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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 (CiGα$_x$, CiGα$_q$, CiGα$_{i1a}$, CiGα$_{i1b}$, and CiGα$_{i2}$) 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, *CiGα$_{i1a}$* and *CiGα$_{i1b}$* mRNAs were specifically expressed in the CNS of the larva, whereas *CiGα$_q$* transcripts were expressed in small parts of the trunk epidermis and the tip of the tail, but not in the CNS. The *CiGα$_x$* 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 CiGα$_{i1a}$ and CiGα$_{i1b}$ are produced by alternative splicing of transcripts from a single gene, *CiGα$_{i1}$*. In the cleavage and gastrula stages, transcripts of *CiGα$_{i1}$* 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 *CiGα$_{i1}$* is expressed in the ocellus, CiGα$_{i1}$ 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

Ascidians, 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-

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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 α, β, and γ 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 α subunit (Gα), resulting in dissociation of the αβγ trimer into an active Gα-GTP monomer and a Gβγ 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 divergent 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 Gα subunits from the ascidian *Ciona intestinalis*. Each Gα 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 Gα isoforms are produced from a single gene by alternative splicing. The CNS-specific Gα 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 α subunits

The total RNA was prepared from the *C. intestinalis* larvae, and a cDNA library was constructed with a λZAP 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 α subunits by polymerase chain reaction (PCR), using a pair of degenerate oligonucleotide primers corresponding to two conserved amino acid sequences, KQM(K/R)IIH and KWI(H/Q)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'-ATACGAGCAAGCACAGCGGGAA-3' for 5'-UTR, and 5'-TATGCATGCGATGACGTCAC-3' for 3'-UTR). The PCR products were subcloned into plasmid vectors and sequenced on both strands with an automatic DNA sequencer (Shinadzu DSQ 1000L, Shimadzu, Kyoto, Japan).

Molecular phylogenetic analysis

The deduced amino acid sequences of Gα encoded by the 500-bp cDNA fragments amplified by PCR from *C. intestinalis* larvae were aligned with the amino acid sequences of Gα 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* Gα isoforms ($G\alpha_i$ XM_011603, $G\alpha_q$ NM_002072, $G\alpha_s$ X04408, $G\alpha_{olf}$ L10665, $G\alpha_z$ XM_009867, $G\alpha_{11}$ AF011497, $G\alpha_{12}$ L01694, $G\alpha_{13}$ L22075, $G\alpha_{15}$ XM_009220, cone transducin D10377, rod transducin X63749), two *Rattus norvegicus* isoforms ($G\alpha_o$ M17526, gustducin X65741), four *Octopus vulgaris* Gα isoforms ($G\alpha_i$ AB025780, $G\alpha_q$ AB025782, $G\alpha_o$ AB025781, $G\alpha_s$ AB025783), and *Drosophila melanogaster* $G\alpha_f$ 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 *CiGα*$_{i2}$, *CiGα*$_q$, and *CiGα*$_x$, the 500-bp cDNA fragments obtained by PCR were used as templates to synthesize the probes. For *CiGα*$_{i1a}$ and *CiGα*$_{i1b}$, the RNA probe was synthesized from the 136-bp coding region (nt 607–742 for *CiGα*$_{i1a}$ and nt 497–632 for *CiGα*$_{i1b}$) corresponding to the alternatively-spliced exon of each transcript. To detect all transcripts from the *CiGα*$_{i1}$ gene, an RNA probe was also synthesized from a cDNA fragment containing 3'-coding and untranslated regions (spanning from the nt 603 to the 1925 of *CiGα*$_{i1a}$).

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 *CiGα*$_{i1}$ 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 *CiGα*$_{i1a}$ and *CiGα*$_{i1b}$ was amplified by PCR using LA Taq with a pair of gene-specific primers 5'-TGGGAGACTGCATGAAACGAAT-3' (corresponding to nt 520–541 of the *CiGα*$_{i1a}$ cDNA), and 5'-GCTACACAGAAGATGATAGCAG-3' (corresponding to nt 808–829 of the *CiGα*$_{i1a}$ 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 DSQ 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 CiGα$_x$, CiGα$_q$, CiGα$_{i1a}$, CiGα$_{i1b}$, and CiGα$_{i2}$. The deduced amino acid sequences of the five isoforms were shown in Fig. 1.

To investigate the structural and evolutionary relationships among CiGα$_x$, CiGα$_q$, CiGα$_{i1a}$, CiGα$_{i1b}$, CiGα$_{i2}$, and known metazoan Gα isoforms, phylogenetic analysis was performed by the neighbor-joining method (Saitou and Nei, 1987; Fig. 2). CiGα$_q$ is most closely related to vertebrate Gα$_q$/Gα$_{11}$ isoforms. CiGα$_x$ is also a member of the G$_q$ class. Within the G$_q$ class, however, CiGα$_x$ is fairly diverged from both the Gα$_q$/Gα$_{11}$ subfamily and the Gα$_{15}$/Gα$_{16}$ subfamily. Therefore, CiGα$_x$ may represent a novel subfamily, the members of which have not been identified in other animals. CiGα$_{i1a}$, CiGα$_{i1b}$, and CiGα$_{i2}$ were closely related to vertebrate and invertebrate Gα$_i$ isoforms. Among these, the *C. intestinalis* Gα$_i$ isoforms CiGα$_{i1a}$ and CiGα$_{i1b}$ are most closely related to each other.

