Int. J. Dev. Biol. 44: 451-456 (2000)

Original Article

Effect of inducers and inhibitors on the expression of *bcs* genes involved in cypris larval attachment and metamorphosis of the barnacles *Balanus amphitrite*

YUICHI OKAZAKI* and YOSHIKAZU SHIZURI

Marine Biotechnology Institute Co. Ltd., Shimizu Laboratory, Shizuoka, Japan

ABSTRACT We examined the expression of six barnacle cypris larva-specific gene (*bcs*) cDNAs (*bcs-1, -2 , -3, -4,- 5,* and *-6*), the *bcs* genes, by using Northern blot analysis under various conditions that induced or inhibited cypris larval attachment and metamorphosis. Inducers of larval attachment and metamorphosis, such as a neurotransmitter, tended to increase the expression of *bcs* mRNAs. All inhibitors of larval attachment and metamorphosis, such as G protein-coupled receptor agonists/antagonists, inhibitors of tyrosine kinase-linked receptors and inhibitors of their signal transduction, suppressed the expression of *bcs-6* mRNA alone, but affected differentially other *bcs* genes. These results strongly suggest that the *bcs-6* product plays a key role in triggering the attachment and metamorphosis of cypris larvae into juvenile barnacles. The roles of four late *bcs* genes (*bcs-3,-4, -5* and *-6*) are discussed.

KEY WORDS: *bcs, expression, receptor, neurotransmitter, signal transduction.*

Introduction

Many environmental factors influence the larval attachment and metamorphosis of marine organisms (Pawlik, 1992). For example, γ-aminobutyric acid induces larval attachment and metamorphosis of red abalone *Haliotis rufescens* (Morse, *et al.,* 1979), L-3,4-dihydroxyphenylalanine induces the attachment of larvae of giant Pacific oyster *Crassostrea gigas* (Coon and Bonar, 1985) and pediveliger larvae of blue mussel *Mytilus edulis* (Cooper, 1983). The effects of various compounds and physiological materials on larval attachment and metamorphosis of barnacles have been reported: an extract of adult barnacle (Larman, *et al.,* 1982), some bacterial films (Maki, *et al.,* 1988), synthetic short-chain peptides (Tegtmeyer and Rittshof, 1989), ions (Rittschof, *et al.,* 1986; 1991), several amine and related compounds (Kon-ya, *et al.,* 1995; Kon-ya and Endo, 1995; Yamamoto, *et al.,* 1996), activators and inhibitors of protein kinase C (PKC) (Yamamoto, *et al.,* 1995), cAMP (Clare, *et al.,* 1995), calmodulin inhibitors (Yamamoto, *et al.,* 1998), hormonal materials(Gomez, *et al.,* 1973; Clare, *et al.,* 1992; Yamamoto, *et al.,* 1997) and agonists and antagonists of biogenic amine receptor (Kawahara, 1997; Yamamoto, *et al.,* 1999). These studies suggest that neurotransmitters and mediators of signal transduction systems, such as cAMP and Ca^{2+}, are involved in the regulation of larval attachment and metamorphosis of marine invertebrates, including barnacles. However, virtually nothing is known about the endogenous molecules involved in larval attachment and metamorphosis of these marine organisms.

Recently, we found useful markers to investigate precisely intracellular molecules in the process of larval attachment and metamorphosis of barnacles, that is, the cypris larva-specific genes (*bcs*) that are expressed specifically in cypris larvae attaching on a suitable substratum and metamorphosing into juvenile barnacles (Okazaki and Shizuri, 2000). During the process of cypris larval attachment and metamorphosis, the sequence of the expression of *bcs* genes was observed. First, the early genes *bcs-1* and *bcs-2* were expressed strongly for several hours just after cypris larvae hatched. The expression of the late genes *bcs-3, bcs-4, bcs-5* and *bcs-6* increased temporarily in this order with the progress of larval attachment and metamorphosis. The expression of *bcs-3, bcs-4, bcs-5* disappeared when the cypris larva attached to the substratum, but the expression of *bcs-6* continued

Abbreviations used in this paper: B. amphitrite, Balanus amphitrite; bcs, barnacle cypris larva-specific gene; G protein, guanine nucleotide-binding protein; TKRs, tyrosine kinase-linked receptors; TK, tyrosine kinase; PKC, protein kinase C; PKA, protein kinase A; NGFR, nerve growth factor receptor; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; InsR, Insulin receptor; PLC, phospholipase C; PI_3K, phoshatidylinositol-3-kinase; MAPK, mitogen activated protein kinase; SSC, Saline Sodium Citrate.

