cDNA structure of an insulin-related peptide in the Pacific oyster and seasonal changes in the gene expression

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Abstract

Insulin-related peptide cDNA was characterized in the Pacific oyster *Crassostrea gigas*. It was determined that three transcripts with differing lengths of 3'-untranslated region (3'-UTR) were expressed in the visceral ganglia. The insulin-related peptide cDNA contained a number of AUUUA motifs that were typical of adenylate/uridylate-rich elements in the 3'-UTR. The deduced preprohormone was a polypeptide of 161 residues and showed a conformation typical of preprohormones of the insulin superfamily, which included conserved amino acids necessary to adopt the globular insulin structure. The expression of the three different transcripts was variable throughout the year, with the highest expression observed in March and lower expression in November and July.

The expression of the shortest mRNA in March was about tenfold higher than in July, while the expression of the longest transcript varied approximately twofold during the year. The accumulation of glycogen in the soft body rapidly increased in October and November, and robust body growth and gametogenetic development occurred in March to May. The period of the highest expression of the oyster insulin-related peptide gene corresponded to the onset of body growth and gametogenetic development, but did not overlap with the period of glycogen accumulation. This is the first report that fully details the structure and expression of the insulin-related peptide gene in bivalves.

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Introduction

Insulin is an important regulator of a number of physiological processes such as glucose uptake, cellular growth and division, and metabolism in mammals. Several peptides structurally related to insulin have also been identified in invertebrates including insects (Nagasawa *et al.* 1986), mollusks (Smit *et al.* 1988), nematodes (Kawano *et al.* 2000) and chordates (McRory & Sherwood 1997). Among these invertebrate insulins, the physiological roles of the peptide have been studied most in insects. Bombyxin is a neurosecretory peptide in the silkworm, *Bombyx mori*, possessing an insulin-like structure (Nagasawa *et al.* 1986). Peptides with a bombyxin-like structure have also been identified in several insect species (Lagueux *et al.* 1990). Bombyxin and the related peptides are reported to be involved in the regulation of carbohydrate metabolism (Satake *et al.* 1997), growth (Nijhout & Grunert 2002), reproduction (Maniere *et al.* 2004) and molting (Nagata *et al.* 1999). On the other hand, genetic studies have revealed that disruption of insulin-like signaling pathways in *Drosophila melanogaster* and *Caenorhabditis elegans* affects growth and longevity in these

organisms. In *D. melanogaster*, overexpression of the insulin-related peptide gene that most closely resembles insulin, DILP2, leads to the generation of large flies that comprise greater numbers of larger cells (Leevers 2001); and ablation of the insulin-producing cells in the brain causes developmental delay, growth retardation, and elevated carbohydrate levels in larval hemolymph (Rulifson *et al.* 2002). In *C. elegans*, mutation of an insulin receptor family member, DAF-2, leads to the formation of the metabolically less active dauer, accompanied by developmental arrest and an increase in life span (Kimura *et al.* 1997). Disruption of the production of the insulin/insulin-like growth factor-like peptide also results in an extended life span (Kawano *et al.* 2000).

As for mollusks, insulin-related peptides have been suggested to control growth and carbohydrate metabolism in gastropods. In the pond snail, *Lymnaea stagnalis*, clusters of neurons called light green cells (LGCs) in the cerebral ganglia produce and release molluscan insulin-related peptides (MIPs) (Smit *et al.* 1988, Li *et al.* 1992). Five MIPs (MIP I, II, III, V and VII) have been identified and characterized (Smit *et al.* 1998). Each of these genes is expressed in LGCs in the cerebral ganglia and in ectopic

LGCs (the canopy cells) in the lateral lobes; in contrast, neurons in the buccal ganglia, which control feeding behavior, exclusively express MIP VII (Smit *et al.* 1996). Cauterization of the LGCs in juvenile snails results in retardation of body and shell growth, which can be restored by implantation of LGC-containing cerebral ganglia from a donor animal (Geraerts 1976). Cauterization of the LGCs also leads to a reduction in food consumption and changes in carbohydrate metabolism in various tissues (Geraerts 1976, 1992). In another model gastropod, the sea hare *Aplysia californica*, insulin is produced in the central region of the cerebral ganglia, mostly within the F and C clusters. The expression of the *Aplysia* insulin (AI) decreases when the animal is deprived of food, and injections of AI reduce hemolymph glucose levels (Floyd *et al.* 1999). These results suggest that insulin-related peptides play important roles in growth control and carbohydrate homeostasis in gastropods.

In bivalves, the presence of an insulin-related peptide has been suggested mainly through immunohistochemical studies. Cells within the intestinal epithelia of *Ostrea edulis* (Martinez *et al.* 1973), *Mytilus edulis* (Fritsch *et al.* 1976), *Anodonta cygnea* and *Unio pictorum* (Plisetskaya *et al.* 1978), and neurosecretory cells in the cerebral ganglia of *M. edulis* (Kellner-Cousin *et al.* 1994) have stained positive for mammalian insulin by immunohistochemistry. In *M. galloprovincialis*, anti-*Anodonta* insulin immunoreactivity was observed in the cerebral ganglia, and the annual cycle of the immunoreactivity in relation to the physiological changes was examined (Danton *et al.* 1996). Recently, recombinant human insulin-like growth factor-I (IGF-I) has been shown to stimulate protein synthesis in mantle edge cells of Pacific oyster, *Crassostrea gigas*, and a partial cDNA sequence of insulin receptor-related receptor has been identified (Gricourt *et al.* 2003).

