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# Central nervous system-specific expression of G protein $\alpha$ 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  $\alpha$  subunits (CiG $\alpha_x$ , CiG $\alpha_q$ , CiG $\alpha_{i1a}$ , CiG $\alpha_{i1b}$ , and CiG $\alpha_{i2}$ ) 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, CiG $\alpha_{i1a}$  and CiG $\alpha_{i1b}$  mRNAs were specifically expressed in the CNS of the larva, whereas CiG $\alpha_q$  transcripts were expressed in small parts of the trunk epidermis and the tip of the tail, but not in the CNS. The CiG $\alpha_x$  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 CiG $\alpha_{i1a}$  and CiG $\alpha_{i1b}$  are produced by alternative splicing of transcripts from a single gene, CiG $\alpha_{i1}$ . In the cleavage and gastrula stages, transcripts of CiG $\alpha_{i1}$  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  $\alpha$  subunits in *C. intestinalis*. Since CiG $\alpha_{i1}$  is expressed in the ocellus, CiG $\alpha_{i1}$  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  $\alpha$ ,  $\beta$ , 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  $\alpha\beta\gamma$  trimer into an active  $G\alpha$ -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 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  $G\alpha$  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  $\lambda$ 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)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'-TATGCATGCGATGACGTAC-3' for 3'-UTR). The PCR products were subcloned into plasmid vectors and sequenced on both strands with an automatic DNA sequencer (Shimadzu DSQ 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  $G\alpha$  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 ( $G\alpha_i$  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*  $G\alpha$  isoforms ( $G\alpha_i$  AB025780,  $G\alpha_q$  AB025782,  $G\alpha_o$  AB025781,  $G\alpha_s$  AB025783), and *Drosophila melanogaster*  $G\alpha_i$  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\_{i2}*, *CiG\alpha\_q*, and *CiG\alpha\_x*, the 500-bp cDNA fragments obtained by PCR were used as templates to synthesize the probes. For *CiG\alpha\_{i1a}* and *CiG\alpha\_{i1b}*, the RNA probe was synthesized from the 136-bp coding region (nt 607–742 for *CiG\alpha\_{i1a}* and nt 497–632 for *CiG\alpha\_{i1b}*) corresponding to the alternatively-spliced exon of each transcript. To detect all transcripts from the *CiG\alpha\_{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 *CiG\alpha\_{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 *CiG\alpha\_{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 *CiG\alpha\_{i1a}* and *CiG\alpha\_{i1b}* was amplified by PCR using LA Taq with a pair of gene-specific primers 5'-TGGGAGACTGCGATGAAACGAAT-3' (corresponding to nt 520–541 of the *CiG\alpha\_{i1a}* cDNA), and 5'-GCTACACAGAAGATGATAGCAG-3' (corresponding to nt 808–829 of the *CiG\alpha\_{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  $G\alpha$  subunits**

Five DNA fragments, each encoding a central part (164–166 aa) of  $G\alpha$  with a distinct amino acid sequence, were amplified from a *C. intestinalis* larval cDNA library by PCR with the degenerate primers. The  $G\alpha$  isoforms encoded by the cDNA fragments were designated as  $CiG\alpha_x$ ,  $CiG\alpha_q$ ,  $CiG\alpha_{i1a}$ ,  $CiG\alpha_{i1b}$ , and  $CiG\alpha_{i2}$ . The deduced amino acid sequences of the five isoforms were shown in Fig. 1.

To investigate the structural and evolutionary relationships among  $CiG\alpha_x$ ,  $CiG\alpha_q$ ,  $CiG\alpha_{i1a}$ ,  $CiG\alpha_{i1b}$ ,  $CiG\alpha_{i2}$ , and known metazoan  $G\alpha$  isoforms, phylogenetic analysis was performed by the neighbor-joining method (Saitou and Nei, 1987; Fig. 2).  $CiG\alpha_q$  is most closely related to vertebrate

