Central Nervous System-specific Expression of G Protein α Subunits in the Ascidian Ciona intestinalis

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Central nervous system-specific expression of G protein α subunits in the ascidian Ciona intestinalis

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ABSTRACT—Heterotrimeric G proteins play crucial roles as mediators of signaling by many extracellular stimuli. The receptors that activate G proteins constitute the largest and most diverse family of cell surface molecules involved in signal transmission of metazoan cells. To investigate G protein signaling in the central nervous system (CNS) of chordates, we isolated cDNA fragments encoding five different G protein α subunits (CiGαx, CiGαq, CiGαi1a, CiGαi1b, and CiGαi2) from larvae of the ascidian, a simple chordate, Ciona intestinalis. In situ hybridization analysis revealed that each isoform had distinct patterns of spatial distribution in embryos. Among them, CiGαi1a and CiGαi1b mRNAs were specifically expressed in the CNS of the larva, whereas CiGαq transcripts were expressed in small parts of the trunk epidermis and the tip of the tail, but not in the CNS. The CiGαx expression was widely observed throughout the trunk and tail of the embryos, and the signals were stronger in the epidermis, mesenchyme, and tail muscle cells. Comparison of cDNA sequences and the exon-intron organization indicate that CiGαi1a and CiGαi1b are produced by alternative splicing of transcripts from a single gene, CiGαi1. In the cleavage and gastrula stages, transcripts of CiGαi1 were widely distributed in embryos, and the expression then became restricted to the CNS of tailbud embryos and larvae. An exhaustive search has failed to find transducin-type α subunits in C. intestinalis. Since CiGαi1 is expressed in the ocellus, CiGαi1 may mediate signals from Ci-opsin1, a visual pigment of the ocellus photoreceptor cells.

Key words: ascidians, heterotrimeric G protein, phototransduction, central nervous system, alternative splicing

INTRODUCTION

Acodians, or sea squirts, are lower chordates, and their simple, tadpole-like larvae share a basic body plan with vertebrates (Corbo et al., 2001). Ascidian embryos have been favored for developmental research because they have low cell numbers, contain only a few different tissue types, develop rapidly, and have a well-known cell lineage (Corbo et al., 2001; Satoh, 2001). The larva of Ciona intestinalis, the cosmopolitan ascidian species, has 2,600 cells, including only 40 notochord cells and 36 muscle cells. The larva has a remarkably simple central nervous system (CNS) with about 330 cells, of which less than one-third are neurons, the remainder being glial cells (Meinertzhagen and Okamura, 2001). An anterior brain vesicle contains two sensory organs, an eyespot (ocellus) and a gravity sense organ (otolith). These two sensory organs are responsible for the swimming behavior of the larva.

It was shown that the larvae were induced to swim upon a step-down of light, and the action spectrum of photic behavior of ascidian larvae was similar to the absorption spectrum of human rhodopsin (Nakagawa et al., 1999; Tsuda et al., 2001). Localization of rhodopsin in the ocellus was shown by the retinal protein imaging method (Ohkuma and Tsuda, 2000). Ci-opsin1, the opsin of Ciona intestinalis, has been identified and shown to be closely related to the vertebrate retinal and pineal opsins (Kusakabe et al., 2001).

Opsins are apoproteins of visual pigments and a model of G protein-coupled receptors (GPCRs). Among signaling receptors, GPCRs are especially important because they constitute the largest and most diverse families of receptor proteins. More than 1000 GPCRs identified in the human genome are involved in the regulation of virtually all physio-
logical processes (Marinissen and Gutkind, 2001). In a project analyzing the expressed sequence tags (EST) of *C. intestinalis* larvae (Kusakabe et al., 2002), we identified several cDNA clones encoding GPCRs. It is expected that tremendous numbers of GPCRs will be discovered in the whole genome sequencing projects of *Ciona intestinalis*, which are currently in progress at the National Institute of Genetics, Japan and Joint Genome Institute, USA.

