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Chapter

Behaviour of a Sialo-Oligosaccharide from Glycophorin in Teleost Red Blood Cell Membranes

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Abstract

Glycophorins (GPs) in red blood cell (RBC) membranes of carp (Cyprinus carpio L.) exhibit bacteriostatic activity against various gram-negative and gram-positive bacteria including fish pathogens. This physiological property also exists in the GPs of yellow tail (Seriola quinqueradiata) and red sea bream (Pagrus major). Thus, we concluded that this antimicrobial activity is not confined to these teleost species but can be found in all fish. This bacteriostatic activity is caused by the sialo-oligosaccharide from these teleost GPs. Only the N-glycolylineuraminic acid (NeuGc) form of sialic acid was detected in the carp. Using NMR and GC–MS, we determined that the structure of the bacteriostatic sialo-oligosaccharide from carp was NeuGcα2→6(Fucα1→4)(Glcα1→3)Galβ1→4GalNAc-ol. The bacteriostatic activity of this monosialyl-oligosaccharide is due to the property of the lectin receptor. It is supposed that some lectin-like proteins exist on the surface of gram-positive bacteria or the flagellum of gram-negative bacteria. Based on the electron microscope observations, teleost GPs containing the sialo-oligosaccharide are released from RBC membranes and then adsorbed onto the surface or the flagellum of invading bacteria in the blood.

Keywords: teleost, carp, yellow tail, red sea bream, red blood cell membranes, glycophorin, antibiotic activity, oligosaccharide, sialic acid

1. Introduction

The blood of mammals such as humans, as well as birds, reptiles and teleosts, contains red blood cells (RBCs; erythrocytes). Human RBCs are the most commonly studied cells for structural and physiological analysis. While human RBCs are approximately 7 μm in diameter, and their centre has a dented discoid shape, teleost RBCs are slightly larger than human RBCs, have nuclei, and have a not dented orbicularity or oval shape (Figure 1) [1, 2].

Studies on biological membranes normally use human RBCs since they have no nuclei and are easy to obtain for RBC membrane preparation after the hemolysis procedure. For the preparation of RBC membrane proteins, it is necessary to use detergents for the solubilization of the phospholipid bilayer. Fairbanks et al. [3]
developed a method in which RBC membranes were solubilized by sodium dodecyl sulfate (SDS), and then the extracted membrane proteins were separated on polyacrylamide gel by electrophoresis (SDS–PAGE). Using the method of SDS-PAGE, major membrane proteins and glycoproteins in RBCs could be detected on SDS gels. However, minor components in human RBC membranes such as substoichiometric proteins (e.g., CD44, CD47, Lu, Kell, Duffy), are not detected clearly on SDS-gels [4].

**Figure 2.** Illustration showing the location of band 3 and GPs in human RBC membranes. This illustration is based on several reviews [4–6]. According to Lux [4], the location of substoichiometric proteins remains unclear. In this illustration, these glycoproteins are omitted, the actin junctional complex (4.1R complex) is simplified, and the topology of band 3 and glycophorins is defined.
2. Membrane proteins of the human and teleost RBC membranes

By using SDS–PAGE, membrane proteins could be detected on SDS gels by staining with Coomassie brilliant blue (CBB). Figure 3 Lane 2 shows typical human RBC membrane proteins separated by SDS–PAGE using the method of Laemmli [7], which was later improved by Fairbanks et al. [3]. The number depicts the nomenclature of each cell membrane protein according to Fairbanks et al. [3]. Band 3, band 4.1 and band 4.2 are currently called the proper names. Band 3, which is an anion transporter as AE1, is detectable as a diffuse band on the SDS-gel due to the microheterogeneity of the oligosaccharides attached to GPs [8]. Although Band 3 is a glycoprotein, it is detectable on SDS gel using protein staining with CBB. This is attributed to a small amount of oligosaccharides in band 3 compared to the protein amount. Approximately 7% of carbohydrates have been contained in Band 3, and contributes to 10% of the total membrane carbohydrate [9].

