ZOOLOGICAL SCIENCE 10: 685–690 (1993)

Immunological Similarity between an Olfactory System-Specific Protein and a Testicular Germ Cell Protein in Kokanee Salmon (*Oncorhynchus nerka*)

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ABSTRACT—An olfactory system-specific protein (N24; molecular weight 24 kDa) was examined for immunological similarity to proteins from various organs (heart, intestine, kidney, muscle, ovary and testis) of mature kokanee salmon (*Oncorhynchus nerka*) by means of Western blotting analysis using a polyclonal antibody to N24. The antibody recognized one 24 kDa protein in the testis but none in the other organs examined. Immunoelectron microscopic analysis revealed that immunoreactive gold particles were mainly concentrated on the nuclei of spermatids and spermatozoa, while no specific gold particles were observed on the nuclei and the cytoplasm of spermatogonia, spermatocytes, Sertoli, Leydig and peritubular cells in maturing and mature testes of kokanee salmon. This is the first description of an antigenic similarity between an olfactory system-specific protein and a testicular germ cell protein in the animal kingdom. This corresponding protein may prove to be a useful molecular marker for studying the mechanism of sperm chemotaxis during fertilization.

INTRODUCTION

Recently we have identified and consequently generated a specific polyclonal antibody to an olfactory system-specific protein, N24 [11], whose molecular weight is 24 kDa in several salmonid species including the kokanee salmon (*Oncorhynchus nerka*). We have also suggested that N24 has some important roles in both olfactory imprinting and discrimination of maternal stream odorants, since its immunoreactivity in fish in the maternal stream was stronger than that in fish in seawater. Although our previous Western blotting and immunocytochemical analyses have indicated that N24 is localized only in the olfactory system (olfactory epithelium, olfactory nerve and olfactory bulb) of the nervous organs [11], information was lacking as to whether N24 might be present in other organs of kokanee salmon, especially in the reproductive system.

The expression of putative olfactory receptor gene family in testicular germ cells has been reported in mammals [9]. This finding is of considerable interest to the hypothesis that common olfactory and germ cell receptors may be involved in the sperm chemotaxis during fertilization. There have been several studies providing evidence that the sperm chemotaxis appears to be a key event in fertilization in the sea urchin [17] and the human [10, 16].

To date, however, no studies have examined whether there are corresponding proteins present in both olfactory systems and testes of any animal species. The present study was therefore conducted to investigate the immunological antigenicity of anti-N24 serum of various organs with special reference to the testis in kokanee salmon by West-

Accepted May 7, 1993
Received March 31, 1993
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ern blotting technique. In addition, immunoelectron microscopic observation was carried out to examine antigenic sites of anti-N24 serum in testicular germ and somatic cells.

MATERIALS AND METHODS

Fish

Maturing and mature kokanee salmon (*Oncorhynchus nerka*) of both sexes, 3 to 5 years old, were caught in Lake Toya from September 22 to November 14, 1992. In Lake Toya, gonadal development in kokanee salmon begins in June, and their spawning period is from October to November.

Fishes were anesthetized with 10% ethyl p-aminobenzoate and their various organs (heart, intestine, kidney, muscle, ovary and testis) were removed. Each organ was rinsed quickly in ice-cold salmon Ringer [11], homogenized in about 2–3 volumes of the same solution, and centrifuged at $10.000 \times g$ for 10 min at 4°C. As has been described previously [11], the supernatant fluid was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and to Western blotting analysis. Briefly, samples containing 50 μg protein were resolved by SDS-PAGE [4], and analysed by Western blotting [13] using anti-N24 serum at a dilution of 1 : 2500.

Immunoelectron microscopy

Small fragments of testes from maturing and mature kokanee salmon were fixed in 2% paraformaldehyde-1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for overnight at 4°C, then rinsed in 0.1 M phosphate buffer containing 10% sucrose, and dehydrated through graded concentrations of ethanol at 4°C. Specimens were embedded in Lowicryl K4M (Polaron Equipment, Watford, England) and polymerized at 4°C with an ultraviolet polymerizer (Dosaka EM, Kyoto, Japan).