***Address correspondence to:** Yuichi Okazaki. Research Institute of Innovative Technology for the Earth. 9-2, Kizugawadai, Kizu-cho, Soraku-gun, Kyoto, 619-0292, Japan. TEL: 81 774 75 2308. FAX: 81 774 75 2321. e-mail: yokazaki@rite.or.jp

0214-6282/2000/$20.00

Printed in Spain
www.ehu.es/ijdb

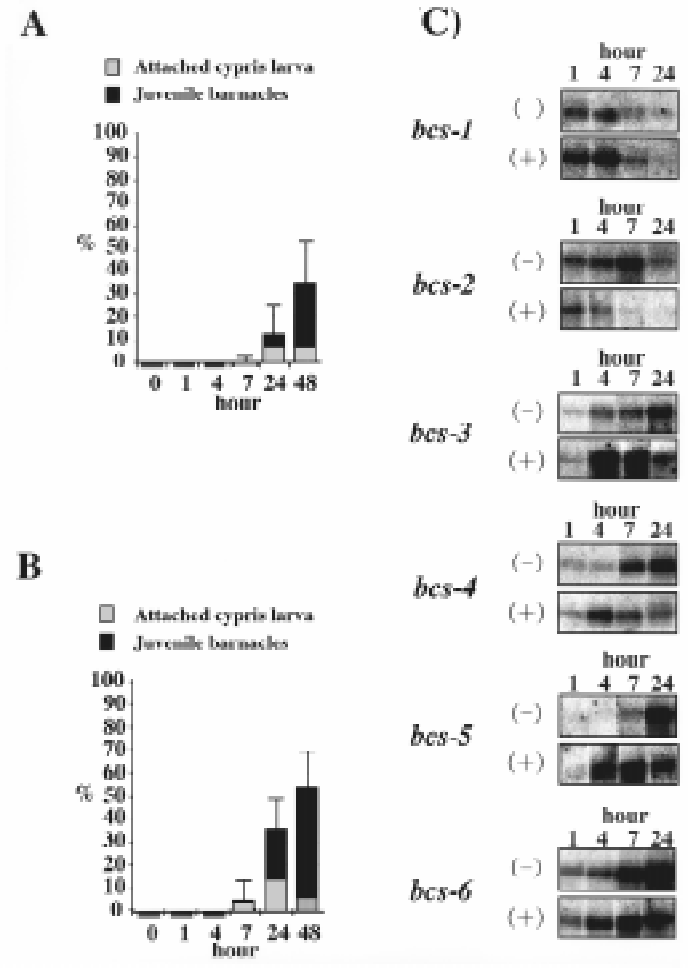

Fig. 1. Cypris larval attachment and metamorphosis, and the expression of six *bcs* genes with or without inducers. *The rate of attachment and metamorphosis of cypris larva cultured* **(A)** *without or* **(B)** *with serotonin at a final concentration of 1x10^{-5} M for 0 (harvesting time), 1, 4, 7, 24 and 48 h. Each bar indicates mean ± S.D.* **(C)** *Northern blot analysis of cypris larva cultured without (-) or with serotonin (+) using six ^{32}P-labeled bcs cDNA probes. The culture periods (hours) are indicated at the top.*

until the cypris larva metamorphosed completely into juvenile barnacles. The predicted peptides of these six genes are: *bcs-1*, an acidic polypeptide with repeating sequences; *bcs-2,* a polypeptide rich in aspartic acid plus glutamic acid residues; *bcs-3*, a polypeptide containing a long DNA binding and dimerization region in its carboxyl terminus; *bcs-4*, a cysteine-rich polypeptide with eight cysteine residues forming two repeat motifs in the core region; *bcs-5*, a polypeptide with eleven alanine-proline and five decapeptide repetitions; and *bcs-6,* a short peptide of twenty-two amino acids. The homology search for these polypeptides indicated that all *bcs* genes were novel, and suggested that the *bcs-3* product probably is a transcription factor belonging to the Elf-1/NTF-1/*grh* and LSF/αCP2/LBP-1c families (Okazaki and Shizuri, 2000).

In this study we investigated the effects of enhancers and inhibitors of cypris larval attachment and metamorphosis of barnacles on the expression of *bcs* genes by Northern blot analysis to obtain information on the mechanisms that control *bcs* gene expression. The results support the idea that the *bcs* gene plays a key role in the attachment and metamorphosis of the cypris larva of barnacles.

Results

Effect of inducers on the expression of six bcs *genes*

Serotonin (Kon-ya, *et al.,* 1995; Yamamoto, *et al.,* 1996) and an extract of adult barnacles (Larman, *et al.,* 1982) stimulate cypris larval attachment and metamorphosis. When cypris larvae were cultured in fresh sea water alone, the rate of larval attachment and metamorphosis was 0 % until 7 h after the start of culture, and it increased to 12 % at 24 h and to 36 % at 48 h (Fig. 1A) (Okazaki and Shizuri, 2000). Figure 1B shows that serotonin increased the attachment and metamorphosis: the rate of larval attachment and metamorphosis was 5 % at 7 h, 35% at 24 h and 54% at 48 h compared with the non-treated group (Fig. 1A). Figure 1C shows the results of Northern blot analysis of six *bcs* genes in the non-treated and serotonin-treated groups, respectively. Serotonin shifted earlier the peak of expression of *bcs-3* and *bcs-4* from 24 h to 4 h after the start of culture, and similarly shifted earlier the peaks of *bcs-5* and *bcs-6* expression from 24 h to 7 h. The expression of *bcs-1* at an early period also tended to be stimulated by the inducers, although the expression of *bcs-2* mRNA was suppressed. The mode of the effect of an extract of adult barnacles on larval attachment and metamorphosis and the expression of *bcs* genes were similar to the mode of serotonin (data not shown).

