, the intensity of reactions between antigen and lectin GNA was reduced, but the intensity of reactions between antigen and mAb-001 was not affected (Fig. 4b, lanes b, c, and d). After sequential digestion with neuraminidase/galactosidase, lectin GNA still recognized two bands (Fig. 4b, lane e). However, after galactose/α-mannosidase and neuraminidase/galactosidase/α-mannosidase digestion, the antigen was not recognized by the lectin GNA.

There was no difference between neuraminidase/galactose oxidase/α-mannosidase sequential digestion and galactosidase/α-mannosidase sequential digestion (Fig. 4b, lanes f and g). This sequential deglycosylation did not affect the reaction of mAb-001 with the polypeptide moiety of the antigen. These results indicate that the carbohydrate chain on the antigen is likely a hybrid-type N-glycan which consists of galactoses and mannose.

The antigen had a very strong reaction with GNA which recognizes terminal mannose with α(1–3) or α(1–6) or α(1–2) link to another mannose. There was a weak reaction with concavalin A, which is specific for mannose and also for glucose. There were no reactions between the antigen and SNA, MAA (for sialic acid); PNA, ECA (for galactose); WGA, PWM (for N-acetylglucosamin); HPA (for N-acetyl-D-galactosamine); lotus agglutinin (for α-L-fucose) and PCA, LcH (for mannose). These provide further confirmation that the N-glycan attached to the antigen has a terminal mannose and probably a galactose as well.

Deacylation. There were significant reductions in ELISA OD values after treatment with 20 mU phospholipase C (Fig. 5). Increasing the concentrations of phospholipase did
FIG. 4. Immunoblots after glycosidase digestion on the epitope screened with lectin GNA. (A) N-Glycosidase digestion: lane a, positive control of glycoprotein; lane b, membrane extraction of *C. salmositica*; lane c, membrane extraction of *C. salmositica* digested with N-glycosidase F (Endo F free); lane d, membrane extraction digested with endoglycosidase F (N-glycosidase free); lane e, membrane extraction digested with endoglycosidase H. (B) Sequential glycosidase digestion (neuraminidase, galactose oxidase, and mannosidase) on the epitope. (A) Screened with lectin GNA: lane a, positive control of glycoprotein; lane b, digested with neuraminidase; lane c, galactosidase; lane d, mannosidase; lane e, neuraminidase and galactose oxidase; lane f, galactosidase and mannosidase; lane g, neuraminidase, galactosidase and mannosidase; The numbers on the left are the molecular weights (kDa) of protein standards (Bio-Rad, high range).

FIG. 5. ELISA OD values after phospholipase C digestion of the epitope. Open circle, positive control without digestion or mild alkaline treatment; Solid circle, digested with different concentration (0.0, 0.05, 0.1, 0.5, and 1.0 U/ml) of phospholipase C; solid square, treated with different concentrations (0.0, 0.05, 0.1, 0.5, and 1.0 M) of KOH; open square, negative control screened with hybridoma culture medium.

not markedly decrease the ELISA OD values. Since phospholipase C is specific for phosphatidylinositol, the result suggests that part of the epitope is phosphatidylinositol, which forms a phospholipid anchor attached to the glycoprotein and anchors the glycoprotein to the cell membrane.

After treatment of antigen with 0.05–1.0 M KOH in 20% methanol, the ELISA OD values were lowered with increasing concentration of KOH. Significant reduction of OD value occurred with 0.1 M KOH, and 0.5 M KOH completely removed the antibody/antigen reaction (Fig. 5). There were no changes in ELISA OD values in control wells without hydrolysis. This indicates that there were fatty acids attached on the antigen and they were removed by mild alkaline treatment.

**DISCUSSION**

The immunological reactivity of the 200-kDa antigen was lost after denaturation, and reduction, indicating that the epitope on Cs-gp200 recognized by mAb-001 has a higher order, conformational structure. Under certain conditions, the reduced protein may be restored to its native conformation and consequently its immunological or biological activity may also be restored. However, if the free SH groups are blocked after reduction, then the protein is unable to fold back to its native conformation (Fries *et al.* 1989).
The polypeptide nature of Cs-gp200 was confirmed by the loss of reactivity by mAb-001 after digestion with protease K (nonspecific protease), trypsin (a lysine and arginine site-specific proteinase), and protease V8 (glutamine and asparagine site specific proteinase).

Periodate oxidation has been used to characterize surface-exposed epitopes on Chlamydomonas flagellar membrane (Woodward et al. 1984) and to denature a wide range of parasite carbohydrate moieties (Lustigman et al. 1990; Omer-Ali et al. 1986; Ravindran et al. 1990; Zihao et al. 1991). An alteration of the electrophoretic mobility and a reaction of protein bands are indications of carbohydrate degradation. In the present study, it was shown that antibody binding decreased with an increased (0.5–5 mM) concentration of sodium periodate and binding was completely lost with 10 mM periodate oxidation. The significant reduction of ELISA OD values in the present study confirms the cleavage of the carbohydrate moiety on the epitope. However, interpretation of periodate sensitivity must include the possibility (although remote) of periodate-mediated oxidation of disulfide bonds (Cecil 1963) to sulphonic acid residues which may alter the antigenicity of the target epitope. In addition, periodate affects the affinity of substrate for enzyme-binding sites (Bergamini et al. 1983) by chemical modification of the active sites.

We have shown that mAb-001 epitope is not sensitive to O-glycosidase but is sensitive to N-glycosidases. This confirms that the carbohydrate component existed as an N-linked glycan attached to the peptide by means of asparagine. Since N-glycosidase F cleaves all types of asparagine bound N-glycans, we assume that both the amino group and the tein.carboxyl group are present in the peptide linkage and that the oligosaccharide has the minimum length of a chitobiose- glycan. The sequential deglycosylation with neuraminidase, galactosidase, and mannosidase or with galactosidase and mannosidase abolished the reaction between the antigen and the lectin, indicating that the carbohydrate chain is composed of mannos and galactose.

ELISA OD values were significantly reduced after the antigen was treated with mild alkali, indicating the presence of covalently linked fatty acids in the epitope. Fries et al. (1989) demonstrated myristic and palmitic acids on the epitope of 25 kDa macrogamete/zygote protein on P. falciparum and these fatty acids are susceptible to mild alkaline treatment. At present, we have no further data on the fatty acid composition of Cs-gp200, although myristic acid is found in VSG in pathogenic trypanosomes of mammals (Ferguson et al. 1985; Lamont et al. 1987) and a mixture of myristic and palmitic acids in the 63-kDa protease of Leishmania major (Etges et al. 1986) and in the 195-kDa merozoite antigen of P. falciparum (see Halder et al. 1985).

The epitope on Cs-gp200 was also sensitive to phospholipase C (phosphatidylinositol-soluble). This suggests the presence of phospholipid which contains a phosphatidylinositol moiety and a glycosyl phosphoryl inositol (GPI) membrane anchor on Cs-gp200 antigen. Fatty acids on the protein with GPI released by phospholipase C have been reported from Leishmania, Trypanosoma, Plasmodium and Toxoplasma (see Halder et al. 1985; Ferguson et al. 1985; Bordier et al. 1986; Nagal and Boothroyd 1989). The cleavage of Cs-gp200 with phospholipase C may result in the generation of a hydrophilic form of the protein. The fatty acids contributed to the structure of the epitope recognized by mAb-001; however, we have not investigated whether the acyl moieties induce or stabilize the conformation of the protein.

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EPITOPE ON 200-kDa MEMBRANE GLYCOPROTEIN OF C. salmositica


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