In spite of these studies, direct evidence for the presence of the insulin-related peptide in bivalves has not been obtained. Therefore, we have conducted cDNA cloning and expression analysis of the oyster insulin-related peptide (oIRP) gene in *C. gigas*, and have evaluated its physiological role by comparing seasonal changes in the gene expression with the annual cycle of glycogen accumulation, growth and gametogenesis.

Materials and Methods

Animals

For cDNA characterization and *in situ* hybridization, natural Pacific oyster, *C. gigas*, ranging from 50 to 80 mm in shell height were collected in July and August 2000 at Nojima, Yokohama, Kanagawa Prefecture, Japan. For observation of seasonal changes in the gene expression and physiological parameters, cultured oysters of commercial size (>1 year old) were purchased from an oyster farm in Okayama Prefecture in September 2001; and were main-

tained in lantern nets hung from rafts set in Ohnoseto, Hiroshima Prefecture, Japan. Forty animals were collected every other month between 5 October 2001 and 16 July 2002. Water temperature in the surface layer in Ohnoseto (recorded with DATASONDE-3 (Hydrolab Inc., Loveland, CO, USA)) was communicated by Dr K. Tarutani of the National Research Institute of Fisheries and Environment of Inland Sea, Japan.

cDNA cloning

Visceral ganglia (VG) were collected from 40 oysters, frozen rapidly and stored in liquid nitrogen until use. A subtraction cDNA library was constructed with Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Tester and driver double-strand cDNA were prepared from mRNA samples extracted from VG (2.5 µg mRNA) and the adductor muscle (2.8 µg mRNA) respectively. The thus constructed cDNA library was used as a template for PCR. Degenerate primers for PCR were designed for amino acid sequences relatively conserved among gastropod insulin-related peptides (B chain, RPHPRGVCG; A chain, CECCMKPC). Gradient PCR was performed on a PTC200 thermal cycler (MJ Research, Inc., Waltham, MA, USA) using FastStart Taq DNA polymerase (Roche) for 30 cycles at 95 °C for 30 s, 38–60 °C for 30 s, and 72 °C for 1 min. PCR products were electrophoresed through agarose and the fragments resulting from PCR reactions performed at the highest annealing temperature were cloned into pCR2.1 vector using TA-cloning kit (Invitrogen). Clones were selected using colony PCR, then cycle-sequenced by dye termination. We selected a clone with Leu and Cys residues predicted at appropriate positions within the B chain, which is important to maintain the conformation of the expressed protein. Then a digoxigenin (DIG)-labeled probe was synthesized using a PCR DIG probe synthesis kit (Roche) and full-length clones were identified in a λZIPLOX cDNA library (Invitrogen) which was constructed from approximately 3 µg VG mRNA.

Northern hybridization

For transcript size and tissue specificity analyses, mRNA was isolated from VG of ten oysters using the Micro FastTrack kit (Invitrogen). FastTrack kit (Invitrogen) was used to isolate mRNA from approximately 1 g of mantle, gill, adductor muscle and labial palp respectively. Fifty percent of mRNA from 10 VG and 2.5 µg mRNA from the other tissues were fractionated on a 3-(N-morpholino)propanesulfonic acid (MOPS)/formaldehyde 1.5% agarose gel and transferred to Hybond-N⁺ (Amersham). The blot was subjected to u.v. cross-linking and hybridized to a DIG-labeled PCR probe, which was synthesized from the cDNA containing the coding region

of oIRP or oyster tubulin α (DDBJ accession no., AB185494) according to the manufacturer's procedure. The blot was hybridized (50% formamide, 0.5% SDS, 5 $\times$ SSPE, 5 $\times$ Denhardt's solution, 0.02% sonicated salmon sperm DNA) with the probe overnight at 45 °C, and then washed twice for 10 min at room temperature with 2 $\times$ SSPE /0.1% SDS, for 20 min at 65 °C with 1 $\times$ SSPE /0.1% SDS, and finally for 20 min at 65 °C with 0.1 $\times$ SSPE /0.1% SDS. An alkaline phosphatase (AP)-conjugated anti-DIG antibody Fab fragment (Roche), a chemiluminescent substrate (CSPD; Roche), and an Image Reader LAS-1000 mini (Fuji Photo Film Co., Ltd, Tokyo, Japan) were used to visualize the specific hybridization. Signal intensity was quantified from a CCD image with Image Gauge Ver.4.0 software (Fuji Photo Film Co.).

Among the 40 oysters collected from the lantern nets at approximately 2-month intervals, 30 animals were used to examine seasonal changes in the gene expression of oIRP. VG mRNA isolation and northern hybridization were conducted as mentioned above, except that 200 ng Neo poly(A) RNA, commercial bacterial RNA (1st Strand cDNA Synthesis Kit for RT-PCR (AMV), Roche), was added to the lysate of VG as an external standard to measure the recovery rate of the mRNA extraction. After the isolation of mRNA from the lysate, 10% was used to detect the Neo poly(A) RNA and the rest was used to detect oIRP mRNA or the oyster small cardioactive peptide precursor (SCP) mRNA (DDBJ accession no., AB185493) as an internal standard.