Expression of six bcs *genes under cold conditions*

Larval attachment and metamorphosis increase simply by storing cypris larva in a dark room at 5°C for 7 days without adding a reagent (Kado, 1991; Clare, *et al.,* 1995). The mechanism of how cold temperatures affect larval attachment and metamorphosis remains to be clarified. Figure 2A shows the attachment and

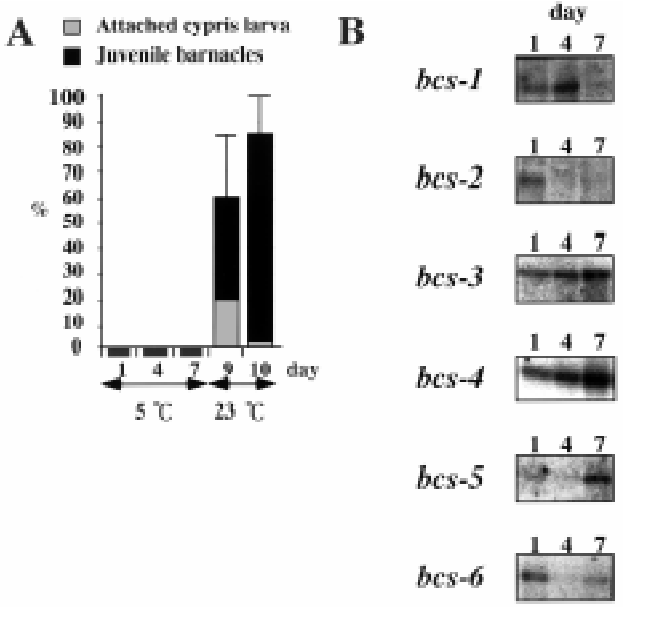

Fig. 2. Expression of six *bcs* genes in the cold. (A) *The rate of attachment and metamorphosis of cypris larva stored at 5°C at days 1, 4 and 7 and incubated at 23°C at days 9 and 10.* **(B)** *Northern blot analysis of cypris larva cultured for 1, 4 and 7 days at 5°C with ^{32}P-labeled bcs-3, bcs-4, bcs-5 and bcs-6 cDNAs as probes.*

metamorphosis of cypris larvae cultured in the cold for 1, 4 and 7 days. In the cold, cypris larvae never attached and metamorphosed, but after transferring to 23°C from 5°C they attached and metamorphosed better than without cold treatment. This behavior suggests that some preparation for attachment and metamorphosis proceeds in the cold. Northern blot analysis (Fig. 2B) showed that *bcs-3* and *bcs-4* mRNAs began to be expressed from day 1 even in the cold and increased toward day 7. The *bcs-5* mRNA was barely detectable on days 1 and 4, but was observed clearly on day 7 in the cold. The expression of *bcs-5* in the cold was markedly delayed compared with warm conditions and the expression of *bcs-6* mRNA was markedly suppressed in the cold compared with warm conditions. The expression of the early genes *bcs-1* and *bcs-2* in the early period was not affected by culturing in the cold.

Effects of inhibitors of tyrosine kinase-linked receptors and their signal transduction pathways on cypris larval attachment and metamorphosis

G protein-coupled receptors and their signal transduction systems are important for larval attachment and metamorphosis of marine organisms, including barnacles (Morse, *et al.,* 1979; Cooper, 1983; Coon and Bonar, 1985; Clare, *et al.,* 1995; Kon-ya, *et al.,* 1995; Yamamoto, *et al.,* 1995, 1996; Kawahara, 1997; Yamamoto, *et al.,* 1998). However, almost nothing is known of tyrosine kinase-linked receptors and their signal transduction. Figure 3 shows the effects of seven reagents on cypris larval attachment and metamorphosis: AG-879, nerve growth factor receptor-tyrosine kinase (NGFR-TK) inhibitor; Tyrphostin 9, platelet-derived growth factor receptor-tyrosine kinase (PDGFR-TK) inhibitor; Tyrphostin 25, epidermal growth factor receptor-tyrosine kinase (EGFR-TK) inhibitor; HNMPA, insulin receptor-tyrosine kinase (InsR-TK) inhibitor; U73122, phospholipase C (PLC) inhibitor; Wortmanin, phoshatidylinositol-3-kinase (PI_3K) inhibitor; and PD-98059, mitogen activated protein kinase (MAPK) kinase inhibitor. These inhibitors tended to suppress cypris larval attachment and metamorphosis in dose dependently. AG-879 and Tyrphostin 9 at 1 μM blocked completely cypris larval attachment and metamorphosis (Fig. 3 A,B). Meanwhile, the inhibitory effects of Tyrphostin 25 and HNMPA were incomplete at a concentration of 100 μM, 100 times higher than those of NGFR-TK and PDGFR-TK inhibitors (Fig. 3 C,D). An experiment to add Tyrphostin 25 at high concentrations (> 100 μM) could not be done because of the insolubility of this reagent. PLCγ, PI_3K and MAPK cascades are signaling transduction pathways by TKRs (Foreman, 1996). U73122 at 50 μM and Wortmanin at 10 μM blocked cypris larval attachment and metamorphosis (Fig. 3 E,F). PD-98059 at a tested highest concentration of 10 μM tended to inhibit larval attachment and metamorphosis although incompletely (Fig. 3G), but could not be examined at concentrations of >10 μM due to its insolubility. These results showed that TKRs and following signal transduction pathways may be involved in larval development.