Most cells contain GPCRs that choose between multiple G proteins to regulate a host of intracellular signaling processes (Gilman, 1987; Tsuda, 1987; Neer, 1995; Hamm and Gilchrist, 1996; Offermanns, 2001). Heterotrimeric G proteins are composed of $\alpha$, $\beta$, and $\gamma$ subunits, and there are multiple isoforms of each subunit (Nürnberg et al., 1995; Hildebrandt, 1997). In response to extracellular signals, the GPCRs exert guanine nucleotide exchange factor activity that substitutes GTP for GDP on the $\alpha$ subunit (G$\alpha$), resulting in dissociation of the $\alpha$GTP monomer and a G$\beta\gamma$ dimer. The activated G proteins, in turn, regulate the activity of a variety of effector proteins, including intracellular enzymes and ion channels. A given GPCR can activate more than one G protein subtype, leading to diverging signaling pathways. The importance of G proteins has been suggested in the regulation of developmental events, such as those regulated by Wnt and Hedgehog signaling (Hammerschmidt and McMahon, 1998; DeCamp et al., 2000; Liu et al., 2001; Malbon et al., 2001; Knust, 2001).

In this study, as an initial step to understand specific coupling between GPCRs and G proteins in ascidian embryos, we isolated and characterized cDNA clones encoding five different G$\alpha$ subunits from the ascidian *Ciona intestinalis*. Each G$\alpha$ isoform showed distinct expression patterns during embryogenesis, suggesting the involvement of G protein signaling in a variety of physiological and developmental processes in ascidian embryos and larvae. We also report that two CNS-specific G$\alpha$ isoforms are produced from a single gene by alternative splicing. The CNS-specific G$\alpha$ isoforms may couple with Ci-opsin1 in the photoreceptor cells of the ocellus.

**MATERIALS AND METHODS**

**Animals and embryos**

Mature adults of *C. intestinalis* were collected from harbors in Murotsu and Aioi, Hyogo, Japan. The adults were maintained in indoor tanks of artificial seawater (Marine Art BR, Senju Seiyaku, Osaka, Japan) at 18°C. The embryos were prepared using gametes obtained from the gonoducts, as described previously (Nakagawa et al., 1999).

**Isolation and sequencing of cDNA clones encoding G protein $\alpha$ subunits**

The total RNA was prepared from the *C. intestinalis* larvae, and a cDNA library was constructed with a ZAP vector (Stratagene, La Jolla, USA), as described previously (Iwasa et al., 2000). The cDNA library was directly used as a template to amplify the cDNA fragments of about 500 bp encoding the G protein $\alpha$ subunits by polymerase chain reaction (PCR), using a pair of degenerate oligonucleotide primers corresponding to two conserved amino acid sequences, KQM(K/R)IHH and KWV(I/G)CF, respectively. The 5'- and 3'-portions of cDNAs were amplified from the cDNA library by PCR using a gene-specific primer and a vector primer, as described previously (Iwasa et al., 2000). Full-length cDNA clones were amplified by PCR using a thermostable DNA polymerase bearing proofreading activity (Takara LA Taq; Takara Shuzo, Japan), with primers corresponding to the 5'- and 3'-untranslated region (UTR) sequences of cDNAs (5'-ATACGAGCAAGGCACGGGGA-3' for 5'-UTR, and 5'-TATGCTATGCGATGCCTAC-3' for 3'-UTR). The PCR products were subcloned into plasmid vectors and sequenced on both strands with an automatic DNA sequencer (Shinadzu DSO 1000L, Shimadzu, Kyoto, Japan).

**Molecular phylogenetic analysis**

The deduced amino acid sequences of G$\alpha$ encoded by the 500-bp cDNA fragments amplified by PCR from *C. intestinalis* larvae were aligned with the amino acid sequences of G$\alpha$s from other animals. A neighbor-joining tree was constructed with the alignment using the Clustal W program (Thompson et al., 1994). The evolutionary distances were estimated using Kimura's empirical method. The sequences used were: eleven Homo sapiens G$\alpha$ isoforms (G$\alpha_6$ XM_011603, G$\alpha_9$ NM_002072, G$\alpha_10$ X04408, G$\alpha_{11}$ L10665, G$\alpha_{12}$ XM_009867, G$\alpha_{14}$ AF011497, G$\alpha_{12}$ L01694, G$\alpha_{12}$ L22025, G$\alpha_{15}$ XM_0099220, cone transducin D10377, rod transducin X63749), two *Rattus norvegicus* isoforms (G$\alpha_2$ M17526, gustducin X65741), four *Octopus vulgaris* G$\alpha_i$ isoforms (G$\alpha_i$ AB025780, G$\alpha_i$ AB025782, G$\alpha_{22}$ AB025781, G$\alpha_{15}$ AB025783), and *Drosophila melanogaster* G$\alpha_{9}$ L09700.