We examined the membrane proteins in the RBCs of carp (Cyprinus carpio L.) (Figure 3 Lane 5), yellow tail (Seriola quinqueradiata) and red sea bream (Pagrus major) (Figure 4 Lanes 2 and 5) by SDS–PAGE. By comparison with molecular mass standards, the prominent protein bands of carp, yellow tail and red sea bream RBC membranes were band 3, band 1 (spectrin α chain) and band 2 (spectrin β chain), and band 4.1, band 5 (actin) and band 7 were designated for human membrane proteins. The general profiles of the CBB stain pattern on the SDS-gels were not strikingly different compared to that of human RBC membranes.

In the red sea bream, the presence of lipids led to broadening of the low-molecular-weight bands (Figure 4 Lane 5). In the yellow tail membranes, spectrin bands were fainter than those in other fish species (Figure 4 Lane 2). It is suggested that the cysteine protease, cathepsin L, hydrolysed these cytoskeletal fibers. Ahimbisibwe et al. [10] reported the presence of cathepsin L in the RBC membranes of several fish species [10].

Figure 3.
SDS-PAGE of carp and human RBC membranes. (a) Coomassie brilliant blue R-250 (CBB)- and PAS-stained human RBC membranes. Lane 1, CBB-stained molecular mass standards: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase b (97 kDa); bovine albumin (66 kDa); egg albumin (45 kDa); and carbonic anhydrase (29 kDa). Lane 2, CBB-stained human RBC membranes. Lane 3, PAS-stained human RBC membranes. (b) CBB- and PAS-stained carp RBC membranes. Lane 4, CBB-stained molecular mass standards. Lane 5, CBB-stained carp RBC membranes. Lane 6, PAS-stained carp RBC membranes. GP, glycophorin. The number denotes the membrane protein designations for human RBC membrane proteins. Approximately 30 μg of membrane protein were applied per lane.
Figure 4. SDS-PAGE of yellow tail and red sea bream RBC membranes. (a) CBB- and PAS-stained yellow tail RBC membranes. Lane 1, CBB-stained molecular mass standards. Lane 2, CBB-stained yellow tail RBC membranes. Lane 3, PAS-stained yellow tail RBC membranes. (b) CBB- and PAS-stained red sea bream RBC membranes. Lane 4, CBB-stained molecular mass standards. Lane 5, CBB-stained red sea bream RBC membranes. Lane 6, PAS-stained red sea bream RBC membranes. GP, glycophorin. The number denotes the membrane protein designations for human RBC membrane proteins. Approximately 30 μg of membrane protein were applied per lane.

Figure 5. Basic structures of O-linked oligosaccharides of GPs of mammalian and carp origins.
teleosts (carp, amberjack and red sea bream), and the specific activity of cathepsin L was highest in the RBC membranes of amberjack, followed by carp and red sea bream. Aoki and Ueno [11] also reported that cathepsin L in mackerel muscle significantly hydrolysed myofibrils.

3. Glycophorins of human RBC membranes

Apart from the membrane proteins that Fairbanks et al. designated, glycophorins (GPs) exist as transmembrane glycoproteins that contain sialic acid. These sialo-oligosaccharide-rich glycoproteins are detectable on SDS-gels by staining with periodic acid-Schiff (PAS) reagent [3, 12]. Figure 3 Lane 3 shows the nomenclature of human RBC membrane sialo-glycoproteins. These GPs are found in the RBC membranes of humans [13–16] and other mammals [17–20] and birds [21, 22]. In human RBC membranes, GP A (dimer) is observed below band 3 on SDS-gels (Figure 3 Lane 3). GP A is a major component of red cell membrane glycoproteins. The electrophoretic migration of GP on SDS gels is relatively low when compared to other membrane proteins because GP is heavily glycosylated. Although the molecular mass of the other membrane proteins can be estimated by migration on SDS–PAGE, each GPs molecular mass cannot be estimated in this manner.