Messenger RNA encoding CiGα$_{i1a}$ and CiGα$_{i1b}$ are produced by alternative splicing from the *CiGα$_{i1}$* gene

Between CiGα$_{i1a}$ and CiGα$_{i1b}$, 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 nucle-

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                 ──────►
CiGαx   KQMRIIHGTGYLHEELAGHIPLIYQNINFSIKTLATAMLELGIPYELEAN  50
CiGαq   KQMKIIHGAGYSDEDKRSFIRLVYQNIVTSIQNMSAAMQTLNLEYEIEEN  50
CiGαi1a KQMKIIHEDGYSEEECLQYKAVVYSNTLQSLITIVRAMGNLKIDFGSSDR  50
CiGαi1b KQMKIIHEDGYSEEECLQYKAVVYSNTLQSLITIVRAMGNLKIDFGSSDR  50
CiGαi2  KQMRIIHESGYSEDECIQYKPVVYSNTIQSMLAIIRAMGTLSIEFANPER  50
        ***.***  **  ..      ..* *   *.  .  **  * . .

CiGαx   KDFGEVSIELPRECENSLD--TTRVLGIMLFWKDEGVQQCYLRRNEFHLL  98
CiGαq   NEHAEEIREVQVDKISSYDDFITNISYIECLWKDTGIQKCYDRRREYQLS 100
CiGαi1a ADDARQLFSLAGSLEDGEMT-QELGDCMKRMWGDKGVQVCFNRSREFQLN  99
CiGαi1b ADDARQLFSLAGSLEDGEMT-QELGDCMKRMWGDKGVQVCFNRSREFQLN  99
CiGαi2  QDDARQLFSLAGSVEDGSFT-PELISIMKRLWQDDTLQQCFQRAREYQLN  99
         .       .                .   * *  .* *. *  *..*

CiGαx   DSAAYYLNNLTRISSASYLPTLQDVLRSRRPTLGITEYLFKIDKFMFGIV 148
CiGαq   DSTYYYLSDLDRIKKPDFLPTQQDILRVRIPTTGIIEYPFDLDQIIFRMV 150
CiGαi1a DSAQYYLDSLDRLVASDYVPTEQDVLRSRVKTTGIVETQFEHKDLHFKMF 149
CiGαi1b DSAQYYLDSLDRLTEPRYVPTQQDVLRTRVKTTGIVEVDFNFKGLTFKMF 149
CiGαi2  DSAGYYLFALERIGAPDYVPTQQDVLRTRVKTTGIVETHFVFKDLHFKMF 149
        **. ***  * *.    ..** **.** *  * ** *  *      * .

                ◄──────
CiGαx   DVGGQKSERRKWIHCF                                  164
CiGαq   DVGGQRSERRKWIHCF                                  166
CiGαi1a DVGGQRSERKKWIHCF                                  165
CiGαi1b DVGGQRSERKKWIHCF                                  165
CiGαi2  DVGGQRSERKKWIHCF                                  165
        *****.***.******
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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 CiGα$_x$ sequence indicate the positions of the two primers used to amplify the cDNA fragments by PCR.