**In situ hybridization**

Digoxigenin-labeled RNA probes were synthesized using a DIG RNA labeling kit (Roche, Japan), according to the manufacturer’s protocol. For CiG$\alpha_{1a}$, CiG$\alpha_{1b}$, and CiG$\alpha_{1o}$, the 500-bp cDNA fragments obtained by PCR were used as templates to synthesize the probes. For CiG$\alpha_{1a}$ and CiG$\alpha_{1o}$, the RNA probe was synthesized from the 136-bp coding region (nt 607–742 for CiG$\alpha_{1a}$ and nt 497–632 for CiG$\alpha_{1o}$). A genomic DNA fragment containing exons for both CiG$\alpha_{1a}$ and CiG$\alpha_{1o}$ was used as a template for PCR amplification. A neighbor-joining tree was constructed with the alignment of the deduced amino acid sequences of G$\alpha$s from the 500-bp cDNA fragments amplified by PCR from *C. intestinalis* larvae and corresponding exons from the genomes of *D. melanogaster* and *H. sapiens*. The tree was rooted with the corresponding exons from the *C. elegans* genome. Whole-mount *in situ* hybridization was carried out basically according to the protocol by Wada et al. (1995). After the coloring reaction, the embryos and larvae were dehydrated in an ethanol series and incubated in ethanol for 5–10 min. Following rehydration with PBST, the embryos were incubated in 25% glycerol in PBST for 5 min, and then transferred to 50% glycerol in PBST. The embryos were photographed and stored in 50% glycerol/PBST.

**Analysis of partial genomic structure of the CiG$\alpha_{1a}$ gene**

The genomic DNA of *C. intestinalis* was extracted from the sperm of one individual, according to a standard method (Sambrook et al., 1989). A genomic DNA fragment containing exons for both CiG$\alpha_{1a}$ and CiG$\alpha_{1o}$ was amplified by PCR using LA Taq with a pair of gene-specific primers 5'-TGGGGACTGTCATGGGACAAATG-3' (corresponding to nt 520–541 of the CiG$\alpha_{1a}$ cDNA), and 5'-GCTACACAGAGATGCGAGG-3' (corresponding to nt 808–829 of the CiG$\alpha_{1a}$ cDNA). The PCR products were cloned into pBluescript II SK (+) (Stratagene). The nucleotide sequence was determined on both strands with an automatic DNA sequencer (Shimadzu DSO 1000L, Shimadzu).
RESULTS

Isolation and characterization of *C. intestinalis* cDNAs encoding Gα subunits

Five DNA fragments, each encoding a central part (164–166 aa) of Gα with a distinct amino acid sequence, were amplified from a *C. intestinalis* larval cDNA library by PCR with the degenerate primers. The Gα isoforms encoded by the cDNA fragments were designated as CiGαx, CiGαq, CiGαi1a, CiGαi1b, and CiGαi2. The deduced amino acid sequences of the five isoforms were shown in Fig. 1.

To investigate the structural and evolutionary relationships among CiGαx, CiGαq, CiGαi1a, CiGαi1b, CiGαi2, and known metazoan Gα isoforms, phylogenetic analysis was performed by the neighbor-joining method (Saitou and Nei, 1987; Fig. 2). CiGαq is most closely related to vertebrate Gαq/Gα11 isoforms. CiGαx is also a member of the Gq class. Within the Gq class, however, CiGαx is fairly diverged from both the Gαq/Gα11 subfamily and the Gα15/Gαi16 subfamily. Therefore, CiGαx may represent a novel subfamily, the members of which have not been identified in other animals. CiGαi1a, CiGαi1b, and CiGαi2 were closely related to vertebrate and invertebrate Gαi isoforms. Among these, the *C. intestinalis* Gαi isoforms CiGαi1a and CiGαi1b are most closely related to each other.