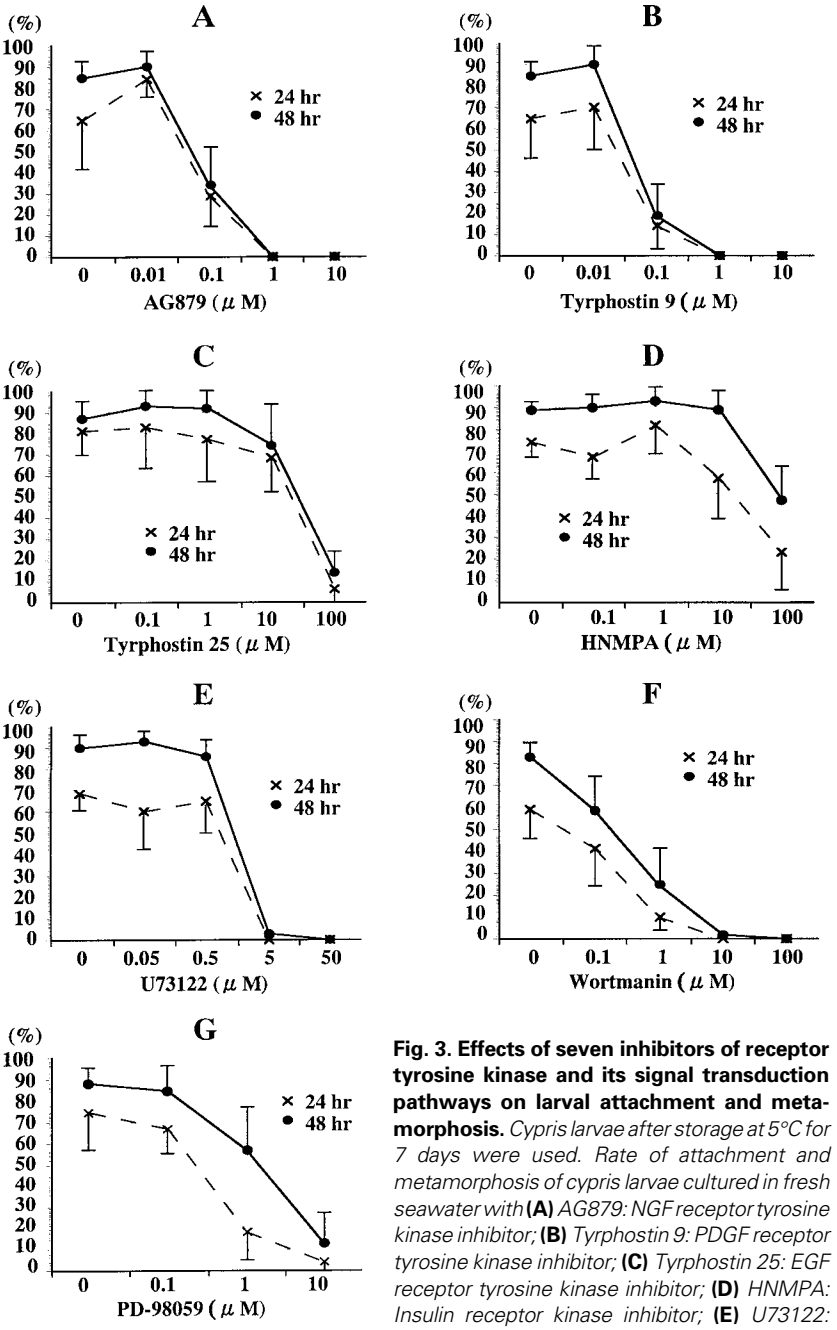

Fig. 3. Effects of seven inhibitors of receptor tyrosine kinase and its signal transduction pathways on larval attachment and metamorphosis. *Cypris larvae after storage at 5°C for 7 days were used. Rate of attachment and metamorphosis of cypris larvae cultured in fresh seawater with* **(A)** *AG879: NGF receptor tyrosine kinase inhibitor;* **(B)** *Tyrphostin 9: PDGF receptor tyrosine kinase inhibitor;* **(C)** *Tyrphostin 25: EGF receptor tyrosine kinase inhibitor;* **(D)** *HNMPA: Insulin receptor kinase inhibitor;* **(E)** *U73122: Phospholipase C inhibitor;* **(F)** *Wortmanin: PI_3K inhibitor; and* **(G)** *PD-98059: MAPK kinase inhibitor, at 24 h (a cross symbol and a dashed line) and 48 h (a closed circle and a solid line). % values on the axis represent Attachment and metamorphosis. Each bar indicates mean ± S.D.*

Effects of inhibitors of tyrosine kinase linked-receptors and their signal transduction pathways on the expression of four late bcs *genes*