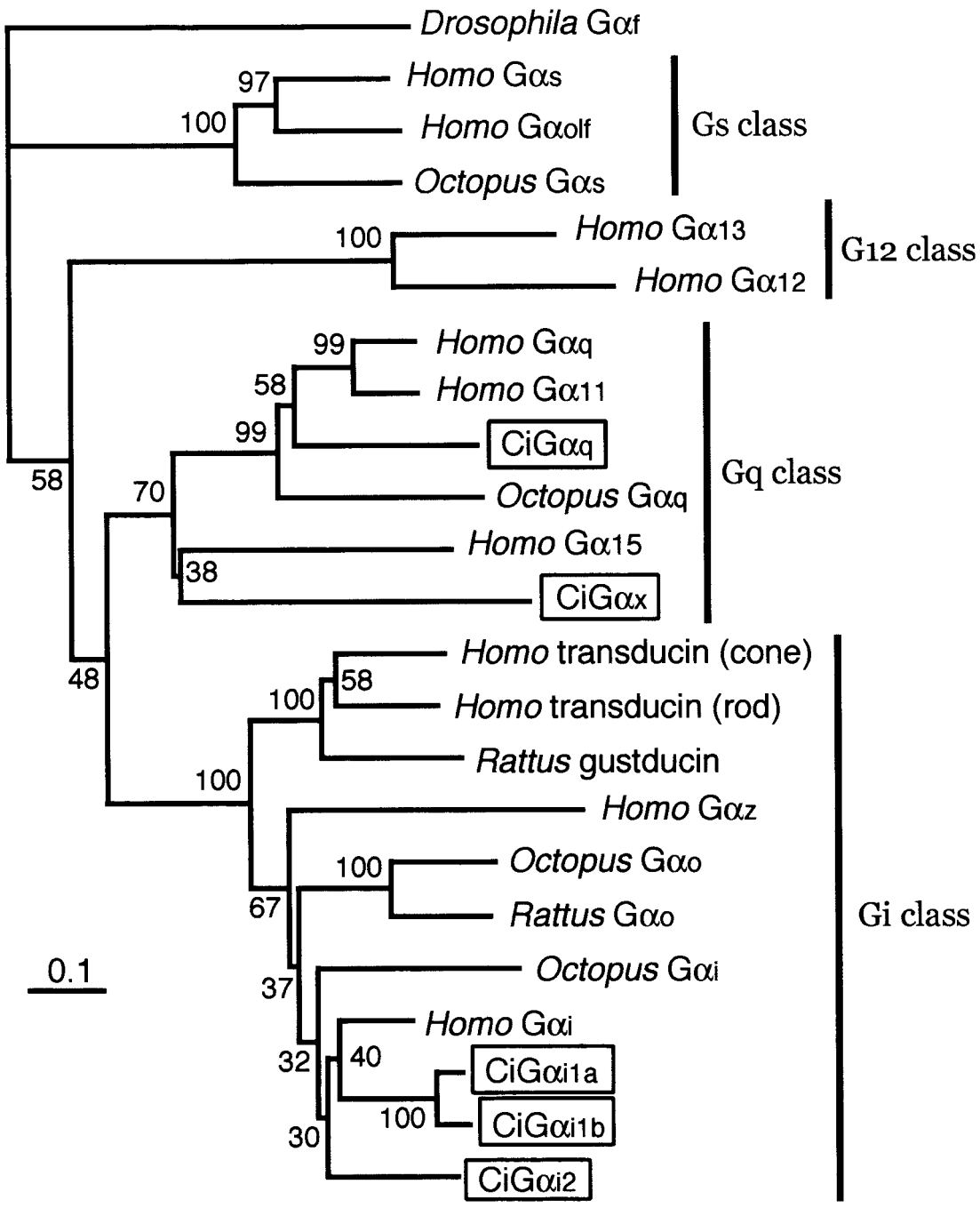

Fig. 2. Molecular phylogenetic tree of G protein α subunits. A phylogenetic tree was inferred from the amino acid sequences by the neighbor-joining method. The scale bar indicates 0.1 amino acid replacements per site. The numbers at the nodes are bootstrap values based on 1,000 replicates. The five *C. intestinalis* Gα isoforms are *boxed*. The four major classes (G_i, G_q, G_s, and G_{12}) of Gα (Simon *et al.*, 1991) are indicated at the right of the corresponding branches.

otide substitutions between their cDNA sequences. These features of CiGα_{i1a} and CiGα_{i1b} 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 CiGα_{i1a} and CiGα_{i1b}. The full-length cDNAs encode a 354-amino acid polypeptide, and the predicted amino acid sequences are identical between CiGα_{i1a} and CiGα_{i1b}, 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 *CiG*α_{i1a} and *CiG*α_{i1b} transcripts (identity >98%, data not shown).

To further confirm that the *CiG*α_{i1a} and *CiG*α_{i1b} 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 *CiG*α_{i1a} and *CiG*α_{i1b} were amplified from the *C. intestinalis* sperm DNA