In situ hybridization

VG collected from oysters in August were fixed with Bouin's fixative (saturated picric acid solution:formaldehyde:acetic acid at 15:5:1) at 4 °C for 1 day. The tissues were dehydrated in serial dilutions of ethanol and embedded in paraffin wax. Sections at 8 μ m thickness were mounted on slides precoated with 3-aminopropyltriethoxysilane, deparaffinized and rehydrated before prehybridization. The sections were treated with 10 μ g/ml Proteinase K (Takara Bio Inc., Otsu, Shiga, Japan)/PBST (10 mM phosphate buffer, 0.15 M NaCl, pH 7.4, 0.1% Tween20) at 37 °C for 15 min and post-fixed with 4% paraformaldehyde/PBS for 10 min. Prehybridization was performed at room temperature with the prehybridization solution (50% formamide, 4 $\times$ SSC, 1 $\times$ Denhardt's solution) for 45 min. Anti-sense or sense DIG RNA probes (approximately 500 bp) were *in vitro* transcribed from the oIRP coding region with Sp6 RNA polymerase promoter, and added to the hybridization solution (50% formamide, 4 $\times$ SSC, 1 $\times$ Denhardt's solution, 0.02% sodium N-dodecanoylsarcosinate, 1 mg/ml t-RNA typeII-C yeast, 5 mM EDTA, 10% sodium dextran sulfate 5000) at 1 μ g/ml. Hybridization was performed at 50 °C for 16 h in a moist chamber. The sections were washed twice for 30 min at 50 °C with 50% formamide/2 $\times$ SSC, 2 $\times$ SSC and

0.2 $\times$ SSC. They were blocked with 2% sheep serum/TBST (10 mM Tris-HCl, 0.15 M NaCl, pH 7.5, 0.2% Tween 20) for 30 min at room temperature, treated with AP-conjugated anti-DIG antibody Fab fragment preabsorbed with VG homogenate overnight at 4 °C, and washed with TBST. The signal was visualized with the phosphate substrate (5-bromo-4-chloro-3-indolyl-phosphate /4-nitro blue tetrazolium chloride) at room temperature.

Analysis of gametogenesis, hemolymph glucose, growth rate and glycogen content

From the oysters maintained in the lantern nets in Ohnoseto, 40 animals were collected at approximately 2-month intervals, and 30 out of these 40 oysters were used to collect VG for the northern hybridization as mentioned above. The remainder were then used to measure hemolymph glucose, glycogen content and growth rate. In addition, among the 30 oysters whose VG had been removed, 10 were used to examine gametogenesis by light microscopy. Pieces of posterior soft body dissected from the 10 oysters were fixed with Bouin's fixative, embedded in paraffin, sectioned at 5 μ m, and stained with Mayer's hematoxylin and eosin. Oocyte development and spermatogenesis were classified into five developmental stages as follows. Stage I: the gonad cavities with a layer of morphologically undifferentiated germ cells were observable. Stage II: in females, oocytes with a prominent nucleolus were identifiable; in males, spermatogonia and primary spermatocytes were identifiable, but spermatozoa were not observed. Stage III: in females, oocytes with yolk globule appeared; in males, active spermatogenesis was observed, and numerous spermatozoa were present in the center of the follicles. Stage IV: in females, numerous fully mature oocytes filled the follicles; in males, spermatozoa filled the entire follicles, and spermatogenesis became less active. Stage V: in both females and males, some amebocytes appeared in the follicles; degenerating oocytes were observable.

For the measurement of glucose, hemolymph samples were collected individually from the adductor muscle of 10 unopened oysters, centrifuged at 500 g for 5 min and supernatants were collected and rapidly frozen on dry ice. Hemolymph glucose was measured by the glucose oxidase method (Glucose CII test Wako, Wako Pure Chemical Industries, Ltd, Osaka, Japan). After the collection of hemolymph, growth of the oysters was estimated by measuring the weight and height of the right shell. Then the whole soft body of each oyster was individually frozen in liquid nitrogen, freeze-dried, weighed and ground to a fine powder. The glycogen content in 0.5 g of the powder was determined by the anthrone method after alkaline digestion (Good *et al.* 1933) with minor modifications. Statistical analysis was conducted using Bonferroni's Tukey-type multiple *t*-test.

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ggcaccgctttgggtgtgaggattaataattcacaagatcactcaacagattttgc
tatgcttacacaacacgtgggatacaagaagcggttaattagggactttgtcccc
gggaactgaaaaactcccattccattctagagg

atgacgtcagaaaaccacgtgggttcattatctccctttgtttgctccagatgggtt
M T S E T T W F I I S L C L L Q M V
tgccctgtcttaagcgggtttgaaaagggtgtgcacgtttgagacgtacaggcgc
C P V L S G F E K V C T F E T Y R R
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G V H Q Q G A C G D N L A D M L R L
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V C R K Y K R S G G T R P D K T Y D
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V L K R A N F T G G G L S P S Q I Q
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A R S A P W K S V L K R M M V S K E
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K A L A F I A N N L D F H R R K K K
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G S P Y N L D K R Y G D I N I V C E
tgctgttaccatagctgcagtggtggcggagtttgaggattattgcgcgagagtga
C C Y H S C S V A E F E D Y C A E *

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Figure 1 Nucleotide and deduced amino acid sequences of the cDNA encoding a prepro oIRP. The nucleotide sequence of the longest oIRP transcript (1.85 kb) is shown. Arrows indicate the starting point of poly-A on the shorter transcripts (0.75 or 0.95 kb). The AUUUA motifs typical of the AREs are underlined. The poly-A signal is shown with a double underline.