The effects of AG-879, Tyrphostin 9, Tyrphostin 25, U73122, Wortmanin and PD-98059 on the expression of the *bcs-3*, *bcs-4*, *bcs-5* and *bcs-6* genes were examined using Northern blot analysis (Fig. 4). The early genes, *bcs-1* and *-2,* were not examined because their expressions were very poor. In non-treated groups, *bcs-3, bcs-4, bcs-5* and *bcs-6* genes were abundantly expressed

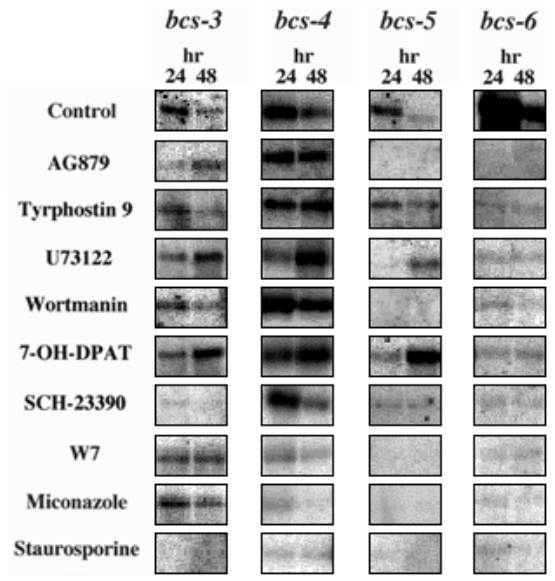

Fig. 4. Effects of nine inhibitors on the expression of six *bcs* genes. *After storage at 5°C for 7 days, cypris larvae were incubated at 23°C for 2 days without (Control) and with nine inhibitors: AG879 (NGF receptor tyrosine kinase inhibitor) at a final concentration of 1 μM; Tyrphostin 9 (PDGF receptor tyrosine kinase inhibitor) at a final concentration of 1 μ M); U73122 (Phospholipase C inhibitor) at a final concentration of 50 μM; Wortmanin (PI_3K inhibitor) at a final concentration of 10 μM; (±)-7-OH-DPAT-HBr (dopamine D_3 receptor agonist) at a final concentration of 10 μM; R(+)-SCH-23390 HCl (dopamine D_1 receptor antagonist) at a final concentration of 10 μM; W7 HCl (calmodulin inhibitor) at a final concentration of 100 μM; Miconazole (adenylyl cyclase inhibitor) at a final concentration of 100 μM and Staurosporine (protein kinase C inhibitor) at a final concentration of 1 μM. RNA blot analysis of cypris larva was carried out using ^{32}P-labeled bcs-3, bcs-4, bcs-5 and bcs-6 cDNAs as probes.*

at 24 h with some decline at 48 h (Fig. 4 Control). AG-879 markedly suppressed both *bcs-5* and *bcs-6* gene expressions. The expression of *bcs-3* mRNA was temporarily suppressed at 24 h, but increased at 48 h (Fig. 4 AG-879). Tyrphostin 9 suppressed *bcs-6* gene expression only (Fig. 4 Tyrphostin 9). Tyrphostin 25, which has a partial inhibitory effect on larval attachment and metamorphosis, did not affect the expression of the *bcs-3*, *bcs-4* and *bcs-5* genes, but suppressed the expression of the *bcs-6* gene compared with the non-treated group (data not shown). U73122 inhibited *bcs-6* gene expression alone with only temporal suppression of *bcs-3*, *bcs-4* and *bcs-5* gene expression (Fig. 4 U73122). Wortmanin strongly suppressed the expressions of both of *bcs-5* and *bcs-6* genes, but did not affect the expression of *bcs-3* and *bcs-4* genes (Fig. 4 Wortmanin). The inhibition of larval attachment and metamorphosis by PD-98059 was incomplete and was accompanied by partial suppression of *bcs-6* gene expression without effect on the expression of *bcs-3*, *bcs-4* and *bcs-5* genes (data not shown).

Effects of inhibitors of G protein-coupled receptors and their signal transduction pathways on the expression of six bcs *genes*

The following reagents inhibit larval attachment and metamorphosis of barnacles: 7OH-DPAT, dopamine D_3 receptor agonist; SCH-23390, dopamine D_1 receptor antagonist (Kawahara, 1997); miconazole, adenylyl cyclase inhibitor (Clare, *et al.,* 1995); W-7, calmodulin inhibitor (Yamamoto, *et al.,* 1998); and staurosporine, PKC inhibitor (Yamamoto, *et al.,* 1995). Ten μM of 7OH-DPAT and SCH-23390, 100 μM of miconazole and W-7, and 1 μM of staurosporine inhibited completely larval attachment and metamorphosis (data not shown). 7OH-DPAT inhibited the expression of *bcs-6* mRNA, and temporally inhibited *bcs-3* and *bcs-5* gene expressions, although the expression of *bcs-4* mRNA was not influenced (Fig. 4 7OH-DPAT). SCH-23390 markedly inhibited the expressions of *bcs-3, bcs-5* and *bcs-6* mRNAs, but not of *bcs-4* mRNA (Fig. 4 SCH-23390). W-7 and Miconazole strongly inhibited the expressions of *bcs-4*, *bcs-5* and *bcs-6,* but not of *bcs-3* mRNAs (Fig. 4 W-7, Miconazole). Because staurosporine inhibited markedly the expression of all four *bcs* genes (Fig. 4 Staurosporine), it might possibly down-regulate non-specifically the expression of most genes in cypris larvae. However, because other inhibitors down-regulated part of the *bcs* genes, their inhibitory effects on the expression of *bcs* genes are considered to be specific.