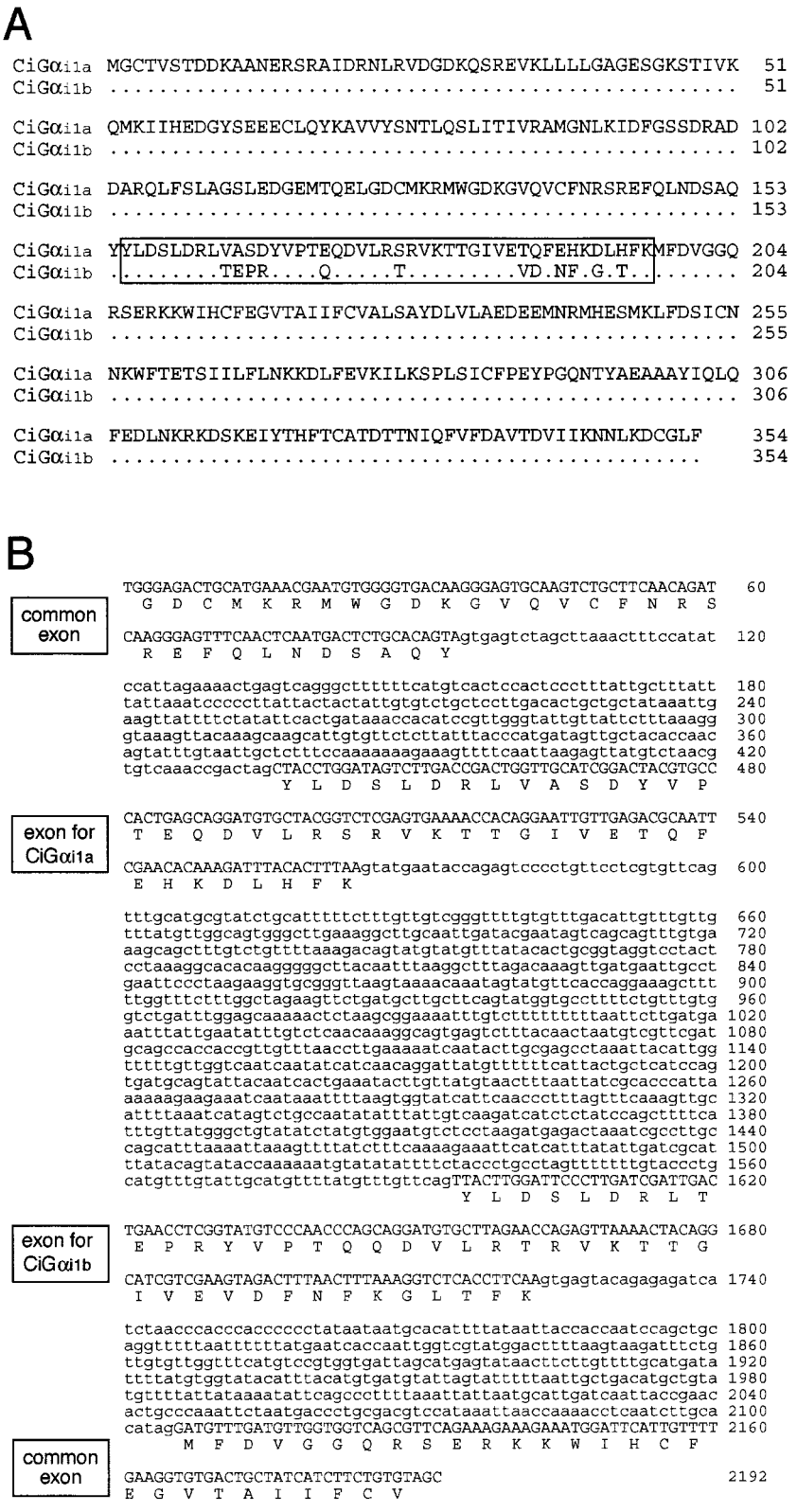

Fig. 3. Two splicing variants of the *CiGα$_{i1}$* gene: *CiGα$_{i1a}$* and *CiGα$_{i1b}$*. (A) The deduced amino acid sequences of CiGα$_{i1a}$ and CiGα$_{i1b}$. The entire sequence of CiGα$_{i1a}$ is shown. *Dots* represent the amino acid residues of CiGα$_{i1b}$ identical to those of CiGα$_{i1b}$, and the letters represent the variable positions in CiGα$_{i1b}$. The variable region encoded by the alternatively spliced exons is *boxed*. (B) Genomic structure of a part of the *CiGα$_{i1}$* gene containing alternatively spliced exons for *CiGα$_{i1a}$* and *CiGα$_{i1b}$*. 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 CiGα_{i1a}, and the downstream exon

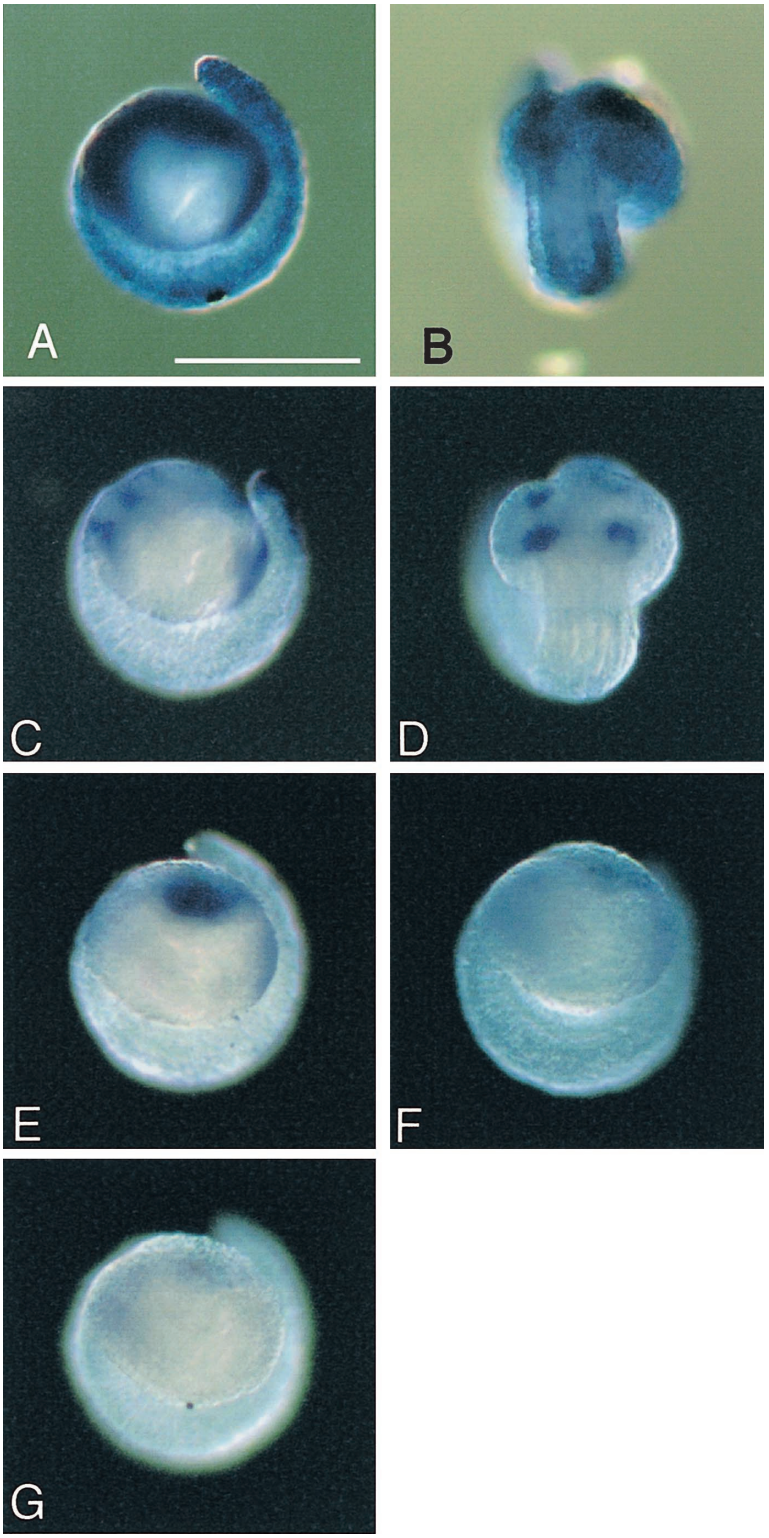

Fig. 4. Spatial expression patterns of mRNAs for five Gα isoforms in *C. intestinalis* tailbud embryos. Transcripts of each Gα isoform were detected at the mid tailbud stage by whole-mount *in situ* hybridization. (A, B) Lateral (A) or dorsal view (B) of an tailbud embryo hybridized with a *CiG*α_x probe. (C, D) Lateral (C) or dorsal (D) view of a tailbud embryo hybridized with a *CiG*α_q probe. (E–G) Lateral view of tailbud embryos hybridized with a *CiG*α_{i1a} (E), *CiG*α_{i1b} (F), or *CiG*α_{i2} (G) probe. See text for details of the expression patterns. Scale bar, 100 μm.