GPs C and D are thought to link to the band 4.1 protein and connect the cytoskeleton structure under the phospholipid bilayer [23–26]. These GPs C, D, and band 3 are associated with the cytoskeleton closely and contributed to the maintenance of the shape and mechanical properties of the RBC after passing through capillary vessels [27]. This information suggested that GPs C and D are anchored to the RBC membrane by the cytoskeleton. In contrast, it is believed that GPs A and B are not associated with the cytoskeleton, thus enabling them to be easily released from the RBC membrane [28].

While GP C and its shorter form, GP D, are antigenically distinct from GPs A and B. GP C carries several blood group antigens (Gerbich, Yus, Wb, Ana, Dha, and others) [29–31]. According to Podbielska et al. [32], O-linked oligosaccharides isolated from GP A carry the A, B, or H blood group antigen. Although these oligosaccharides reacted with ABH blood group antigens, the reaction was estimated at a relatively low level. Moreover, GP A-deficient RBCs did not clearly demonstrate the physiological role of GP A [33].

4. Glycoproteins of teleost RBC membranes

We examined the GPs in the RBCs of carp (Figure 3 Lane 6), yellow tail and red sea bream (Figure 4 Lanes 3 and 6) by SDS–PAGE under the same conditions as the human preparation, followed by staining with PAS reagent.

The PAS-stained bands in all teleost preparations were stained poorly in the same way as avian GPs [34]. The carp and yellow tail RBC membrane preparations yielded one major band on the SDS-gels (Figures 3 Lane 6 and 4 Lane 3). The PAS stain pattern on the SDS-gels suggests that the carp and yellow tail RBC membranes had fewer forms of GP than the human RBC membrane. The main carp GP was located near the position of the carp and human band-3 proteins. In addition, the main carp GP was positioned near human GP A (dimer).
In the red sea bream, one major band and a faint band with lower molecular weight were observed (Figure 4 Lane 6). Aoki et al. [35] detected some GP bands in rainbow trout preparation. There was one major band and two faint bands with lower molecular weights were detected. It was presumed that the major band was the GP dimer, while the faint band beneath the major band and the faint band with lower molecular weight were the incomplete polymer and monomer forms respectively. It is suggested that the major band in the red sea bream was a polymer form.

While membrane protein patterns (CBC-stained band patterns) are generally similar to those in humans, GP patterns (PAS-stained band patterns) are different in humans. These differences are caused by the components containing sialo-oligosaccharides.

5. Structure of a sialo-oligosaccharide from carp RBC membranes

We examined the amino acid composition of carp GP followed by the kind of sialic acid. While the amino acid composition was not strikingly different compared to that of human GP A, with the exception of valine, lysine and arginine, only the N-glycolyneuraminic acid (NeuGc) form of sialic acid was detected in the carp GP by TLC and a colorimetric method [36].

There are several reports on the sialic acid component of mammals sources of GP. In humans, GP contains N-acetylneuraminic acid (NeuAc), whereas the presence of NeuGc has been reported in GPs of horses [37], bovine [38], pig [39], and monkeys (Macaca fuscata) [19] and others. However, to date, little is known about sialic acid in teleost GPs. Compared to the sialic acids from nonhuman sources, this result suggested that teleosts contained the NeuGc, not as NeuAc. Interestingly, NeuGc has also been reported in the eggs of rainbow trout, chum salmon and land-locked cherry salmon [40].

The carbohydrate fraction of carp GP was separated into two components (P-1 and P-2) using a Glyco-Pak DEAE column with a continuous linear gradient of 0–100 mM NaCl. This fraction contained at least two kinds of O-linked oligosaccharides. Based on the chromatogram obtained using a NeuAc oligomer (α,2→8), the electro-negativity suggested that P-1 contained one sialic acid residue, whereas P-2 contained two residues [36].

We obtained ca. 190 μg P-1 and ca. 70 μg P-2 (as total carbohydrate) by HPLC from carp GP (ca. 4.0 mg protein). Using the graphite carbon column with ammonium bicarbonate in acetonitrile solution as an eluent, the yield of desalted oligosaccharide was satisfactory (P-1: ca. 90% and P-2: ca. 100%) [36].