Immunoglobulin (IgG)-gold technique [15] was used for the present immunoelectron microscopy. All procedures were done at room temperature. Ultrathin sections indicating gold interference color were collected on uncoated 150-mesh nickel grid and placed for 10 min on a dorp containing 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to reduce non-specific adsorption. Sections were immunoreacted with anti-N24 serum diluted at 1 : 2500 in 0.1% BSA-PBS for 2 hr. After rinsing in PBS, the grids were reacted with goat anti-rabbit IgG-coated 15 nm colloidal gold (E.Y Labs, San Mateo, CA, USA) diluted at 1:100 in 0.1% BSA-PBS for 1 hr. After rinsing in PBS and then in distilled water, the grids were stained with 5% uranyl acetate in distilled water for 5 min, and examined on a Hitachi H7000 electron microscope. The specificity of the present immunolabeling was confirmed by the substitution of normal rabbit serum or PBS for the anti-N24 serum.

RESULTS

Figure 1A shows the electrophoretic patterns of soluble extracts of the heart, intestine, kidney, muscle, ovary and testis from a mature kokanee salmon as resolved by SDS-PAGE and stained with Coomassie Blue. A polyclonal antiserum to N24 at a dilution of 1 : 2500 recognized the 24 kDa protein only in the testis; it was absent from all other organs examined by Western blotting analysis (Fig. 1B).

Sites reacting with anti-N24 serum in maturing and mature kokanee salmon testes were detected by immunoelectron microscopic analysis. No specific gold particles were observed on the nuclei or the cytoplasm of spermatogonia, Sertoli and peritubular cells (Fig. 2A), spermatocytes (Fig. 2B), and Leydig cells (data not shown). On the contrary, gold-labeled N24 immunoreactivity was preferentially located on the nuclei of spermatids (Fig. 2C) and spermatozoa (Fig. 2D). In the spermatid, the gold particles were concentrated on electron-dense portion of the nucleoplasm, and some were observed on the cytoplasm (Fig. 2C). Few gold particles were localized on the tail of spermatozoa (Fig. 2D).

In control sections, no specific gold particles were present on the cytoplasm nor on the nuclei of any spermatogenic or somatic cells.