encodes that of CiGα_{i1b}. The positions of the introns adjacent to the isoform-specific exons of *CiG*$\alpha_{i1a/b}$ are conserved with respect to the Gα sequences in mammals and insects (Fig. 3A,B). The genomic organization of the *CiG*α_{i1} gene strongly suggests that the *CiG*α_{i1a} and *CiG*α_{i1b} 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 *CiG*α_x 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 *CiG*α_q 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 *CiG*α_q-expressing regions seem to contain the developing adhesive organ. On the dorsal trunk epidermis, the *CiG*α_q-expressing regions were bilaterally located as two pairs of patches (Fig. 4D). Both of the two splicing variants of *CiG*α_{i1}, *CiG*α_{i1a}, and *CiG*α_{i1b}, were specifically expressed in the brain and adhesive organ (Fig. 4E, F). A difference in expression patterns was not clear between *CiG*α_{i1a} and *CiG*α_{i1b}, although the hybridization signals were much weaker for *CiG*α_{i1b}. We failed to detect clear hybrid-

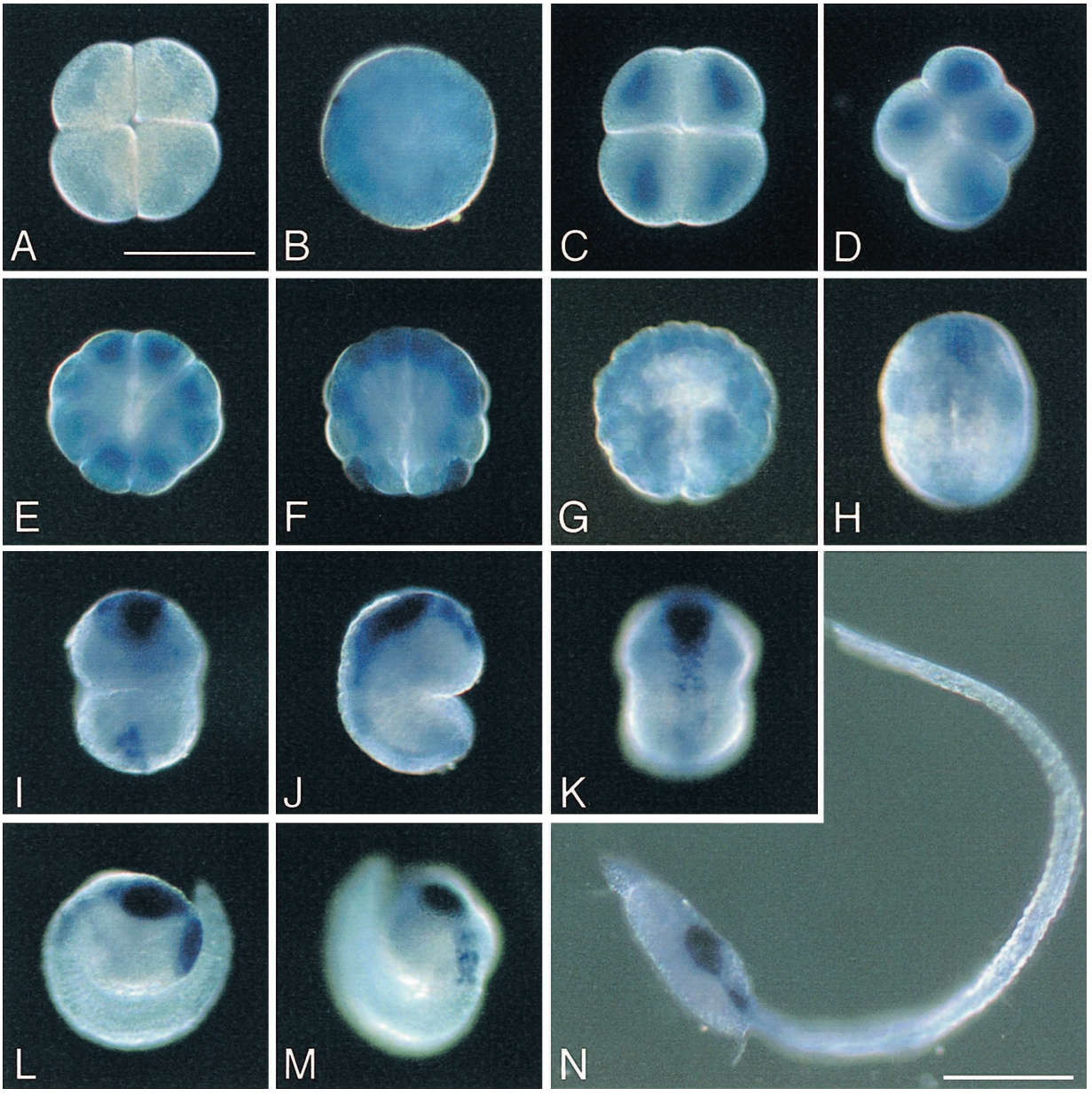

Fig. 5. Whole-mount *in situ* hybridization using a *CiG*α_{i1} sense (A) or antisense (B–N) probe. (A, C) Four-cell embryo. (B) Fertilized egg. (D) Eight-cell embryo. (E) 16-cell embryo. (F) 64-cell embryo. (G) Gastrula. (H) Neurula. (I–K) Ventral (I), lateral (J), or dorsal (K) view of an early tailbud embryo. (L, M) Lateral (L) or dorsal (M) view of mid tailbud embryo. (N) Tadpole larva. Scale bar, 100 μm.

ization signals for *CiGα$_{i2}$*, even after observing the staining reaction for three days; only faint signals were observed in the brain (Fig. 4G).