Discussion

The results of this study of Northern blot analysis indicate that the expression of the *bcs* genes changed, which was associated intimately with larval development of barnacles depending on the conditions to induce or inhibit attachment and metamorphosis. An increase in the expressions of *bcs-3*, *-4* and *-5* mRNAs at both 5°C and 23°C suggests that some preparations for larval attachment and metamorphosis proceed in the cold (at 5°C) accompanied by accumulation of these gene products. In our previous study (Okazaki and Shizuri, 2000), we suggested the product of the *bcs-3* gene is a transcriptional factor involved in regulating cellular proliferation and differentiation during attachment and metamorphosis because this gene encoded a DNA binding region belonging to the Elf-1/NTF-1/*grh* and LSF/αCP2/LBP-1c families. The expression of *bcs-4* mRNA in the cold may also be a preparation for rapid attachment observed after change to a warmer temperature. This *bcs-4* gene product has some similarity with an adhesive protein of blue mussels in their amino acid sequence (Okazaki and Shizuri, 2000), suggesting that *bcs-4* gene codes for a similar adhesive protein. The expression of *bcs-6* mRNA was suppressed in the cold compared with in the warm. This result is very interesting because cypris larval attachment and metamorphosis does not proceed in the cold. We speculate that the *bcs-6* gene that encodes a short peptide with twenty-two amino acids (Okazaki and Shizuri, 2000) may trigger the final step of attachment and metamorphosis. This speculation is consistent with the results that the expression of the *bcs-6* mRNA was always suppressed by all the inhibitors tested for larval attachment and metamorphosis.

From these results and the existence of both dopamine in cypris larvae (Yamamoto, *et al.,* 1999) and of G protein-coupled receptor genes in barnacles (Isoai, *et al.*, 1996; Kawahara *et al.*, 1997), we speculates the following. After the cypris larva hatches, *bcs-3* mRNA, the expression of which may be involved in G protein - coupled receptor such as dopamine D_1 receptor, may lead to accelerate larval development as a transcriptional factor. Subsequently, an increasing expression of the *bcs-5* mRNA, the product of which has some similarity with polypeptides that negatively control cellular proliferation (Okazaki and Shizuri, 2000) follows.

Both G protein-coupled receptor such as dopamine D_1 receptor and TKR such as NGFR might be necessary to activate the expression of the *bcs-5* mRNA at a later phase. After completing the expression of a series of *bcs* genes involved in attachment and metamorphosis, the *bcs-6* gene may trigger the final step toward to the change in cypris larva to a juvenile barnacle because the expression of the *bcs-6* mRNA was always suppressed under inhibitory conditions. Although the expression of the *bcs-6* mRNA at low levels was observed from the early period of a cypris larva, the activation of various receptors, such as dopamine D_1 receptor, NGFR, PDGFR and so on, may be necessary for this gene to be fully expressed to initiate development of the cypris larva. The involvement *bcs-6* mRNA in these complicated receptor systems may be consistent with the *bcs-6* gene having some isoforms (Okazaki and Shizuri, 2000). Also, the effect of the dopamine D_3 receptor agonist suggests that a cypris larva may have a repression system against the expression of the *bcs-6* mRNA, such as the dopamine D_3 receptor, to maintain the plankton larva until the cypris larva finds a suitable base for attachment and metamorphosis.

A more detailed analysis of the *bcs* genes, particularly the *bcs-6* gene, will give us more information to understand the precise molecular mechanism of larval attachment and metamorphosis of barnacles.

Materials and Methods

Preparation and assay of attachment and metamorphosis of cypris larvae

Cypris larvae of the barnacle *Balanus amphitrite (B. amphitrite*) cultured in our laboratory were used in all experiments. The rate of larval attachment and metamorphosis was calculated by the method of Kon-ya and Miki (1994). Briefly, ten cypris larvae were inoculated into a Petri dish, and the number of attached cypris larvae and juvenile barnacles was counted. The rate of larval attachment and metamorphosis was calculated by [number of attached cypris larva and juvenile barnacles / number of inoculated cypris larva (i.e. 10)] X 100.

