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Diverse isoforms of guanylyl cyclases in the gonads of echinoderms and medaka fish

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For over 20 years it has been known that cGMP concentrations are increased by a wide variety of agents. The formation of cGMP from GTP is catalysed by guanylyl cyclase. Guanylyl cyclase is found in various cellular compartments of most organisms including animals, plants and bacteria, in soluble and/or membrane-bound forms (Drewett & Garbers, 1994). Membrane-bound guanylyl cyclase (mGC) is a single polypeptide which was first established by cloning and sequencing of the cDNA encoding a sea urchin sperm protein crosslinked to a sperm-activating peptide (SAP) IIA (Chinkers & Garbers, 1991). Soluble guanylyl cyclase (sGC) consists of two different subunits (alpha and beta). mGC is composed of an extracellular, a single transmembrane and an intracellular domain that is further divided into a protein-kinase-like domain and a cyclase catalytic domain. The primary structure of the catalytic domain of both mGC and sGC is highly conserved among vertebrates and invertebrates (Suzuki *et al.*, 1999).

In the past decade we have isolated 74 SAPs from the egg jelly of 17 species of sea urchins distributed over five taxonomic orders, and classified these into five groups: SAP-I to SAP-V (Suzuki, 1995). These peptides show essentially the same biological effects on sea urchin spermatozoa via their specific receptors. The binding of a specific SAP to sperm surface receptors causes a marked and rapid increase and then a subsequent rapid decrease in cGMP concentrations in sea urchin spermatozoa. The transient increase in cGMP concentrations is attributed to the transient activation and subsequent inactivation of mGC, which is closely linked to the state of the enzyme. The addition of a specific SAP to sperm homogenate also results in an increase in cGMP concentration. *Hemicentrotus pulcherrimus* sperm mGC contains 26 phosphorylated amino acids per mole of enzyme, and by treatment of the spermatozoa with SAP-I, the enzyme-bound phosphates decrease to 4 mol per mole of enzyme. Recently, we identified the positions of 14 phosphoserine residues in the phosphorylated form of the sperm mGC, all of which are located in or near the 'turn' region of the predicted secondary structure within the intracellular region (kinase-like and catalytic domains) (Furuya *et al.*, 1998). Of 14 phosphoserine residues, four serine residues in the catalytic domain are conserved among various vertebrate mGCs. Loss of phosphate from mGC is dependent on external sodium and calcium ion concentrations. A specific protein phosphatase inhibitor such as calyculin A inhibits the dephosphorylation and decrease in the activity of SAP-I-treated sea urchin sperm mGC, suggesting that a specific protein phosphatase sensitive to calyculin A is responsible for dephosphorylation of the mGC (Suzuki, 1999).

mGCs have been reported to serve as a cell-surface receptor for ligands such as SAP-IIA, natriuretic peptides and heat-stable enterotoxins. These peptide ligands crosslink to a specific mGC in the respective target tissue in the presence of a chemical crosslinking reagent. In the presence of the crosslinker, the radioiodinated SAP-I crosslinks specifically to a 77 kDa protein in *Strongylocentrotus purpuratus* spermatozoa and to a 71 kDa protein in *H. pulcherrimus* spermatozoa. The two proteins are more than 95% identical. Radioiodinated SAP-IIB from *Glyptocidaris crenularis*, which belongs to the same order as *Arbacia punctulata*, crosslinks mainly to a 62 kDa protein in *G. crenularis* spermatozoa. On the other hand, radioiodinated SAP-III crosslinks to three proteins of 126, 87 and 64 kDa in *Clypeaster japonicus* spermatozoa. Structural analysis of these three proteins revealed that the 126 kDa protein would be a mGC and the 87 kDa protein corresponds to the 71 kDa *H. pulcherrimus* protein. The 64 kDa protein corresponds to the GPI-anchored protein found in *S. purpuratus* and *H. pulcherrimus* spermatozoa. Therefore, we presume that the receptor for a SAP consists of three different proteins, at least one of them being a mGC. Although it is variable which proteins are responsible for binding to the peptide, the binding of the peptide to the receptor always results in activation of the mGC (Suzuki, 1999).