The distinct expression of the *CiGα$_{i1}$* 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 *CiGα$_{i1b}$* (Fig. 4E,F). Therefore, we used a longer probe that can be hybridized with both *CiGα$_{i1a}$* and *CiGα$_{i1b}$* mRNAs (see Materials and Methods). The *CiGα$_{i1}$* 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 *CiGα$_{i1}$* mRNA was observed until the gastrula stage (Fig. 5G). At the neurula stage, the *CiGα$_{i1}$* expression was restricted to the anterior ectoderm, especially the presumptive brain vesicle, although the hybridization signal was ambiguous (Fig. 5H). The *CiGα$_{i1}$* expression became restricted to the palps (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 palps, 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; Iwasa *et al*., 2000). Metazoan Gα subunits can be classified into G_s, G_i, G_q, and G_{12} classes (Simon *et al*., 1991; Suga *et al*., 1999). The present analysis identified two members of the G_q class and three of the G_i class in *C. intestinalis* embryos. Although we have not found $G\alpha_s$ isoforms in *Ciona*, we recently identified a cDNA clone encoding Gα closely related to vertebrate $G\alpha_s$ isoforms in another ascidian species, *Halocynthia roretzi* (Iwasa *et al*., 2001). To date, however, no Gα isoforms have been assigned to the G_{12} class in ascidians.

Among the G_q class members, $CiG\alpha_q$ is closely related to the vertebrate $G\alpha_q$ and $G\alpha_{11}$. However, the vertebrate $G\alpha_q$ and $G\alpha_{11}$ are more closely related to each other than to $CiG\alpha_q$, suggesting that the vertebrate $G\alpha_q$ and $G\alpha_{11}$ isoforms originated by gene duplication during vertebrate evolution after the divergence between the vertebrate and the urochordate. The metazoan G_q-class isoforms are further classified into $G\alpha_q$ and $G\alpha_{15/16}$ subfamilies. Another *Ciona* G_q-class isoform, $CiG\alpha_x$, is fairly diverged both from the $G\alpha_q$ and $G\alpha_{15/16}$ subfamilies. Therefore, $CiG\alpha_x$ may be a member of a novel Gα subfamily. It will be interesting to see whether Gα closely related to $CiG\alpha_x$, and is present in other animals, including vertebrates.

Based on their primary structure, G_i-class isoforms are further classified into four distinct subfamilies: $G\alpha_i$, $G\alpha_o$, $G\alpha_t$, and $G\alpha_z$ (Suga *et al*., 1999). All three G_i class members of *Ciona* belong to the $G\alpha_i$ subfamily, and so far no ascidian Gα isoforms have been assigned to the $G\alpha_t$, $G\alpha_o$, and $G\alpha_z$ subfamilies. Since $G\alpha_o$ isoforms have been reported in diverse invertebrate phyla, including sponges (Suga *et al*., 1999), arthropods (Thambi *et al*., 1989; Horgan *et al*., 1995), and molluscs (Kojima *et al*., 1997; Iwasa *et al*., 2000), it is likely that ascidians also have this isoform. G_o is abundant in the CNS both in vertebrates (Strathmann *et al*., 1990) and in insects (Thambi *et al*., 1989; Horgan *et al*., 1995), and play important roles in the function and development of the nervous systems (Offermanns 2001). Therefore, future studies are needed to clarify whether $G\alpha_o$ isoforms are present in ascidians.

Alternative splicing of *CiGα$_{i1}$* transcripts

A comparison of the *CiGα$_{i1a}$* and *CiGα$_{i1b}$* cDNA sequences and the genomic structure of the *CiGα$_{i1}$* gene strongly suggest that *CiGα$_{i1a}$* and *CiGα$_{i1b}$* are products of alternative splicing. In vertebrate, splice variants of $G\alpha_s$, $G\alpha_i$, and $G\alpha_o$ are known. Alternative splicing of the mammalian $G\alpha_o$ gene generates two different isoforms of Gα (Tsukamoto *et al*., 1991). These isoforms, $G\alpha_{o1}$ and $G\alpha_{o2}$, are identical to each other in the N-terminal 248 amino acids; the sequences thereafter diverge. $G\alpha_{o1}$ and $G\alpha_{o2}$ result from alternative splicing of exons 7 and 8. The diverged carboxyl-terminus contains an effector-interacting domain. Therefore, $G\alpha_{o1}$ and $G\alpha_{o2}$ exhibit different properties in signal transduction (Kleuss *et al*., 1991). There are four splice variants of mammalian $G\alpha_s$ (Kozasa *et al*., 1988). These $G\alpha_s$ variants directly activate adenylate cyclases and calcium channels (Mattera *et al*., 1989). The relative proportion and tissue distribution of the two variants of $G\alpha_s$ change during cellular differentiation, development, aging, and adaptive processes (Ihnatovych *et al*., 2001). Mammalian $G\alpha_i$ proteins are encoded by three different genes, *Gα$_{i1}$*, *Gα$_{i2}$*, and *Gα$_{i3}$*, which are closely-related to each other (Itoh *et al*., 1988). Transcripts of the mammalian *Gα$_{i2}$* 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 $G\alpha_{i2}$ variants exhibit differential cellular localization and function. Multiple Gα isoforms are also produced by alternative splicing of a single $G\alpha_o$ 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 *CiGα$_{i1}$* and vertebrate Gα 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 CiGα$_{i1a}$ and CiGα$_{i1b}$ in the regulation of signal transduction during ascidian development.