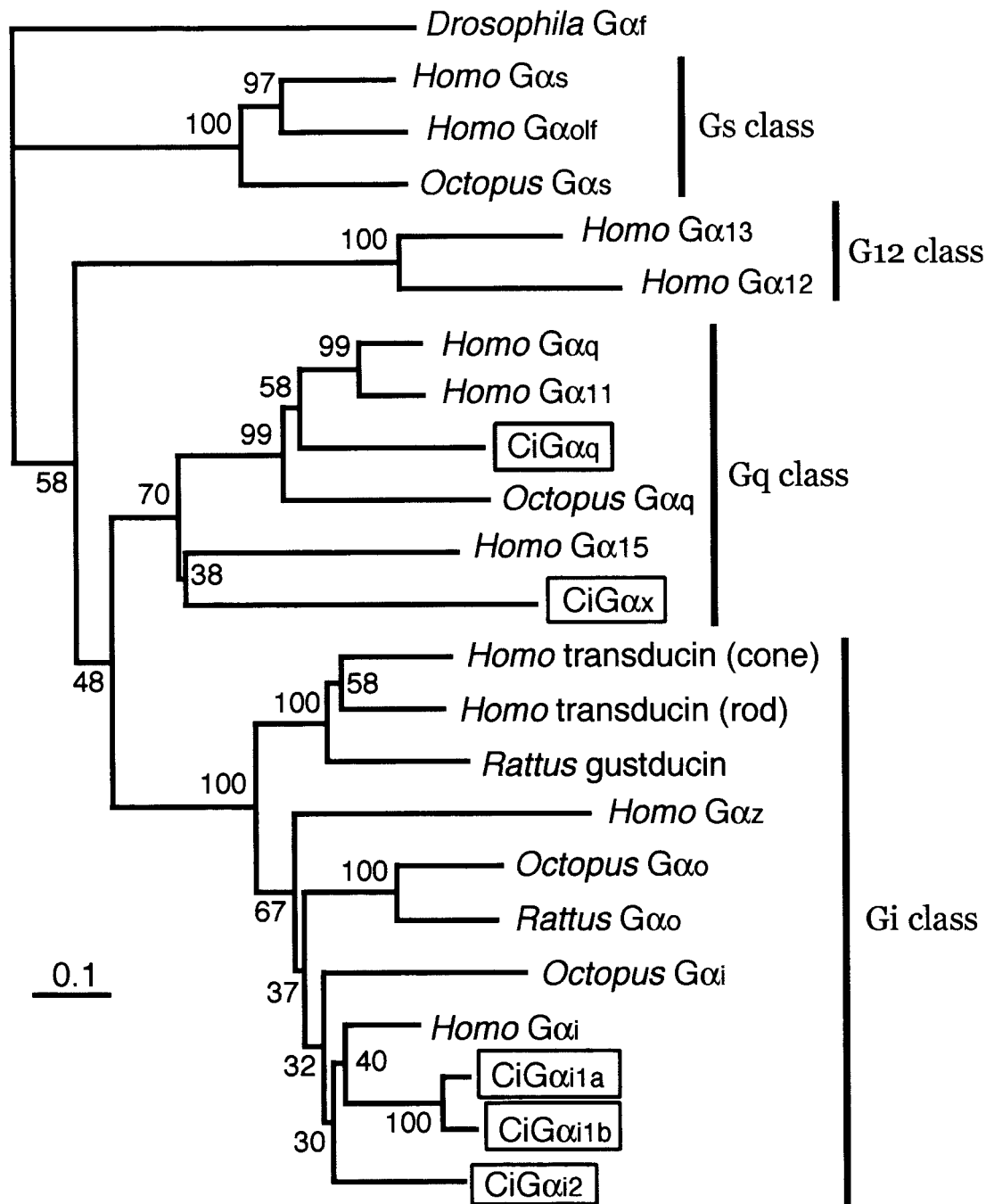
$G\alpha_q/G\alpha_{11}$  isoforms.  $CiG\alpha_x$  is also a member of the  $G_q$  class. Within the  $G_q$  class, however,  $CiG\alpha_x$  is fairly diverged from both the  $G\alpha_q/G\alpha_{11}$  subfamily and the  $G\alpha_{15}/G\alpha_{16}$  subfamily. Therefore,  $CiG\alpha_x$  may represent a novel subfamily, the members of which have not been identified in other animals.  $CiG\alpha_{i1a}$ ,  $CiG\alpha_{i1b}$ , and  $CiG\alpha_{i2}$  were closely related to vertebrate and invertebrate  $G\alpha_i$  isoforms. Among these, the *C. intestinalis*  $G\alpha_i$  isoforms  $CiG\alpha_{i1a}$  and  $CiG\alpha_{i1b}$  are most closely related to each other.