Results

Isolation and sequencing of the oIRP cDNA

Since the dissected VG contained adjacent adductor muscle tissue, cDNA obtained from VG was subtracted

with the adductor muscle cDNA so as to concentrate VG-specific cDNA. Thus prepared VG cDNA was used as a template for PCR. PCR was performed using degenerate sense and anti-sense primers designed to regions of the gastropod insulin-related peptide genes that

Signal sequence

oIRP	----- MTSETT-WFIISLCLLQMVCPLS ----
MIP I	-----MAGVRLVFTKA--FMVTVLLTLLLNIGVKPAEG
MIP II	-----MVGVRLVFTNA--FVVTVLLTLLLDVVVKPAEG
MIP III	-----MASVHLTLTKA--FMVTVFLLTLLNVSIT--RG
MIP V	-----MAGVRLVFTKA--FMVTVLLTLLLNIGVKPAEG
MIP VII	-----MNASVESCLT--FTF-VLVALCVGLTIG----
Aplysia	-----MSKFLLQSHSANACLLTLLTTLASNLDISLA--
Tunicate	-----MNSSS--YSVVLNKEVMAKE-----
Human	-----MALWMRLLP-----LLALLALWGPDAAA----

B chain

oIRP	GF EKV CT FET Y RRGV HQ Q AC GN LAD ML RLV CR KY KRS GG TR PD KTY DVL
MIP I	--QFSACNINDRP--HRRGVCGSALADLVDFACSSSNQPAMV-----
MIP II	--QSSCSLSRP--HPRGICGSNLAGFRAFCISNQNSPSMV-----
MIP III	TTQHTCSILSRP--HPRGLCGSTLANMVQWLCSTYTTSSKV-----
MIP V	--QFSACSFSSRP--HPRGICGSDLADLRAFCISRRNQAMV-----
MIP VII	QQVNTCTMFSRQ--HPRGLCGNRLARAHANLCFLLRNTYDFIFPR----
Aplysia	NFEHSCNGYMRP--HPRGLCGEDLHVIIISNLCSSLGNNRFLAKYMV--
Tunicate	-----QNEYLCGSHLADVLSFVCGNRGYFFEPRT-----
Human	-----F--VNQHLCGSHLVEALYLVCGERGFFYTPKT-----

C peptide

oIRP	-----KRAFTGGGLSP--SQIQARSAPWKS V-LKRMM
MIPI	-----KRNAETD-LDDP-LRNIK
MIPII	-----KRDAETGWLLPETMVKRNAETD-LDDP-LRNIK
MIPIII	-----KRQAEPDEEDDA-MSKIM
MIPV	-----KRDAETGWLLPETMVKRNAQTD-LDDP-LRNIK
MIPVII	KRSVDNTFEKVYSIPLSVLAELDLSDDDWGAYVSKKDIPIRSETNGLSGANFESSA
Aplysia	-----KR--DTENVNDK-LRGIIL
Tunicate	-----RRSVDDRAVDFI-SEQQA
Human	-----RREAED--LQVGQVELGGGPG--AGS-LQPLA

oIRP	VS KE KALAF I ANN L DFHRR KK KGSP YN LDKR
MIP I	LSSESALTYLTKR-----
MIP II	LSSESALTYLTKR-----
MIP III	ISKKRALS Y LTKR-----
MIP V	LSSESALTYLTKR-----
MIP VII	FDKQLELPAMKSTTSQ L FRILKLRGSRLKR-
Aplysia	L NKKEA FSYLTKR-----
Tunicate	KDYM GAMPH IRKRR-----
Human	LEGS-----LQKR-----

A chain

oIRP	--YGDIN IVCECCYHS CS VAE FE DYCAE -----
MIP I	--QGT TNI VC EC CMKPCTLS EL RQYCP-----
MIP II	--QRT TNL VC EC CFNYCTPDVVRKYCY-----
MIP III	--ESR PSI VC EC CFNQCTVQ ELL AYC-----
MIP V	--QRT TNL VC EC CYNVCTVDVFY EY CY-----
MIP VII	EVMA EPSL VC DC CCYNECSVR KLA TYC-----
Aplysia	--EASGS IT CE CF NQCRIF ELA QYCR LPD HFFSRIS
Tunicate	-----G IV EQCCYRVCS L RO LE TYCN-----
Human	-----G IV EQCC TSI CS LY Q LE NYCN-----

Figure 2 Comparison of the predicted amino acid sequence of oIRP with other insulin superfamily peptides. Invertebrate IRPs (oyster, *Lymnaea*, *Aplysia*, tunicate) and human insulin are aligned for each domain with ClustalW. oIRP is indicated in bold letters. The positions that have an identical residue or similar amino acids in more than seven out of the nine indicated sequences are shaded with dark or light gray respectively. The residues essential for the tertiary structure, such as the disulphide bridges and the hydrophobic core, are boxed.

showed the greatest amino acid sequence homology with other insulin superfamily genes. PCR yielded a single band in electrophoresis, and the PCR product was cloned into pCR2.1 vector. The inserts of the selected clones were sequenced, and Leu and Cys residues were predicted at appropriate positions. The insert was labeled with DIG and used to identify the full-length clone of oIRP from a

λZIPLOX VG cDNA library. Twenty-three positive clones were isolated and sequenced. Clones of three different insert sizes (0.75, 0.95 and 1.85 kb) were screened and found to comprise the same coding region with different lengths of 3'-untranslated region (3'-UTR). They consisted of a 483 bp open reading frame that encodes a 161 amino acid precursor (Fig. 1).

