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Purification and identification of a glycoprotein that induces the attachment of oncomiracidia of *Neobenedenia girellae* (Monogenea, Capsalidae)

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Abstract

Neobenedenia girellae, a monogenean skin parasite, shows low host specificity. *N. girellae* is an important pathogen in marine cultured fish such as yellowtail and amberjack. An effective control method is required but none has yet been established. To clarify the mechanisms of host specificity, we purified and identified the attachment-inducing substances of oncomiracidia from tiger puffer fish. The attachment-inducing substances were mainly included in skin mucous extract. Skin mucous extract lost its ability to induce attachment after boiling and/or exposure to the reducing agent dithiothreitol, suggesting that attachment-inducing substances are of a proteinaceous nature. Since lectins such as Con A, WGA, PHA-L, and PSA inhibited the induction of attachment, attachment-inducing proteins were suspected to be glycoproteins. Glycoproteins specifically interacting with Con A were collected and purified by anion exchange chromatography, resulting in two active peaks (peaks 3-A and 6). The active component in peak 3-A was identified as Wap 65-2 by N-terminal amino acid sequencing, while the glycoprotein in peak 6 could not be identified. These results suggested that oncomiracidia recognised Wap 65-2 and another glycoprotein of their host.

Keywords: *Neobenedenia girellae*; Monogenea; Host specificity; Host recognition; Attachment; Glycoprotein; Wap 65-2

1. Introduction

The ectoparasitic flatworm *Neobenedenia girellae*, a capsalid monogenean, infects the body surface of marine fish, such as the tiger puffer, *Takifugu rubripes*, the Japanese flounder, *Paralichthys olivaceus*, and the amberjack, *Seriola dumerili* (Ogawa and Yokoyama, 1998). Infection of Japanese cultured marine fish was first noted in 1991 (Ogawa et al., 1995). Ogawa et al. (1995) confirmed that *N. girellae* was introduced to Japan through imported amberjack. This parasite is now recognised as a crucial pathogen of commercially relevant fish due to high mortality rates of the host species, low host specificity, and the parasite's wide distribution. Heavily infected fish may stop eating, the colour of their bodies darkens and they swim erratically and rub against the net, which may result in dermal ulceration and subsequent bacterial invasion (Leong and Colorni, 2002).

Monogeneans generally show high degrees of host specificity and many studies have been conducted to clarify the mechanisms of host specificity (Bakke et al., 1992; Whittington et al., 2000; Buchmann and Lindenstrøm, 2002). Oncomiracidia are able to distinguish their hosts from other organisms and inorganic substrates. Kearn (1967) demonstrated that when oncomiracidia of the capsalid *Entobdella soleae* were offered a choice of epidermis on scales taken from their host, the common sole, *Solea solea*, or those from fish species closely or distantly related to this species, the parasite showed much greater

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affinity for the epidermis of *S. solea* than for other fish species. Oncomiracidia of the capsalids *N. girellae* and *Benedenia seriolae*, responded and attached to lyophilised extracts of skin epithelia of various fish species, including species other than their natural hosts (Yoshinaga et al., 2000, 2002). Besides these instances in capsalid monogeneans, circumstantial evidence has accumulated which indicates that some polystomatid and gryodactylid monogeneans are able to recognise suitable hosts utilising chemical signals (Du Preez and Kok, 1997; Buchmann and Bresciani, 1998). Chemical, mechanical, and behavioural mechanisms have been suggested to explain host specificity (Whittington et al., 2000; Buchmann and Lindenstrøm, 2002). Host chemicals are considered to offer a promising explanation for host specificity among monogeneans (Yoshinaga et al., 2000, 2002; Buchmann, 2001; Hirazawa et al., 2003; Jorndrup and Buchmann, 2005). Therefore, host recognition seems to play an important role in the host specificity of monogeneans. Although many researchers have described the host specificity of monogeneans, the substance that oncomiracidia recognise and that induces attachment to the host has not been identified.

In this study, to clarify the mechanism of host specificity in *N. girellae*, we focused on attachment-inducing substances of the tiger puffer fish, a species in which the genome has been sequenced. We examined these substances using an in vitro assay and we purified and identified an attachment-inducing substance.