These O-linked oligosaccharides (P-1 and P-2) were composed of glucose, galactose, fucose, N-acetylglactosamine (GalNAc) and NeuGc. Using NMR and GC–MS, we determined that the structures of P-1 and P-2 were NeuGcα2→6(Fucα1→4) (Glcα1→3) Galβ1→4GalNAc-ol [41] and NeuGcα2→6(Fucα1→4)(Glcα1→3)(NeuGcα2→2) Galβ1→4GalNAc-ol, respectively (Figure 5). These O-linked oligosaccharides were unique to vertebrates with respect to the hexosamine and hexose linkages and their nonchain structure.

Human GPs contain O-linked sialo-oligosaccharides, and the structure of these oligosaccharides has been analysed [42]. The most commonly elucidated GP oligosaccharides from mammals sources are reported as below: tetra-saccharide core, NeuAcα2→3Galβ1→3(NeuAcα2→6)GalNAc-ol; tri-saccharide cores, Galβ1→3(NeuAcα2→6)GalNAc-ol or NeuAcα2→3Galβ1→3GalNAc-ol (Figure 5).
O-linked oligosaccharides containing NeuGc have also been reported among horse, pig, and rabbit GPs, and the most commonly reported structure is a trisaccharide, \( \text{Gal} \beta 1 \rightarrow 3(\text{NeuGc} \alpha 2 \rightarrow 6) \text{GalNAc-ol} \) [20]. Other derivatives are synthesized by attaching NeuGc and Gal residues to the trisaccharide core [28].

Although Glc residue in O-linked oligosaccharides has not been reported to detect in mammalian [20] and chicken GPs [43], Guérardel et al. [44] reported that O-glycans synthesized by nematodes contained the Glc residue, whereas the Fuc residue was detected in the O-linked oligosaccharides of human GP A [32].

From the NMR spectra obtained using the asialo P-1 fraction, the characterized proton signals revealed an overall downfield shift in the resonance of \( \alpha \text{Glc} \) and \( \alpha \text{Fuc} \), except for the H-1 signals [41]. This O-linked oligosaccharide indicates a globule form rather than chain-like structure. Furthermore, the linkage between Gal and GalNAc-ol is 1→4, unlike the 1→3 standard linkage for O-linked oligosaccharides. The 1→4 linkage of GalNAc is unique compared with other O-linked oligosaccharides of mammals sources of GP. Interestingly, the glycosidic linkage of xylan from the seaweed cell wall is the \( \beta 1 \rightarrow 3 \) unlike the standard \( \beta 1 \rightarrow 4 \) linkage of xylan from land plants [45]. It seems possible to detect the \( \beta 1 \rightarrow 4 \) linkage of GalNAc in marine organisms.

6. Physiological activity of GPs from carp, yellow tail and red sea bream

We performed a sensitivity test using GP preparations from the carp RBC membranes. The sensitivity test for the growth of test bacteria was performed using the disc-plate method [36, 46]. All of the test bacteria (gram-positive bacteria: \textit{Micrococcus luteus} and \textit{Bacillus subtilis}, gram-negative bacteria: \textit{Aeromonas hydrophila}, \textit{Vibrio anguillarum}, \textit{Pseudomonas fluorescens}, \textit{Edwardsiella tarda} and \textit{Escherichia coli}) formed inhibition zones around the paper disc containing the GP fraction (Figure 6). For \textit{E. tarda}, the inhibition zones were observed over a light box to discern the production of FeS from SS agar medium (Figure 6f). \textit{M. luteus} and \textit{E. coli} produced yellow pigments and white pigments, respectively (Figure 6d and g). While the outer zone of \textit{M. luteus} and \textit{E. coli} did not produce pigments, the inner zone represented growth inhibition. In contrast, the inhibition zone was not formed around the paper discs containing PBS (Figure 6g).