The 3'-UTRs of the three oIRP transcripts contained a number of AUUUA motifs (Fig. 1) that are typical of adenylate/uridyate-rich elements (AREs).

The deduced amino acid sequence showed a pre-prohormone structure typical for the insulin superfamily and consisted of a signal peptide, a B chain, a C peptide and an A chain. The predicted amino acid sequence of oIRP is aligned with MIPs and AI, and tunicate and human insulin in Fig. 2. The alignment demonstrated the conservation of the amino acids essential for the tertiary structure, such as disulphide bridges and the hydrophobic core. In the A and B chains of oIRP, six cysteines were present in positions typical for the insulin superfamily. The presence of two extra cysteines, which has been suggested as a peculiarity of the mollusk insulins, was also confirmed in oIRP. However, the position of an extra cysteine in the B chain differed between oIRP and MIPs or AI. Despite the conservation of the residues involved in the maintenance of tertiary structure, the overall amino acid homology was low. The sequence identity and similarity of oIRP with MIP III were 22 and 39% respectively, excluding signal peptide; sequence identity and similarity with human insulin were 16 and 29% respectively.

Expression of the oIRP gene in VG

Northern analysis using a DIG-labeled PCR probe specific for oIRP indicated the presence of transcripts of three different lengths in VG (Fig. 3). The sizes of these transcripts corresponded to those of the three clones with different 3'-UTR length isolated from the library. Tissue specificity analysis showed that oIRP mRNA was present exclusively in VG, while oyster tubulin α mRNA was detected in all tissues examined (Fig. 3).

Histological sections of VG were subjected to *in situ* hybridization using a DIG-labeled cRNA probe specific for oIRP. The results showed that the oIRP gene was expressed in some of the neurons and axons in VG (Fig. 4). The diameter of the neurons expressing the oIRP gene (oIRP neuron) was about 10 μ m. The oIRP gene expression was restricted to a limited population of neurons, but observed in various parts of VG with a lateral symmetry. Detailed studies using serial horizontal sections revealed the distribution of oIRP neurons along a dorso-ventral axis (Fig. 4). In the dorsal part of the VG, the oIRP neurons formed clusters, and the presence of at least two pairs of clusters was confirmed (Fig. 4b and d). In the middle part of the VG, the oIRP neurons were scattered near the epidermal surface of the VG (Fig. 4e). In the ventral part, the oIRP neurons spread over an area from near to the epidermal surface to a central part of the VG (Fig. 4f).

Seasonal variation in oIRP expression, growth rate, glycogen content, hemolymph glucose and gametogenesis

The water temperature in Ohnoseto varied from a maximum of 25 °C in September to a minimum of 10 °C in

Figure 3 Northern analysis of oIRP mRNA. oIRP, hybridization with a probe to the oIRP mRNA; tubulin, hybridization with a probe to the oyster tubulin α ; the size and positions of the RNA markers are shown in the upper panel. V, visceral ganglion; M, mantle; G, gill; A, adductor muscle; L, labial palp.

February during this study. Growth rate was evaluated by the increase in right shell height, right shell weight, and dry body weight. The mean value of each of these parameters increased during the observation period, except for the dry body weight in July (Fig. 5). A significant increase was observed from March to May in the right shell weight ($n=10$, $P<0.05$) and the dry body weight ($n=10$, $P<0.01$). Shell growth was more apparent in the weight than in the height. The increase and decrease of the dry body weight were closely related to gonad maturation. As the mature gonad is an organ comprising a large part of the oyster body, the significant increase of the dry body weight from March to May was due partly

Figure 4 Localization of the oIRP mRNA in the visceral ganglia by *in situ* hybridization. (a) Section in the dorsal part of the visceral ganglia. Abbreviations: VG, visceral ganglion; M, adductor muscle; K, kidney. (b) Higher magnification of the region indicated by the box in panel a; arrow indicates a cluster of oIRP neurons in the dorsal part of the left visceral ganglion; arrowheads indicate scattered oIRP neurons. (c) Control hybridization with sense oIRP cRNA probe. (d) Another cluster of oIRP neurons; the distance from the cluster shown in panel b is about 100 μ m. No cluster of the oIRP neurons was observed in several serial sections between those in panels b and d; arrow indicates a cluster of oIRP neurons. (e) Distribution of oIRP neurons in the middle part of the left visceral ganglion. (f) Distribution of oIRP neurons in the ventral part of the left visceral ganglion. Scale bars: 1 mm in panel a; 100 μ m in panels b–f.

to gonad development, and the significant decrease in July ($n=10$, $P<0.01$) resulted from the release of gametes on spawning.