**Fig. 1.** Comparison of amino acid sequences among *C. intestinalis* Gα isoforms. Amino acid sequences encoded by the 500-bp cDNA fragments of five *C. intestinalis* Gα are aligned. Dashes indicate gaps introduced in the sequence to optimize the alignment. Asterisks indicate the positions where all isoforms exhibit the same amino acid. Dots indicate the positions where all of the residues are similar to each other. Arrows above the CiGαx sequence indicate the positions of the two primers used to amplify the cDNA fragments by PCR.

 messenger RNA encoding CiGαi1a and CiGαi1b are produced by alternative splicing from the CiGαi gene.

Between CiGαi1a and CiGαi1b, only 12 residues were different out of 165 amino acids encoded by the cDNA fragment described above. The diverged positions were limited within a 33-amino acid portion of the polypeptides, and outside this variable region there were few synonymous nucleotides.

**Fig. 2.** Phylogenetic tree of *C. intestinalis* Gα isoforms CiGαx, CiGαq, CiGαi1a, CiGαi1b, and CiGαi2, and known metazoan Gα isoforms. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). The amino acid sequences were aligned with ClustalW (Thompson et al., 1994).
otide substitutions between their cDNA sequences. These features of CiGα1a and CiGα1b indicate the possibility that transcripts for these isoforms are splicing variants of a single gene. To assess this possibility, we first determined the entire cDNA sequences of CiGα1a and CiGα1b. The full-length cDNAs encode a 354-amino acid polypeptide, and the predicted amino acid sequences are identical between CiGα1a and CiGα1b, except for the 12 positions found by the analysis of the PCR fragments (Fig. 3A). The 5' and 3' UTR sequences are almost identical between the CiGα1a and CiGα1b transcripts (identity >98%, data not shown).

To further confirm that the CiGα1a and CiGα1b transcripts originate from a single gene, we then examined the structure of the gene. Genomic DNA containing the coding regions that had different sequences between CiGα1a and CiGα1b were amplified from the C. intestinalis sperm DNA.
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Fig. 3. Two splicing variants of the CiGαi1 gene: CiGαi1a and CiGαi1b. (A) The deduced amino acid sequences of CiGαi1a and CiGαi1b. The entire sequence of CiGαi1a is shown. Dots represent the amino acid residues of CiGαi1a identical to those of CiGαi1b, and the letters represent the variable positions in CiGαi1b. The variable region encoded by the alternatively spliced exons is boxed. (B) Genomic structure of a part of the CiGαi1 gene containing alternatively spliced exons for CiGαi1a and CiGαi1b. The exons are indicated by uppercase letters, whereas the introns are indicated by lowercase letters. The predicted amino acid sequences are indicated beneath the corresponding codons by the single-letter codes.
by PCR. A 2.2-kb genomic DNA fragment was amplified and cloned. The entire nucleotide sequence of the genomic DNA fragment revealed that two homologous exons were tandemly aligned on the same DNA strand (Fig. 3B). One of the exons that locates upstream to the other encodes the variable region of CiGα1b, and the downstream exon

Fig. 4. Spatial expression patterns of mRNAs for five Gα isoforms in C. intestinalis tailbud embryos. Transcripts of each Gα isoform were detected at the mid tailbud stage by whole-mount in situ hybridization. (A, B) Lateral (A) or dorsal view (B) of a tailbud embryo hybridized with a CiGαx probe. (C, D) Lateral (C) or dorsal (D) view of a tailbud embryo hybridized with a CiGαq probe. (E–G) Lateral view of tailbud embryos hybridized with a CiGα1a (E), CiGα1b (F), or CiGα2 (G) probe. See text for details of the expression patterns. Scale bar, 100 μm.
encodes that of CiGαi1b. The positions of the introns adjacent to the isoform-specific exons of CiGαi1a/b are conserved with respect to the Gα sequences in mammals and insects (Fig. 3A,B). The genomic organization of the CiGαi1 gene strongly suggests that the CiGαi1a and CiGαi1b mRNAs are produced by alternative splicing.