To clarify the physiological activity of teleost fish GPs other than those from carp, we performed a sensitivity test for the growth of \textit{M. luteus} using GP preparations from the RBC membranes of yellow tail and red sea bream (Figure 7) [47]. These results showed that not only carp GP preparations but also yellow tail and red sea bream GPs had antibiotic activities.

Compared with the profile of forming an inhibition zone, these results also suggested that the yellow tail or red sea bream GPs have a broad-spectrum antibiotic activity similar to that of carp GP. While carp are freshwater fish, yellow tail and red sea bream are marine red-flesh fish and white-flesh fish, respectively. Thus, it is assumed that the antimicrobial activity of sialo-origosaccharide from GP is not confined to these teleost species but can be found in all fish.

Then, we examined which GP fraction demonstrates bacteriostatic activity by using a sensitivity test [36]. The carp RBC membrane preparation, GP preparation, carbohydrate and P-1 fractions also exhibited bacteriostatic activity (Figure 8a–f). The P-2 fraction exhibited bacteriostatic activity within the area of the disc paper (Figure 8e and f). In contrast, the inhibition zones were not observed using the GP
fraction that lacked sialic acid or the human GP. These results suggest that the test bacteria are sensitive to monosialyl-oligosaccharides from teleost GPs.

Based on electron microscope observations [36], the carp GP molecules attach to the flagellum of \textit{V. anguillarum} rather than the cell itself (Figure 9a). Conversely, the GP molecules attach to the cell surface (contained cleavage line) on \textit{M. luteus} (Figure 9b). Carp GP exists with the size of various molecules and has a diameter of 40–220 nm from TEM images (Figure 9c-3). It seems that the smallest GP molecules selectively possessed bacteriostatic activity.
Figure 7. Sensitivity test for *M. luteus* by the disc-plate method. (a) GP preparation from carp (ca. 15 μg protein/disc). GP, carp GP; control, PBS; (b) GP preparation from yellow tail (ca. 10 μg protein/disc). GP, yellow tail GP; control, PBS; (c) GP preparation from red sea bream (ca. 10 μg protein/disc). GP, red sea bream GP; control, PBS.

Figure 8. Sensitivity test for the growth of *E. tarda* and *M. luteus*. (a) Carp RBC membranes (ca. 5 mg·protein/disc); (b) carp GP fraction without streptomycin treatment (ca. 17 μg·protein/disc); (c) carp GP fraction (ca. 15 μg·protein/disc); (d) carbohydrate fraction from carp GP (ca. 4 μg/disc); (e, f) P-1 and P-2 fractions (ca. 8 μg/disc each); upper left disc, P-1; upper right disc, P-2; lower disc, PBS. (a–e) Plates containing *E. tarda*; (f) plate containing *M. luteus*. 
These bacteriostatic activities of teleost GP are caused by the contained monosialyl-oligosaccharide and are attributed to the property of the lectin receptor. It is supposed that some lectin-like proteins exist on the surface of gram-positive bacteria or the component of flagellum of gram-negative bacteria. Based on the obtained observations, (1) the teleost GPs are released from RBC membranes and aggregated with each other by hydrophobic areas within the protein moiety of GP. (2) The sialo-oligosaccharides are exposed on the outer layer of the aggregated GP molecules. (3) Aggregated GP molecules are adsorbed onto the surface or the flagellum of invading bacteria in the blood plasma. (4) The bacteria attached to GP molecules will be led to a bacteriostatic state (Figure 10) [28]. The bacteriostatic activity of sialo-oligosaccharides from carp GP is attributed to pentose formation. This may be related to the bacteriostatic activity caused by the pento- or hexa-saccharides obtained from chitin [48]. In the bacteriostatic reaction by teleost GP, it is supposed that the size of the oligosaccharide corresponds to that of the cleft occurs in the lectin-like protein and also might contribute to the negative charge of sialic acid. In teleost blood, IgG does not exist unlike human blood, and other antibodies exist at low levels [49]. It is suggested that GP may exist as a substitute for antibodies such as IgG in teleost blood on the immune system. Although the physiological function of human GP has not yet been clarified, the structure of human GP’s O-linked tetra-oligosaccharide is a simpler form than that of carp’s pentose. And NeuAc in human GP is also simpler than carp’s NeuGc. IgG is considered a major component in the human immune system, and the bacteriostatic activity of human GPs has been lost in the process of evolution.