The level of expression of the three different sizes of oIRP mRNA changed markedly during the period examined (Fig. 6a). In general, levels were elevated in March, prior to growth and germ cell development, and lower in July after active spawning. There was also a difference that depends on the transcript sizes. The expression of the shortest mRNA was tenfold higher in March than in July, whereas the expression of the longest transcript varied only twofold during the same period (Fig. 6b). The level of the external standard, Neo poly(A) mRNA, was not influenced by the extractions (data not shown). The level of expression of the internal standard, SCP mRNA, also varied seasonally, but it had a different

expression pattern from oIRP, with its expression peaking in May and at its lowest in January (Fig. 6a). The results of the external and the internal standards confirmed that the annual changes in oIRP mRNA were not artifacts from mRNA extraction or northern hybridization processes.

Glycogen content in the soft body increased significantly from October to November ($n=10$, $P<0.01$), remained almost stable until March and declined in May and July (Fig. 7). However, the decrease was not statistically significant. Averages of the hemolymph glucose showed some increase from January to May, but the difference was not significant (Fig. 7).

The results of the histological observations on gonad development are summarized in Table 1. Most of the oysters in late October were in stage V, where many amoebocytes appeared in the gonad cavity and

Figure 5 Seasonal variations of right shell heights, right shell weights and dry body weights. ▲, right shell heights; ■, right shell weights; ◆, dry body weights. Results are expressed as means $\pm$ S.D. of ten animals. Different letters denote significant differences (Bonferroni's Tukey-type multiple *t*-test).

phagocytosed degenerating oocytes or remaining spermatozoa (Fig. 8 h and i). The gonad regression progressed in accordance with the glycogen accumulation in the soft body. In late November, all oysters sampled possessed the gonad cavity lined with immature germ cells, and the sex of the oysters was not apparent (stage I, Fig. 8a). In January, oocytes with a prominent nucleolus became visible in some females, and spermatogonia and spermatocytes were observed in one male (stage II, Fig. 8b and c). In March, oocytes in early yolk globule formation appeared in a female, and spermatozoa became observable in some males (stage III, Fig. 8d and e). Thereafter, the oysters matured rapidly. In May, all oysters were in stage IV. Female gonad cavities were filled with fully mature oocytes, and male gonads were filled with spermatozoa (Fig. 8f and g). In July, all females and males were in stage V again, and some amoebocytes were observable in the gonad cavity.

To summarize, glycogen accumulated from October to November, and growth and gametogenesis progressed rapidly from March to May. The expression of the oIRP gene became highest prior to the rapid growth and gametogenesis, and was low when glycogen accumulation progressed.

Discussion

In this study, we clarified the cDNA structure of the oIRP gene and the presence of the three different sizes of the transcript. We also compared the oIRP gene expression pattern with seasonal variation of growth, gametogenesis and glycogen storage of the oysters under natural conditions. This is the first report that fully details the structure and expression of the IRP gene in bivalves.

The deduced prepro oIRP contains four putative dibasic processing sites (Lys-Arg), although they have yet to be confirmed by purification and sequencing of the peptide. The predicted oIRP amino acid sequence exhibited low sequence similarity with known vertebrate insulins or invertebrate IRPs, such as MIPs and AI. However, residues important for the maintenance of the basic insulin core structure were well conserved. The spacing between cysteine residues was conserved, suggesting that the characteristic disulfide bridges in the insulin molecules have been conserved. The hydrophobic core residues responsible for globular insulin structure were either identical to other members of the insulin superfamily or replaced by residues of equivalent hydrophobicity. Vertebrate insulin contains three pairs of cysteines, which form two interchain and one intrachain disulfide bonds. The prepro oIRP contains eight cysteine residues, as are observed in MIPs and AI. An extra pair of cysteines in the mollusk sequences suggests the ability to form an extra disulfide bond (Smit *et al.* 1988). Interestingly, the position of an extra cysteine in the B chain of the oIRP differed with MIPs or AI. This difference might be a characteristic for bivalves, but further clarification of IRP cDNA from other bivalves is needed.

The Pacific oyster expressed oIRP transcripts of three different lengths, the levels of which varied seasonally. To investigate whether the three oIRP transcripts are encoded by a single gene, Southern blot analysis was performed on EcoRI and HindIII digests of the oyster genomic DNA. A single fragment was detected on both digests using a probe located within the coding region (data not shown), indicating that these transcripts are expressed from a single gene. Until now, three mechanisms have been shown to generate multiple transcripts from the same gene: the selection of an alternative polyadenylation site, the use of alternative transcription start sites and the differential splicing of pre-mRNA. Considering the complete sequence identity among oIRP transcripts, the most probable mechanism is that the oIRP transcripts in different lengths are produced by the alternative polyadenylation site selection. Recently, much attention has been focused on the regulatory function of 3'-UTR in gene transcription and translation. Alternatively polyadenylated mRNAs have been shown to establish tissue-specific or developmental stage-specific expression of different products from the same gene (Galli *et al.* 1988). The presence of oIRP transcripts in three different lengths might indicate functional diversity of the oIRP peptide, for example as neurohormone and neurotransmitter (Smit *et al.* 1998). A difference in translation efficiency between alternatively polyadenylated mRNAs has also been reported (Qu *et al.* 2002). In relation to the translation efficiency, an interesting finding is the presence of a number of AUUUA motifs typical of the AREs in the 3'-UTRs of the oIRP transcripts. The AREs represent the most common determinant of RNA stability in