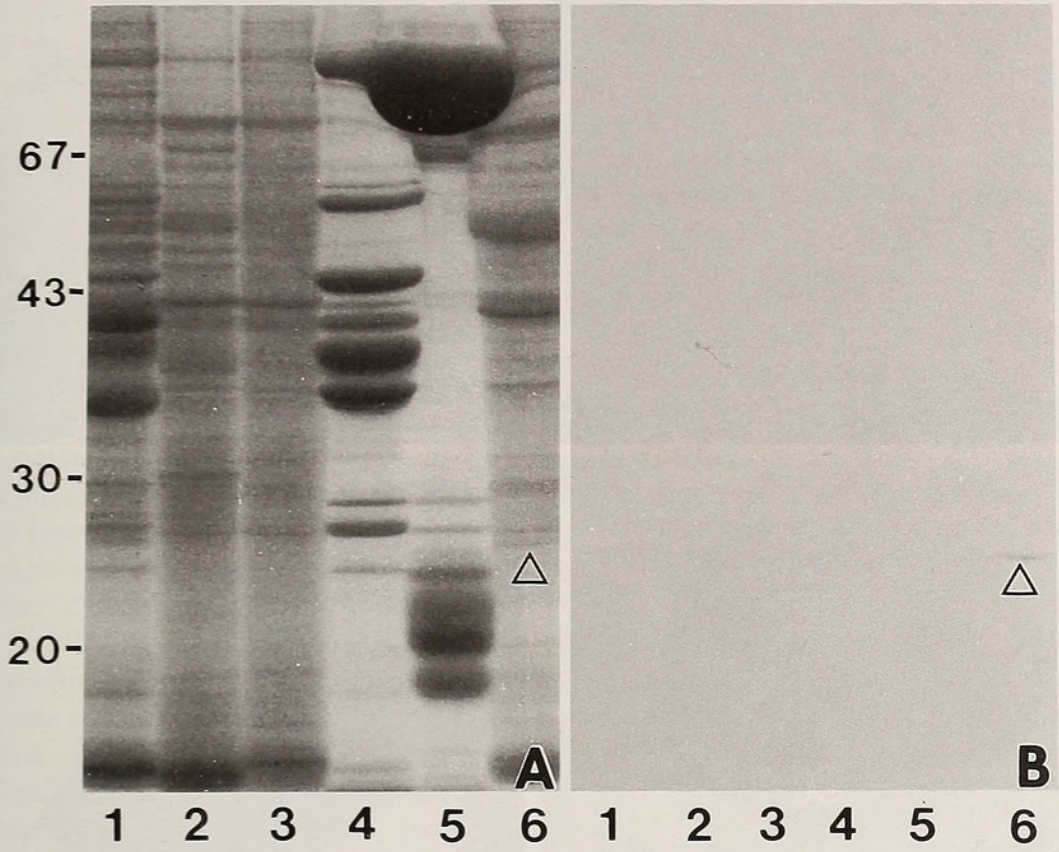

FIG. 1. SDS-PAGE (A) and Western blotting (B) of soluble extracts of heart (1), intestine (2), kidney (3), muscle (4), ovary (5) and testis (6) from mature kokanee salmon. Anti-N24 serum (1:2500) recognizes a band of molecular weight 24 kDa in the testis but not in the other organs. Arrowheads identify a testicular 24 kDa protein. Numbers at the left are Mr standard $\times 10^3$.

DISCUSSION

The two immunological analyses in the present study clearly demonstrate the immunological similarity between an olfactory system-specific protein and a testicular germ cell protein in kokanee salmon. Although we were not able to separate testicular constituent cells into spermatogenic and somatic cells by the Western blotting analysis, the immunoelectron microscopic analysis has revealed that the antigenic sites reacting with anti-N24 serum were observed only in spermatids and spermatozoa, localized mainly on their nuclei. The immunoreactivity were concentrated on their nucleoplasm, but not specific to their nuclear membrane. The testes from maturing and mature fish were used for the present observation, but we have not examined the sperm after spermiation yet. Further studies are necessary to investigate the antigenic site of spermiating sperm. The significance of some immunoreactivities on the cytoplasm of spermatid also needs for further investigations. For all that, the present paper accounts for the first time the existence of a corresponding protein that is found in both the olfactory system and the testis of animals.

Parmentier *et al.* [9] have reported that a common receptor gene family encodes for olfactory and germ cell receptors, and that these receptors may be involved in the sperm chemotaxis during fertilization. The molecular basis for the specificity of interactions between egg and sperm during fertilization has been studied in both the animals and plants [3]. The chemoattraction of sperm to egg, involving receptors of the guanylate cyclase family, has been established in the sea urchin [3, 17]. And the sperm chemotaxis to ovarian follicular factors has also been demonstrated in the