Vertebrates possess both sGCs and mGCs. mGCs can be grouped into three major subfamilies: (1) natriuretic peptide receptors, (2) enterotoxin/guanylin receptors and (3) sensory-organ-specific guanylyl cyclases (Seimiya *et al.*, 1997). Sea urchins also contain many isoforms of guanylyl cyclases including soluble

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and membrane-bound enzymes (Suzuki *et al.*, 1999). Many mGCs found in sea urchins seem to be similar to natriuretic peptide receptors/mGCs. Other echinoderm species also possess many isoforms of guanylyl cyclase. The starfish *Asterina pectinifera* contains six isoforms. The brittle star *Ophioplocus japonicus* possesses 10 isoforms. The holothurian *Stichopus japonicus* contains nine isoforms. Phylogenetic analysis of all the echinoderm mGCs with other guanylyl cyclases indicates that the echinoderm mGCs are divided into several groups: (1) SAP receptor-types, (2) group A, which are similar to natriuretic peptide receptors/mGCs, and (3) others. The analysis also indicates that every echinoderm species contains one mGC belonging to group A (Suzuki *et al.*, 1999).

Six years ago we started research on medaka fish guanylyl cyclases, especially on the expression of mGCs and sGCs in the course of development. The medaka fish is a freshwater teleost found in East Asia. Historically speaking, medaka fish has been developed as a model animal in Japan for studying vertebrate genetics and development (Ishikawa *et al.*, 1997; Ozato & Wakamatsu, 1994). Compared with the zebrafish, medaka fish has several advantages as a model animal: (1) its smaller genome size – 800 Mb, about one-half that of zebrafish, (2) the availability of many natural mutants and several inbred lines, (3) ease of culture both in the laboratory and outdoors and (4) the fact that the eggs can be used every day. In the last 6 years we have isolated cDNA clones for seven mGCs (*OlGC1-7*) from various medaka fish cDNA libraries, which are further divided into three subfamilies (*OlGC2* and *OlGC7* correspond to mammalian GC-A and GC-G; *OlGC1* to GC-B; *OlGC3*, *OlGC4* and *OlGC5* to GC-D, GC-E and GC-F; *OlGC6* to GC-C) (Seimiya *et al.*, 1997; Mantoku *et al.*, 1999; Takeda & Suzuki, 1999). Genomic Southern analysis demonstrated that *OlGC1* and *OlGC7* exist as a single copy, but *OlGC2* is two copies, suggesting that *OlGC2* is duplicated. Since natriuretic peptide receptors/mGCs are found in many organisms including *Caenorhabditis elegans*, GC-A (in the present study *OlGC2*) may be a prototype of mGC and duplication of *OlGC2* may be a key step in the appearance of many isoforms of mGC in the course of evolution.

Medaka fish contains nitric-oxide-sensitive guanylyl cyclase (sGC) activity, which increases from the day 9 embryos and is high in the adult brain. We isolated cDNA clones for both subunits (*OlGCS-alpha-1* and *OlGCS-beta-1*) of a medaka fish sGC, corresponding to mammalian heterodimeric sGC, from a day 9 embryo cDNA library and determined their nucleotide sequences. Messenger RNAs (mRNA) of both subunits are expressed in many organs including brain, eye, spleen, intestine, ovary and testis. These mRNAs are also detected in unfertilised eggs. *OlGCS-alpha-1* of medaka fish sGC exists as a single copy in the genome. We also isolated genomic DNA clones for both subunits of the sGC from a medaka fish (white strain) genomic library and determined the complete nucleotide sequences. *OlGCS-alpha-1* consists of nine exons and *OlGCS-beta-1* of 13 exons. In the genome the two subunit genes are organised in tandem. The two genes are only about 1 kbp apart and span approximately 34 kbp in the order of alpha-1 and beta-1 subunits. To determine the promoter activity of the sGC genes, several fusion genes containing the 5′-upstream region of the respective gene with *pEGFP-1* vector were constructed and injected into the cytoplasm of both blastomeres of a medaka fish 2-cell stage embryo using a micropipette. The injected embryo was cultured and GFP fluorescence detected under a fluorescence microscope. In the embryo injected with a fusion gene containing the 5′-upstream region of alpha-1 subunit, GFP fluorescence was detected in the brain, but not at all in the embryo injected with a fusion gene containing the intervening sequence between alpha-1 and beta-1 subunits. These results suggest that the 5′-upstream region of *OlGCS-alpha-1* can affect the expression of *OlGCS-beta-1*. Therefore, we presume that the expression of the two subunit genes is coordinated.

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