Figure 6 Seasonal variations of oIRP mRNA expression in the visceral ganglia. (a) Northern analysis of oIRP mRNA prepared from samples collected at 2-month intervals. oIRP, hybridization with a probe to the oIRP mRNA; SCP, hybridization with a probe to the oyster SCP; the size and positions of the RNA markers are shown to the upper left. (b) Graphic representation of relative chemiluminescence intensities from the northern analysis of oIRP mRNA shown in panel a. Open columns, the expression of the 0.75 kb transcript; lightly shaded columns, the 0.95 kb transcript; darkly shaded columns, the 1.85 kb transcript.

mammalian cells, and have been found in the 3'-UTR of proto-oncogenes, nuclear transcription factors and cytokines (Chen & Shyu 1995). Specific proteins bind to the AREs to form complexes that lead to either degradation or protection of the mRNA (Bevilacqua *et al.* 2003). The sequence characteristics of the 3'-UTR of oIRP mRNA suggest that the oyster possesses a post-transcriptional regulatory mechanism for oIRP. Recently, a mechanism important for insulin mRNA stability has been reported in

rat, in which a polypyrimidine tract-binding protein (PTB), depending on the glucose concentration, binds to pyrimidine-rich sequences located in the 3'-UTR of the rat insulin mRNA (Tillmar *et al.* 2002). However, the PBT binding sequence was not present in the 3'-UTR of the oIRP mRNA.

In *L. stagnalis*, seven structurally related but distinct MIP genes are present in the genome, and the expression of five of these has been confirmed (Smit *et al.* 1998).

Figure 7 Seasonal variations of the glycogen content in the soft body and hemolymph glucose concentration. ●, glycogen content; ×, hemolymph glucose concentration. Results are expressed as means ± s.d. of ten animals. Different letters denote significant differences (Bonferroni's Tukey-type multiple t-test). The differences in hemolymph glucose concentrations were not significant throughout the observation.

Bombyxin from the silkworm *Bombyx mori*, the *Drosophila* insulin-like peptide and the *C. elegans* insulin-like peptide are also members of multigene families that encode several structurally related but distinct peptides (Kondo *et al.* 1996, Duret *et al.* 1998, Rulifson *et al.* 2002). As it was anticipated that heterogeneous molecules also exist

Table 1 Gonad development from October to July. Numbers of oysters at each stage of gonad development are presented

		Stages of gonad development*					
Month	Sex	n	I	II	III	IV	V
Oct	F	4	—	1	—	—	3
	M	5	—	—	—	—	5
	UD	1	1	—	—	—	—
Nov	F	—	—	—	—	—	—
	M	—	—	—	—	—	—
	UD	10	10	—	—	—	—
Jan	F	3	—	3	—	—	—
	M	1	—	1	—	—	—
	UD	6	6	—	—	—	—
Mar	F	1	—	—	1	—	—
	M	8	—	2	6	—	—
	H	1	—	—	1	—	—
May	F	3	—	—	—	3	—
	M	7	—	—	—	7	—
Jul	F	3	—	—	—	—	3
	M	7	—	—	—	—	7

F, female; M, male; UD, sex undistinguishable; H, hermaphrodite with both oocytes and spermatocytes; n, number of oysters. In May and July, sex was identifiable for all the specimens.
*See text for details.

in Pacific oyster, we attempted to clone other oIRP family members by low stringency hybridization. But we were unable to find other oIRP members. Since the sequence similarity of insulin superfamily members is generally low, the possibility that other members of oIRP exist still remains.

IRP-producing neurons in *L. stagnalis* and *A. californica* are located in a bilaterally symmetrical area and formed a limited number of clusters in the cerebral ganglia. In this study at least two clusters of oIRP neurons were identified. But at the same time, oIRP mRNA was detected in nerve cell bodies and axons scattered in a wide area in the epidermal side of the ventral part of VG. The presence of oIRP transcripts in the isolated neurons is interesting since, in mollusks, IRPs are supposed to act not only as hormones but also as neurotransmitters on target neurons in the neural ganglia (Smit *et al.* 1998). Clarification is needed, by whole-mount *in situ* hybridization, of the exact number of the clusters and the relationship between the clusters and the dispersed oIRP-expressing neurons.

Although oIRP cDNA was identified in VG of Pacific oyster, the cerebral ganglia (CG) have been suggested to produce IRP in *M. edulis* and *M. galloprovincialis* (Kellner-Cousin *et al.* 1994, Danton *et al.* 1996). In addition, insulin-like immunoreactivity has been reported to localize to the digestive tract of some bivalves using an antibody raised against mammalian insulin (Fritsch *et al.* 1976, Plisetskaya *et al.* 1978). Therefore we tried to examine oIRP gene expression in CG and the digestive tract of the oyster. For CG, which are a pair of small organs located at the base of the labial palps in *C. gigas*, the organs were too small to be dissected out in a reproducible manner. For this reason, we could not examine the oIRP gene expression in CG. For the digestive tract, we examined the oIRP gene expression in some parts of the digestive tract by northern or *in situ* hybridization, but we did not observe any positive signal in the tissue (data not shown). From the present results, the oIRP gene expression in VG is apparent, but we cannot exclude the possibility of oIRP gene expression in CG or the digestive tract of *C. gigas*.