Spatially restricted expression of *Ciona* Gα genes and roles of G-protein signaling in ascidian embryos and larvae

The present study demonstrated that multiple Gα 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* Gα isoforms may participate in these cellular activities.

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

From fertilization to the gastrula stage, *CiGα$_{i1}$* mRNA is present ubiquitously as maternal messages. Therefore, CiGα$_{i1}$ may mediate signaling between blastomeres during the cleavage stages. Later in embryogenesis, the *CiGα$_{i1}$* expression is restricted to the CNS and the adhesive organ. This expression pattern suggests that CiGα$_{i1}$ 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_t (transducin) are responsible for signal transduction in the photoreceptor cells of the retina by coupling with rhodopsin, while invertebrate opsins activate G_q and G_o (Tsuda and Tsuda, 1990). However, our extensive search has failed to find Gα$_t$ in the *Ciona* EST and genome sequence databases. Interestingly, both G_t 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 *CiGα$_{i1}$* is expressed in the brain vesicle including photoreceptor cells. Therefore, CiGα$_{i1}$ may interact with Ci-opsin1 in phototransduction of the ascidian larval ocellus. Since no Gα$_t$ has been reported in invertebrates to date, Gα$_t$ may have appeared during early vertebrate evolution after the separation between vertebrates and urochordates.

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REFERENCES

Corbo JC, Di Gregorio A, Levine M (2001) The ascidian as a model organism in developmental and evolutionary biology. Cell 106: 535–538

Darras S, Nishida H (2001) The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. Dev Biol 236: 271–288

DeCamp DL, Thompson TM, de Sauvage FJ, Lerner MR (2000) Smoothened activates Gαi-mediated signaling in frog melanophores. J Biol Chem 275: 26322–26327

de Sousa SM, Hoveland LL, Yarfitz S, Hurley JB (1989) The *Drosophila* Goα-like protein gene produces multiple transcripts and is expressed in the nervous systems and in ovaries. J Biol Chem 264: 18544–18551

Gilman AG (1987) G proteins: transducers of receptor-generated signals. Annu Rev Biochem 56: 615–649

Hamm HE, Gilchrist A (1996) Heterotrimeric G proteins. Curr Opin Cell Biol 8: 189–196

Hammerschmidt M, McMahon AP (1998) The effect of pertussis toxin on zebrafish development: a possible role for inhibitory G-proteins in Hedgehog signaling. Dev Biol 194: 166–171

Hildebrandt JD (1997) Role of subunit diversity in signaling by heterotrimeric G proteins. Biochemical Pharmacol 54: 325–339

Horgan AM, Lagrange MT, Copenhaver PF (1995) A developmental role for heterotrimeric protein Go$_\alpha$ in a migratory population of embryonic neurons. Dev Biol 172: 640–653

Ihnatovych I, Hejnová L, Kostrnová A, Mares P, Svoboda P, Novotny J (2001) Maturation of rat brain is accompanied by differential expression of the long and short splice variants of $G_s\alpha$ protein: identification of cytosolic forms of $G_s\alpha$. J Neurochem 79: 88–97

Itoh H, Toyama R, Kozasa T, Tsukamoto T, Matsuoka M, Kaziro Y (1988) Presence of three distinct molecular species of Gi protein alpha subunit. Structure of rat cDNAs and human genomic DNAs. J Biol Chem 263: 6656–6664

Iwasa T, Yanai T, Nakagawa M, Kikkawa S, Obata S, Usukura J, Tsuda M (2000) G protein α subunit genes in octopus photoreceptor cells. Zool Sci 17: 711–716

Iwasa T, Kanehara K, Watari A, Ohkuma M, Nakagawa M, Tsuda M (2001) Heterotrimeric G protein α and β subunit genes of the ascidian, *Halocynthia roretzi*. In "The Biology of Ascidians" Ed by Sawada H, Yokosawa H, Lambert CC, Springer-Verlag, Tokyo, New York, pp 147–152

Kleuss C, Hescheler J, Ewel C, Rosenthal W, Schultz G, Wittig B (1991) Assignment of G protein subtypes to specific receptors inducing inhibition of calcium currents. Nature 353: 43–48

Knust E (2001) G protein signaling and asymmetric cell division. Cell 107: 125–128

Kojima D, Terakita A, Ishikawa T, Tsukahara Y, Maeda A, Shichida Y (1997) A novel G_o-mediated phototransduction cascade in scallop visual cells. J Biol Chem 272: 22979–22982

Kozasa T, Itoh H, Tsukamoto T, Kaziro Y (1988) Isolation and char-

acterization of the human Gs gene. Proc Natl Acad Sci USA 85: 2081–2085

Kusakabe T, Kusakabe R, Kawakami I, Satou Y, Satoh N, Tsuda M (2001) *Ci-opsin1*, a vertebrate-type opsin gene, expressed in the larval ocellus of the ascidian *Ciona intestinalis*. FEBS Lett 506: 69–72