For assays of inducing larval attachment and metamorphosis, the rate of attachment and metamorphosis of cypris larva cultured with inducers was examined at 0 (at harvesting), 1, 4, 7 and 24 h after culture. As inducers of larval attachment and metamorphosis, serotonin HCl (Research Biochemicals International, Massachusetts) and an extract of adult barnacles (Larman, *et al.,* 1982) were added to fresh seawater containing cypris larvae (0 h) at a final concentration of 10 μM or 0.1 OD_{280}, respectively. The extract of adult barnacles was prepared by homogenizing whole tissues of adult barnacles in sterilized phosphate buffered saline (-), and induced larval attachment and metamorphosis at a final concentration of 0.05 OD_{280} or more. The rate of larval attachment and metamorphosis was examined in the cold. The harvested cypris larvae were incubated at 5°C for 7 days and then were cultured for two days at 23°C. The number of cypris larvae that attached and metamorphosed were counted at days 1, 4 and 7 in the cold followed by counting at days 9 and 10.

For assays of inhibiting larval attachment and metamorphosis, cypris larvae stored at 5°C in a dark room for 7 days after harvesting were used. The following compounds were used as inhibitors: R(+)-7Chloro-8-hyroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (R(+)-SCH-23390 HCl), (±)-2-Dipropylamino-7-hydroxy-1,2,3,4-tetrahydro idenaphthalene ((±)-7OH-DPAT HBr), *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7HCl) (Research Biochemicals International, Massachusetts), 1-[2,4-Dichloro-β-([2,4-dichlorobenzyl]oxy)-phenethyl]imidazole ((±)-Miconazole) (Sigma Chemical co. MO) or Staurosporine (Sigma Chemical co. MO), 1-[6-[[(17β)–3–Methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-1H-pyrrole-2,5 dione (U73122) (BIOMOL Research Labs. Inc., PA), [1S-(1α,6*b*α,9*a*β,11α,11*b*β)]11–(Acetyloxy)-1,6*b*,7,8,9*a*,10,11,11*b*-octahydro-1-(methoxymethyl) 9*a*, 11b-dimethyl-3*H*-furo-[4,3,2-*de*]indeno[4,5-*h*]-2-benzopyran-3,6,9-trione (Wortmanin) (Wako Pure Chemical Industries, Ltd., Osaka), 2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD-98059) (BIOMOL Research Laboratories, Inc., PA), AG-879 (BIOMOL Research Laboratories, Inc., PA), hydroxy-2-naphtalenylmethylphosphonic acid (HNMPA, BIOMOL Research Laboratories, Inc., PA), Tyrophostin 9 (RG-50872, BIOMOL Research Laboratories, Inc., PA), Tyrphostin 25 (RG-50875, Sigma Chemical co., MO). These compounds were added to fresh seawater containing cypris larvae when they were transferred to 23°C after incubation for 7 days at 5°C. The rates of attachment and metamorphosis of cypris larvae cultured for two days at 23°C after adding the any reagents were calculated. All inhibitors were dissolved at concentrations of 5 mM-100 mM in dimethylsulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Osaka). A sample with DMSO alone was prepared as a control because DMSO has a weak capability to induce larval attachment and metamorphosis.

Northern blot analysis

Poly(A)$^+$ RNA from cypris larvae cultured under several kinds of conditions and periods were extracted (Micro mRNA purification kit, Pharmacia Biotech AB) and 500 ng of Poly(A)$^+$ RNA were run on agarose-formaldehyde gel electrophoresis and were transferred on to a nylon membrane (Hybond N$^+$, Amersham, UK). The Poly(A)$^+$ RNA on the membrane was hybridized with ^{32}P-radiolabeled cDNA fragments digested by *Eco*RI/*Xho*I of *bcs-3* (clone #9-8), *bcs-4* (clone #10-3), *bcs-5* (clone #2-17), *bcs-6* (clone #8-3) as a probe in a rapid hybridization buffer (Amersham, UK) at 65°C for 3 h, and was washed three times in 1x sodium chloride/sodium citrate (SSC) and 0.1x SSC at 65°C for 20 min. (Okazaki and Shizuri, 2000). The membrane was briefly dried, wrapped in plastic wrap and was exposed to a Fuji imaging plate (Fuji photo film co., Ltd., Tokyo) overnight in a lead box at room temperature. The imaging plate was developed using a Bio imaging analyzer (BAS 1000, Fuji photo film co., Ltd., Tokyo).

Acknowledgments

We wish to thank S. Kikuta, M. Umehara, S. Ajioka, Marine Biotechnology Institute, for their technical assistance. We also thank Dr. M. Sugimoto, AGENE Research Institute., for his critical discussions. This work was performed as a part of The Industrial Science and Technology Frontier Program supported by the New Energy and Industrial Technology Development Organization.

References

CLARE, A.S., RITTSCHOF, D. and COSTLOW, J.D., JR. (1992). Effects of the nonsteroidal ecdyson mimic RH5849 on larval Crustaceans. *J. Exp. Zool.* 206: 436-440.

CLARE, A.S., THOMAS, R.F. and RITTSCHOF, D. (1995). Evidence for the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement. *J. Exp. Biol.* 198: 655-664.

COON, S.L. and BONAR, D.B. (1985). Induction of settlement and metamorphosis of the Pacific oyster, *Crassostrea gigas* (thunberg), by L-DOPA and catecholamines. *J. Exp. Mar. Biol. Ecol.* 94: 211-221.

COOPER, K. (1983). Potential for application of the chemical DOPA to commercial bivalve settling systems. *J. Shellfish Res.* 3: 110-111.