To gain insight into the function of oIRP, we monitored the accumulation of glycogen, growth rate, gametogenesis and the expression of oIRP. The expression of the oIRP gene became highest in March prior to the rapid growth and gametogenesis, and was low in November when glycogen accumulation had progressed. Thus the oIRP gene expression was closely coupled to a specific phase of the annual physiological cycle of the animal. Apparent seasonal changes in the oIRP gene expression also indicate that the gene expression might be controlled, directly or indirectly, by environmental factors with a seasonal periodicity. Until now, only the effects of feed condition on the gene expression of molluskan IRP have been examined (Smit *et al.* 1998, Floyd *et al.* 1999). Although we cannot always consider that active gene

Figure 8 Histological changes in oocyte development and spermatogenesis. Developmental stages are divided into five stages (I to V) which are indicated on the left side. The left panels are male and the right panels are female. See text for detailed description of the stages. (a) Stage I in November. (b) Stage II male in January; arrow indicates spermatocytes; arrowhead indicates spermatids. (c) Stage II female in January; arrow indicates an oocyte with a prominent nucleolus. (d) Stage III male in March. (e) Stage III female in March; arrow indicates an oocyte in early yolk globule stage. (f) Stage IV male in May. (g) Stage IV female in May. (h) Stage V male in October. (i) Stage V female in October. VC, vesicular cells; SP, spermatzoa; AM, ameobocyte. Scale bars: 50 µm in panels a, b, c and e; 100 µm in panels d, f, g, h and i.

expression indicates active production or release of oIRP, the highest gene expression in March implies that the oIRP has some functional relationship with the physiological activities occurring in early spring. Physiological changes of *C. gigas* reported to occur in early spring include glycogen mobilization (Mathieu & Lubet 1993) and an increase of tissue protein content (Berthelin *et al.* 2000), in addition to the onset of body growth and gametogenesis confirmed in this study. Those processes must be controlled and connected with each other by several hormonal factors, and oIRP might be one of these factors. At present we do not have enough information to speculate which physiological process oIRP is most likely to be involved in. The peptide hormones hitherto reported to control these processes, including putative ones, and their possible relationships with oIRP are discussed below.

Although the decrease of glycogen in accordance with gonad development and body growth was not statistically significant in the present results, mobilization of glycogen reserves is generally accepted to correlate with gametogenesis in bivalves (Mathieu & Lubet 1993). Therefore one possibility for oIRP function is the stimulation of glycogen mobilization. A putative glycogen mobilization hormone (GMH) has been characterized in *M. edulis* (Robbins *et al.* 1991); this is a peptide present in CG with a molecular mass of >20 kDa. oIRP might be involved in the process of glycogen mobilization together with GMH and other endocrine factors. On the other hand, stimulation of glycogen synthesis by vertebrate insulin or molluscan IRPs has been reported or implied in some bivalves (Plisetskaya *et al.* 1978, Danton *et al.* 1996). But considering the annual gene expression pattern of oIRP, it might be difficult to regard oIRP as a major factor in promoting seasonal glycogen accumulation. Injection of glucose to observe changes in oIRP gene expression would be important to realize the function of oIRP in glycogen metabolism, but we could not conduct the experiment because the injection method that ensures rapid and reproducible dispersal of the injected glucose into body fluids was not available.

The roles of IRPs in the reproductive activities of mollusks are not well clarified. In *L. stagnalis*, the number of egg masses produced by the LGC-cauterized snails became significantly smaller than in the controls (Geraerts 1976). On the other hand, the relative weight of the reproductive organ to the whole body weight was unaffected by the LGC removal (Geraerts 1992). In *Aplysia*, the bag cells, neurosecretory cells that produce egg-laying hormone (ELH), contain insulin receptors and application of mammalian insulin to the bag cells results in a large increase in the intracellular Ca^{2+} concentration and triggers the secretion of ELH (Jonas *et al.* 1997). Considering these results, we cannot rule out a role for oIRP in the regulation of gametogenetic development. Investigation of the sexual differences in oIRP gene expression might help us realize the function of oIRP in reproduction.

The significant shell growth observed in the spring specimens suggests the possible involvement of oIRP in the regulation of body growth. In *L. stagnalis*, cauterization of LGCs results in retardation of body and shell growth, while glycogen content of various tissues and hemolymph glucose increase after the cauterization (Geraerts 1976). Based on the results of the LGC-cauterization experiments, Geraerts (1992) hypothesized that food consumption and the resulting increase in hemolymph glucose stimulate LGCs to release MIPs, and then MIPs activate the glucose uptake by various tissues and support normal body growth in all organs. Similar observations are reported for AI, where the expression of the AI gene decreases when the animal is deprived of food, and injections of AI reduce hemolymph glucose levels (Floyd *et al.* 1999). Although direct experimental evidence has not been obtained, MIPs are thus hypothesized to maintain normal body growth through activation of cellular metabolism, such as glucose incorporation, in various tissues. The importance of metabolic activity control by IRP is more clearly demonstrated in *C. elegans*, in which disruption of the insulin signaling pathway results in the metabolically less active dauer, accompanied by developmental arrest and an increase in life span (Kimura *et al.* 1997). Oysters accumulate glycogen in vesicular cells during winter, and start to utilize the accumulated glycogen in spring as an energy source for growth and reproduction (Mathieu & Lubet 1993). These physiological processes are essential for normal progression of the annual physiological cycle and adaptation to seasonal environmental changes. oIRP might constitute a part of the physiological system that controls the metabolic shift that occurs in spring.

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