Expression patterns of Gα mRNAs in C. intestinalis embryos

The expression patterns of mRNAs for C. intestinalis Gα isoforms were examined in tailbud embryos by whole-mount in situ hybridization (Fig. 4). The CiGαx expression was widely observed throughout the trunk and tail of the embryos, and the signals were stronger in the epidermis, mesenchyme, and tail muscle cells (Fig. 4A,B). The CiGαq transcripts were expressed in the anterior and dorsal trunk epidermis as well as the dorsal side of the tip of the tail (Fig. 4C). The anterior CiGαq-expressing regions seem to contain the developing adhesive organ. On the dorsal trunk epidermis, the CiGαq-expressing regions were bilaterally located as two pairs of patches (Fig. 4D). Both of the two splicing variants of CiGαi1, CiGαi1a, and CiGαi1b, were specifically expressed in the brain and adhesive organ (Fig. 4E, F). A difference in expression patterns was not clear between CiGαi1a and CiGαi1b, although the hybridization signals were much weaker for CiGαi1b. We failed to detect clear hybrid-
ization signals for CiGα12, even after observing the staining reaction for three days; only faint signals were observed in the brain (Fig. 4G).

The distinct expression of the CiGα1f gene in the nervous system of tailbud embryos prompted us to further examine the expression patterns of this gene throughout embryonic development. Probably due to the small size (136 bp) of the splice variant-specific probes, the hybridization signals were very weak, especially for CiGα1b (Fig. 4E,F). Therefore, we used a longer probe that can be hybridized with both CiGα1a and CiGα1b mRNAs (see Materials and Methods). The CiGα1a transcripts were present in the eggs and cleavage stage embryos as maternal messages (Fig. 5B–F). The transcripts were ubiquitously distributed in the eggs and early embryos (Fig. 5B–G). The ubiquitous distribution of the CiGα1a mRNA was observed until the gastrula stage (Fig. 5G). At the neurula stage, the CiGα1a expression was restricted to the anterior ectoderm, especially the presumptive brain vesicle, although the hybridization signal was ambiguous (Fig. 5H). The CiGα1a expression became restricted to the pals (adhesive organ), the central nervous system (CNS), including the brain vesicle and the visceral ganglion, and the dorso-distal part of the tail (Fig. 5I–K). At the mid tailbud stage, the hybridization signals were very strong and were restricted to the pals, the entire brain vesicle, and the visceral ganglion (Fig. 5L,M). The gene expression persisted to the CNS until the tadpole larva stage (Fig. 5N).

**DISCUSSION**

**Diversity of Gα isoforms in ascidians**

In the present study, we showed that mRNAs encoding at least five different Gα isoforms are present in ascidian embryos. Mammals have at least 17 functional Gα genes, several of which are spliced alternatively, that encode 23 distinct protein products (Nürnberg et al., 1995). The presence of multiple Gα isoforms has also been demonstrated in various invertebrates, including insects, nematodes, octopus, hydra, and sponges (Wilkie and Yokoyama, 1994; Suga et al., 1999). Among the Gα isoforms present in ascidians, CiGα is closely related to vertebrate Gαq isoforms, which are products of alternative splicing. In vertebrate, splice variants of Gαo gene generate two different isoforms of Gα (Tsukamoto et al., 1991). These isoforms, Gαo1 and Gαo2, are identical to each other in the N-terminal 248 amino acids; the sequences thereafter diverge. Gαo1 and Gαo2 result from alternative splicing of exons 7 and 8. The diverged carboxyl-terminus contains an effector-interacting domain. Therefore, Gαo1 and Gαo2 exhibit different properties in signal transduction (Kleuss et al., 1991). There are four splice variants of mammalian Gαo (Kozasa et al., 1988). These Gαo variants directly activate adenylate cyclases and calcium channels (Mattera et al., 1989). The relative proportion and tissue distribution of the two variants of Gαo change during cellular differentiation, development, aging, and adaptive processes (Ihnatovych et al., 2001). Mammalian Gαo proteins are encoded by three different genes, Gαo1, Gαo2, and Gαo3, which are closely-related to each other (Itoh et al., 1988). Transcripts of the mammalian Gαo2 gene undergo alternative splicing, which in turn give rise to two distinct proteins with different carboxyl-terminal amino acid sequences (Montmayeur and Borrelli 1994). The Gαo2 variants exhibit differential cellular localization and function. Multiple Gα isoforms are also produced by alternative splicing of a single Gαo gene in Drosophila (de Sousa et al., 1989). Therefore, alternative splicing seems to be a common mechanism to produce diversity of Gα isoforms in a wide variety of animals. Interestingly, the way alternative
splicing occurs differs between \( CiG_{i1} \) and vertebrate \( \alpha \) genes, suggesting that these alternative splicing events evolved independently in ascidians and in vertebrates. In future studies, it will be very important to investigate and learn the functional differences between \( CiG_{i1a} \) and \( CiG_{i1b} \) in the regulation of signal transduction during ascidian development.