FOREMAN, J.C. and JOHANSEN, T. (Eds.) (1996). *Textbook of Receptor Pharmacology.* CRC Press, Inc. London, Bombay, Tokyo.

GOMEZ, E.D., FAULKNER, D.J., NEWMAN, W.A. and IRELAND, C. (1973). Juvenile hormone mimics: Effect on cirriped crustacean metamorphosis. *Science* 179: 813-814.

ISOAI, A., KAWAHARA, H., OKAZAKI, Y. and SHIZURI, Y. (1996) Molecular cloning of a new member of the putative G protein-coupled receptor gene from barnacle *Balanus amphitrite. Gene* 175:95-100.

KADO, R. (1991). Effect of light on the larval development of *Balanus amphitrite Darwin* (Cirripedia). *Nippon Suisan Gakkaishi* 57: 1821-1825.

KAWAHARA, H. (1997). Study on the Biogenic Amine Receptors in Barnacle, *Balanus*

amphitrite. Ph.D. thesis, Hokkaido University.

KAWAHARA, H., ISOAI, A. and SHIZURI, Y. (1997) Molecular cloning of a putative serotonin receptor gene from barnacle, *Balanus amphitrite*. *Gene* 184: 245-50.

KON-YA, K. and MIKI, W. (1994). All-seasonal assay for antifouling substances using reared barnacle larvae. *J. Mar. Biotechnol.* 1: 193-195.

KON-YA, K. and ENDO, M. (1995). Catecholamines as settlement inducers of barnacle larvae. *J. Mar. Biotechnol.* 2: 79-81.

KON-YA, K., MIKI, W. and ENDO, M. (1995). L-tryptophan and related compounds induce larval settlement of the barnacle *Balanus amphitrite Darwin*. *Fisheries Science* 61: 800-803.

LARMAN, N.V., GABBOT, P.A. and EAST, J. (1982). Physicochemical properties of the settlement factor proteins from the barnacle *Balanus balanoides*. *Comp. Biochem. Physiol.* 72B: 329-338.

MAKI, J.S., RITTSCHOF, D., COSTLOW, J.D. and MITCHELL, R. (1988). Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films. *Mar. Biol.* 97: 199-206.

MORSE, D.E., HOOKER, N., DUNCAN, H. and JENSEN, L. (1979). γ–aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis. *Science* 204: 407-410.

OKAZAKI, Y. and SHIZURI, Y. (2000). Structures of six cDNAs expressed specifically at cypris larva of barnacles, *Balanus amphitrite*. *Gene* 250:127-35**.**

PAWLIK, J.R. (1992). Induction of marine invertebrate larval settlement: Evidence for chemical cues. In *Ecological Roles of Marine Natural Products.* (Ed. V.J. Paul). New York, Cornell University Press, pp.189-236.

RITTSCHOF, D., MAKI, J., MITCHELL, R. and COSTLOW, J.D. (1986). Ion and neuropharmacological studies of barnacle settlement. *Netherl. J. Sea. Res.* 20: 269-275.

RITTSCHOF, D., SCHMIDT, A.R., HOOPER, I., GERHART, D.J., GUNSTER, D. and BONAVENTURA, J. (1991). Molecular medition of settlement of selected invertebrate larvae. In *Bioactive Compounds from Marine Organisms* (Eds. M.-F. Thompson, R. Sarojini and R. Nagabhushanam). New Delhi, Bombay, Calcutta, Oxford and IBH publishing Co. Pvt. Ltd., pp.317-330.

TEGTMEYER, K. and RITTSHOF, D. (1989). Synthetic peptide analogs to barnacle settlement pheromone. *Peptides* 9: 1403-1406.

YAMAMOTO, H., TACHIBANA, A., MATSUMURA, K. and FUSETANI, N. (1995). Protein kinase C (PKC) signal transduction system involved in larval metamorphosis of the barnacle, *Balanus amphitrite*. *Zoological Science* 12: 391-96.

YAMAMOTO, H., TACHIBANA, A., KAWAI, S., MATSUMURA, K. and FUSETANI, N. (1996). Serotonin involvement in larval settlement of the barnacle, *Balanus amphitrite*. *J. Exp. Zool.* 275: 339-345.

YAMAMOTO, H., OKINO, T., YOSHIMURA, E., TACHIBANA, A., SHIMIZU, K. and FUSETANI, N. (1997). Methyl farnesoate induces larval metamorphosis of the barnacle, *Balanus amphitrite* via protein kinase C activation. *J. Exp. Zool.* 278: 349-355.

YAMAMOTO, H., TACHIBANA, A., SAIKAWA, W., NAGANO, M., MATSUMURA, K. and FUSETANI, N. (1998). Effects of calmodulin inhibitors on cyprid larvae of the barnacle, *Balanus amphitrite*. *J . Exp. Zool.* 280: 8-17.

YAMAMOTO, H., SHIMIZU, K., TACHIBANA, A. and FUSETANI, N. (1999). Roles of dopamine and serotonin in larval attachment of the barnacle, *Balanus amphitrite*. *J. Exp. Zool.* 284:746-758.

Received: July 2000
Accepted for publication: August 2000