Kusakabe T, Yoshida R, Kawakami I, Kusakabe R, Mochizuki Y, Yamada L, Shin-i T, Kohara Y, Satoh N, Tsuda M, Satou Y (2002) Gene expression profiles in tadpole larvae of *Ciona intestinalis*. Dev Biol 242: 188–203

Liu T, DeCostanzo JA, Liu X, Wang H-y, Hallagan S, Moon RT, Malbon CC (2001) G protein signaling from activated rat Frizzled-1 to the β catenin-Lef-Tcf pathway. Science 292: 1718–1722

Marinissen MJ, Gutkind SJ (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmcol Sci 22: 368–376

Mattera R, Graziano MP, Yatani A, Zhou Z, Graf R, Codina J, Birnbaumer L, Gilman AG, Brown AM (1989) Splice variants of the alpha subunit of the G protein Gs activate both adenylyl cyclase and calsium channels. Science 243: 804–807

Malbon CC, Wang H-y, Moon RT (2001) Wnt signaling and heterotrimeric G-proteins: strange bedfellows or a classic romance? Biochem Biophys Res Commun 287: 589–593

Meinertzhagen IA, Okamura Y (2001) The larval ascidian nervous system: the chordate brain from its small beginnings. Trends Neurosci 24: 401–410

Montmayeur J-P, Borrelli E (1994) Targeting of $G\alpha_{i2}$ to the Golgi by alternative spliced carboxy-terminal region. Science 263: 95–98

Nakagawa M, Miyamoto T, Ohkuma M, Tsuda M (1999) Action spectrum for the photophobic response of *Ciona intestinalis* (Ascidiacea, Urochordata) larvae implicates retinal protein. Photochem Photobiol 70: 359–362

Nakayama A, Satou Y, Satoh N (2001) Isolation and characterization of genes that are expressed during *Ciona intestinalis* metamorphosis. Dev Genes Evol 211: 184–189

Neer EJ (1995) Heterotrimeric G proteins: organizers of transmembrane signals. Cell 80: 249–257

Nürnberg B, Gudermann T, Schultz G (1995) Receptors and G proteins as primary components of transmembrane signal transduction. Part 2. G proteins: structure and function. J Mol Med 73: 123–132

Offermanns S (2001) *In vivo* functions of heterotrimeric G-proteins: studies in Gα-deficient mice. Oncogene 20: 1635–1642

Ohkuma M, Tsuda M (2000) Visualization of retinal proteins in the cerebral ganglion of ascidian, *Halocynthia rotetzi*. Zool Sci 17: 161–170

Saitou N, Nei M (1987) The neighbor-joining method: a new method of reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning, Cold Spring Harber Laboratory Press, Cold Spring Harber, New York

Satoh N (2001) Ascidian embryos as a model system to analyze expression and function of developmental genes. Differentiation 68: 1–12

Simon MI, Strathmann MP, Gautam N (1991) Diversity of G proteins in signal transduction. Science 252: 802–808

Strathmann M, Wilkie TM, Simon MI (1990) Alternative splicing produces transcripts encoding two forms of the α subunit of GTP-binding protein Go. Proc Natl Acad Sci USA 87: 6477–6481

Suga H, Koyanagi M, Hoshiyama D, Ono K, Iwabe N, Kuma K-i, Miyata T (1999) Extensive gene duplication in the early evolution of animals before the parazoan-eumetazoan split demonstrated by G proteins and protein tyrosine kinases from sponge and hydra. J Mol Evol 48: 646–453

Terakita A, Yamashita T, Nimbari N, Kojima D, Shichida Y (2002) Functional interaction between bovine rhodopsin and G protein transducin. J. Biol Chem 277: 40–46

Thambi NC, Quan F, Wolfgang WJ, Spiegel A, Fotre M (1989) Immunological and molecular characterization of Go_{α}-like proteins in the *Drosophila* CNS. J Biol Chem 264: 18552–18560

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Tsuda M (1987) Photoreception and phototransduction in invertebrate photoreceptors. Photochem Photobiol 45: 915–931

Tsuda M and Tsuda T (1987) Two distinct light regulated G-proteins in octpus photoreceptors. Biochim Biophys Acta 1052: 204–210

Tsuda M, Kawakami I, Miyamoto T, Nakagawa M, Shiraishi S, Gouda, M (2001) Photoresponse and habituation of swimming behavior of ascidian larvae, *Ciona intestinalis*. In "The Biology of Ascidians" Ed by Sawada H, Yokosawa H, Lambert CC, Springer-Verlag, Tokyo, New York, pp 153–157

Tsukamoto T, Toyama R, Itoh H, Kozasa T, Matsuoka M, Kaziro Y (1991) Structure of the human gene and two rat cDNAs encoding the α chain of GTP-binding regulatory protein Go: Two different mRNAs are generated by alternative splicing. Proc Natl Acad Sci USA 88: 2974–2978

Wada H, Satoh N (2001) Patterning the protochordate neural tube. Curr Opin Neurobiol 11: 16–21

Wada S, Katsuyama Y, Yasugi S, Saiga H (1995) Spatially and temporally regulated expression of the LIM class homeobox gene *Hrlim* suggests multiple distinct functions in development of the ascidian, *Halocynthia roretzi*. Mech Dev 51: 115–126

Wilkie TM, Yokoyama S (1994) Evolution of the G protein α subunit multigene family. Soc Gen Physiol Ser 49: 249–270

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