Spatially restricted expression of \( Ciona \) \( \alpha \) genes and roles of G-protein signaling in ascidian embryos and larvae.

The present study demonstrated that multiple \( \alpha \) genes are expressed with distinct expression patterns in \( Ciona \) embryos. Although autonomous cell-fate specification is a dominant mechanism in ascidian embryos, the importance of cell-cell communications has increasingly become evident in the determination and differentiation of embryonic cells in ascidians, especially in their nervous systems (Meinertzhagen and Okamura 2001; Wada and Satoh 2001; Darras and Nishida 2001). It is quite probable that \( G \) proteins mediate signaling in these cell-cell interactions during ascidian development. It is also known that \( G \) proteins are located on intracellular membranes, and are involved in membrane trafficking and vesicular transport mechanisms of the cell (Nürnberg et al., 1995). Therefore, some of the \( Ciona \) \( \alpha \) isoforms may participate in these cellular activities.

Among the four \( \alpha \) genes identified in this study, \( CiG_{i4} \) and \( CiG_{i1} \) showed distinct and spatially restricted expression patterns. In the tailbud stage, \( CiG_{i4} \) is expressed in the anterior and dorsal trunk epidermis and the tip tail. Trunk regions expressing \( CiG_{i4} \) seem to include the future adhesive organ and siphon rudiments (Nakayama et al., 2001). The \( CiG_{i4} \) protein may be involved in cell signaling during development of these organs.

From fertilization to the gastrula stage, \( CiG_{i1} \) mRNA is present ubiquitously as maternal messages. Therefore, \( CiG_{i1} \) may mediate signaling between blastomeres during the cleavage stages. Later in embryogenesis, the \( CiG_{i1} \) expression is restricted to the CNS and the adhesive organ. This expression pattern suggests that \( CiG_{i1} \) isoforms play important roles in the development and function of the nervous systems which require intracellular signaling in various aspects.

Recently, we have reported that \( Ciona \) larvae express a vertebrate-type opsin gene \( Ci-opsin1 \) in the photoreceptor cells of the ocellus (Kusakabe et al., 2001). In vertebrates, \( G_{i} \) (transducin) are responsible for signal transduction in the photoreceptor cells of the retina by coupling with rhodopsin, while invertebrate opsins activate \( G_{s} \) and \( G_{o} \) (Tsuda and Tsuda, 1990). However, our extensive search has failed to find \( G_{o} \) in the \( Ciona \) EST and genome sequence databases. Interestingly, both \( G_{s} \) and \( G_{o} \) belong to the \( G_{i} \) class and the vertebrate rhodopsin can activate \( G_{i} \) in vitro (Terakita et al., 2002). We have shown that \( CiG_{i1} \) is expressed in the brain vesicle including photoreceptor cells. Therefore, \( CiG_{i1} \) may interact with \( Ci-opsin1 \) in phototransduction of the ascidian larval ocellus. Since no \( G_{o} \) has been reported in invertebrates to date, \( G_{o} \) may have appeared during early vertebrate evolution after the separation between vertebrates and urochordates.

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