2. Materials and methods

2.1. Source of N. girellae oncomiracidia

A population of *N. girellae* was maintained in juvenile spotted halibut, *Verasper variegates*, at the Marine Biological Technology Center, Nippon Suisan Kaisha, Ltd. Eggs laid were collected by putting 5 cm^2 nylon nets (mesh size, 5 mm) into the tank. The eggs, which were entangled in the nets, were collected and incubated in a 300 ml plastic beaker at 25 °C for 5–7 days. Filtered seawater was changed daily during incubation. The water was changed each 12 h for collection of oncomiracidia, and the oncomiracidia were used for experiments within 12 h after hatching.

2.2. Assay of attachment-inducing ability

Oncomiracidia (within 12 h after hatching) were exposed to 500 μl of seawater including various substances (e.g. skin mucous extract, purified peak, lectin) for 3 h at 25 °C in 24-well culture plates (Ina-optika corporation). Attached oncomiracidia were judged to be those with an unfolded haptor attached to the bottom of the culture plate. Attachment-inducing ability of substances was indicated by the percentage of oncomiracidia that attached to the bottom of each well. All assays were carried out in triplicate in each experiment.

2.3. Collection of mucous extract, gill extract and serum of tiger puffer fish

Mucous containing epithelial cells was collected from the skin of tiger puffer fish by scraping using glass slides. Mucous and gill samples were homogenised in TBS (50 mM Tris–HCl, 0.1 M NaCl, pH 7.2) and centrifuged at 3,000*g* for 30 min at 4 °C. The supernatant was re-centrifuged at 10,000*g* for 1 h at 4 °C, and these supernatants (mucous and gill extracts) were kept at 4 °C until use in in vitro assays. Blood was kept at 4 °C overnight and centrifuged at 3,000*g* for 10 min at 4 °C; serum was kept at 4 °C until use in in vitro assays. Mucous, gill extracts and serum were diluted in seawater to a final protein concentration of 400 μg/ml. The attachment-inducing ability of serum and these extracts was examined. Significant differences in the percentage attached compared with controls are shown.

2.4. Denaturation of mucous extract

Mucous extract was boiled at 100 °C for 10 min and kept at 4 °C until use in in vitro assays. The extract was then reduced with 100 μM dithiothreitol (DTT) at 4 °C, 25 °C or 100 °C and kept at 4 °C until use in in vitro assays. Also, the denatured mucous extracts were diluted in seawater to a final protein concentration of 400 μg/ml. The attachment-inducing ability of denatured mucous extracts was examined. Significant differences in the percentage attachment compared with skin mucous extract are shown.

2.5. Screening of the attachment-inducing proteins with lectins

Attachment-inducing capacities were assayed in seawater containing 400 μg/ml of skin mucous extract with or without 100 μg/ml of 12 lectins. Concentrations of lectins were determined with reference to previous studies (Yoshinaga et al., 2000, 2002). The lectins used in the experiments were wheat germ agglutinin (WGA), *Sophora japonica* agglutinin (SJA), *leuco-agglutinating* isolectin of *Phaseolus vulgaris* agglutinin (PHA-L), *erythro-agglutinating* isolectin of PHA (PHA-E), *Lens culinaris* agglutinin (LCA), *Pisum sativum* agglutinin (PSA), *Gerardia savaglia* lectin (GSL-I), Con A, peanut agglutinin (PNA), soybean agglutinin (SBA), *Ulex europaeus* agglutinin (UEA-I), and *Ricinus communis* agglutinin (RCA-I). These lectins and carbohydrates are listed in Table 1. The attachment-inducing ability of lectins and/or skin mucous extract was examined.

In these experiments, Con A, WGA, PHA-L, and PSA clearly suppressed attachment-inducing capacities in skin mucous extract. Therefore, the effect of these lectins at various concentrations was examined. Attachment-inducing ability of these lectins (50, 100, or 200 μg/ml) was examined.

In these experiments, Con A suppressed the attachment-inducing capacities of skin mucous extract. Therefore, the