Figure 9. Electron microscope images of the bacteria and carp GP. (a) *V. anguillarum* and carp GP. 1, *V. anguillarum* without carp GP; 2, *V. anguillarum* with carp GP. An equal volume of glycophorin solution (ca. 0.4 μg·protein/20 μL) was added to the cell suspension (ca. 3 × 10^6 cfu/20 μL) at 25°C. (b) *M. luteus* and carp GP. 1, *M. luteus* without carp GP; 2, *M. luteus* with carp GP. An equal volume of glycophorin solution (ca. 0.4 μg·protein/20 μL) was added to the cell suspension (ca. 3 × 10^6 cfu/20 μL) at 25°C. (c) Carp GP. 1, SEM image under the same conditions of *V. anguillarum* with carp GP; 2, SEM image under the same conditions of *M. luteus* with carp GP; 3, TEM image.

7. Behaviour of a sialo-oligosaccharide from GP in RBC membranes

These bacteriostatic activities of teleost GP are caused by the contained monosialyl-oligosaccharide and are attributed to the property of the lectin receptor. It is supposed that some lectin-like proteins exist on the surface of gram-positive bacteria or the component of flagellum of gram-negative bacteria. Based on the obtained observations, (1) the teleost GPs are released from RBC membranes and aggregated with each other by hydrophobic areas within the protein moiety of GP. (2) The sialo-oligosaccharides are exposed on the outer layer of the aggregated GP molecules. (3) Aggregated GP molecules are adsorbed onto the surface or the flagellum of invading bacteria in the blood plasma. (4) The bacteria attached to GP molecules will be led to a bacteriostatic state (Figure 10) [28]. The bacteriostatic activity of sialo-oligosaccharides from carp GP is attributed to pentose formation. This may be related to the bacteriostatic activity caused by the pento- or hexa-saccharides obtained from chitin [48]. In the bacteriostatic reaction by teleost GP, it is supposed that the size of the oligosaccharide corresponds to that of the cleft occurs in the lectin-like protein and also might contribute to the negative charge of sialic acid. In teleost blood, IgG does not exist unlike human blood, and other antibodies exist at low levels [49]. It is suggested that GP may exist as a substitute for antibodies such as IgG in teleost blood on the immune system. Although the physiological function of human GP has not yet been clarified, the structure of human GP’s O-linked tetra-oligosaccharide is a simpler form than that of carp’s pentose. And NeuAc in human GP is also simpler than carp’s NeuGc. IgG is considered a major component in the human immune system, and the bacteriostatic activity of human GPs has been lost in the process of evolution.
Figure 10. Schematic representation of the teleost GP interaction with invading bacteria in fish blood.

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References


[2] Saitō K. Biochemical studies on the fish blood-XIV. On the mean corpuscular constant and shape of red corpuscule. Memoirs of Faculty of Fisheries Kagoshima University. 1959;7:192-197


glycophorin. Archives of Biochemistry and Biophysics. 1991;291:76-88


[27] Staricoff MA, Tanner MJA. Role of band 3 and glycophorin C in the maintenance of the shape and mechanical properties of the human red blood cell. Cellular & Molecular Biology Letters. 1996;1:151-161


[29] Anstee DJ, Ridgwell K, Tanner MJA, Daniels GL, Parsons SF. Individuals lacking the Gerbich blood-group antigen have alterations in the human erythrocyte membrane sialoglycoproteins β and γ. Biochemical Journal. 1984;221:97-104


[33] Lesley JB, Groves JD, Okubo Y, Thilaganathan B, Tanner JA. Altered
band 3 structure and function in glycophorin A- and B-deficient (MkMk) red blood cells. Blood. 1994;84:916-922

[34] Jackson RC. The exterior surface of the chicken erythrocyte. Journal of Biological Chemistry. 1975;250:617-622