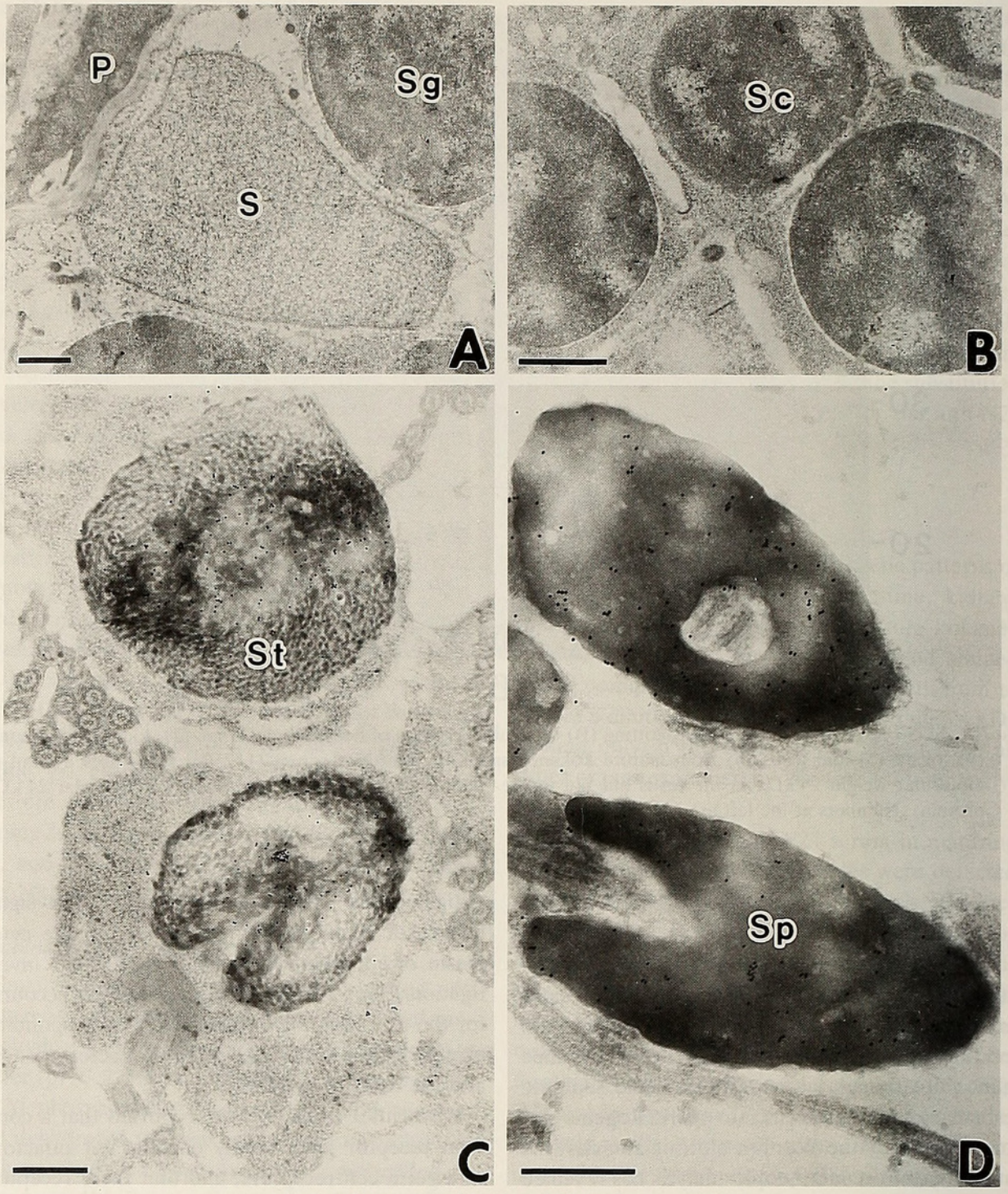

FIG. 2. Immunoelectron micrographs of maturing (A, B and C) and mature (D) testes of kokanee salmon. The immunoreactive gold particles to N24 are mainly concentrated on the nuclei of spermatids (St in C) and spermatozoa (Sp in D), but not on those of spermatogonia (Sg in A), Sertoli cells (S in A), peritubular cells (P in A) and spermatocytes (Sc in B). Scales: A and B, 1 μm; C and D, 0.5 μm.

human [10, 16]. These studies supply evidence to the sperm chemoattraction in response to some defined molecules of egg origin in the animal. However, no attempts have been made to investigate any factors of sperm origin chemoattractive to eggs.

In the present study, we could not provide any direct evidences for the involvement of sperm

N24-immunoreactive protein in the fertilization process. The significance of the concentration of the protein on the nuclei of spermatid and spermatozoa could be partly explained by the fact that salmonid sperm does not possess acrosome, so it is reasonable to assume that the nucleus itself plays some important roles in the mechanism underlying the sperm-egg communication. If the sperm possesses species-specific proteins which have chemotactic ability, cross-species fertilization would be effectively prevented or at least diminished.

Another interesting correlation between the olfactory system and the reproductive system concerns sex pheromones. Several electrophysiological studies have shown that some steroid hormones and prostaglandins function as potent reproductive pheromones in teleost fishes [1, 6, 7, 12]. Female goldfish release the gonadal steroid 17α,20β-dihydroxy-4-pregnen-3-one, which is the maturation inducing steroid in female amago salmon [8] and is involved in the process of spermiation in male amago salmon and goldfish [14], stimulates gonadotropin and milt productions in male goldfish and functions as a potent sex pheromone [2]. Li *et al.* [5] have reported that cholecystokinin acts on cholecystokinin-B receptors as a mediator for olfactory influence on reproductive physiology in mice. Peptides may prove to be more efficient than steroids or prostaglandins as species-specific pheromones. The role of urine in sex discrimination in the spawning behavior of goldfish has also been described [18]. The possibility of pheromonal function of N24-immunoreactive protein in sperm should be investigated.

In summary, this is the first report on the antigenic similarity between an olfactory system-specific protein and a testicular germ cell protein in the animal kingdom. The testicular protein may provide a useful molecular marker in studying the basic mechanism underlying the sperm chemotaxis. The development of an *in vitro* assay for the sperm chemotaxis would shed more light on the sperm-egg communication during fertilization.

ACKNOWLEDGMENTS

We thank Professor Howard A. Bern, University of California, for reading the manuscript. We also thank Mr Hiroyuki Haruna and Mrs Rirako Yamada, Toya Lake Station for Environmental Biology, for their technical assistance. This study was supported in part by the Special Grant-in-Aid for Promotion of Education and Science in Hokkaido University, provided by the Ministry of Education, Science and Culture of Japan, and by a research grant from the Akiyama Foundation to H.U.

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