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

Between  $CiG\alpha_{i1a}$  and  $CiG\alpha_{i1b}$ , 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-

$CiG\alpha_x$	KQMRIIHGTGYLHEELAGHIPLIYQNINFSIKTLATAMLELGIPYELEAN	50
$CiG\alpha_q$	KQMKIIHGAGYSDEDKRSFIRLVYQNIIVTSIQNMSAAMQTLNLEYEIEEN	50
$CiG\alpha_{i1a}$	KQMKIIHEDGYSEEECLQYKAVVYSNTLQSLITIVRAMGNLKIDFGSSDR	50
$CiG\alpha_{i1b}$	KQMKIIHEDGYSEEECLQYKAVVYSNTLQSLITIVRAMGNLKIDFGSSDR	50
$CiG\alpha_{i2}$	KQMRIIHESGYSEDECIQYKPVVYSNTIQSMLAIIRAMGTLSEFANPER	50
	***.*** ** .. ..* * *. . ** * . .	
$CiG\alpha_x$	KDFGEVSIELPRECENSLD--TTRVLGIMLFWKDEGVQQCYLRRNEFHLL	98
$CiG\alpha_q$	NEHAAEIREVQVDKISSYDDFITNISYIECLWKDTGIQKCYDRRREYQLS	100
$CiG\alpha_{i1a}$	ADDARQLFSLAGSLEDGEMT-QELGDCMKRMWGDKGVQVCFNRSREFQLN	99
$CiG\alpha_{i1b}$	ADDARQLFSLAGSLEDGEMT-QELGDCMKRMWGDKGVQVCFNRSREFQLN	99
$CiG\alpha_{i2}$	QDDARQLFSLAGSVEDGSFT-PELISIMKRLWQDDTLQQCFQAREYQLN	99
	. . . * * . * * . * * . *	
$CiG\alpha_x$	DSAAYYLNNLTRISSASYLPTLQDVLRSRRPTLGITEYLFKIDKFMFGIV	148
$CiG\alpha_q$	DSTYYYLSLDRIKKPDLPTQQDILRVRIPTTGIIIEYPFDLQIIIFRMV	150
$CiG\alpha_{i1a}$	DSAQYYLDSLDRLVASDYVPTQDVLRSRVKTTGIVETQFEHKDLHFHMF	149
$CiG\alpha_{i1b}$	DSAQYYLDSLDRLTEPRYVPTQDVLRTRVKTTGIVEVDFNFKGLTFHMF	149
$CiG\alpha_{i2}$	DSAGYYLFALEIRIGAPDYVPTQDVLRTRVKTTGIVETHFVFKDLHFHMF	149
	** . *** * * . . ** ** * * * * * * *	
$CiG\alpha_x$	DVGGQKSERRKWIHCF	164
$CiG\alpha_q$	DVGGQSRERRKWIHCF	166
$CiG\alpha_{i1a}$	DVGGQSRERKKWIHCF	165
$CiG\alpha_{i1b}$	DVGGQSRERKKWIHCF	165
$CiG\alpha_{i2}$	DVGGQSRERKKWIHCF	165
	***** . *** .*****	

**Fig. 1.** Comparison of amino acid sequences among *C. intestinalis*  $G\alpha$  isoforms. Amino acid sequences encoded by the 500-bp cDNA fragments of five *C. intestinalis*  $G\alpha$  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  $CiG\alpha_x$  sequence indicate the positions of the two primers used to amplify the cDNA fragments by PCR.



**Fig. 2.** Molecular phylogenetic tree of G protein  $\alpha$  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*  $G\alpha$  isoforms are boxed. The four major classes ( $G_i$ ,  $G_q$ ,  $G_s$ , and  $G_{12}$ ) of  $G\alpha$  (Simon *et al.*, 1991) are indicated at the right of the corresponding branches.

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

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

## A

Ci $\alpha_{11a}$	MGCTVSTDDKAANERSRAIDRNLRVDGDKQSREVKLLLLGAGESGKSTIVK	51
Ci $\alpha_{11b}$	.....	51
Ci $\alpha_{11a}$	QMKIIHEDGYSEEECLQYKAVVYSNTLQSLITIVRAMGNLKIDFGSSDRAD	102
Ci $\alpha_{11b}$	.....	102
Ci $\alpha_{11a}$	DARQLFSLAGSLEDGEMTQELGDCMKRMWGDKGVCFNRSREFQLNDSAQ	153
Ci $\alpha_{11b}$	.....	153
Ci $\alpha_{11a}$	YLDLSLRLVASDYVPTEQDVLRSRVKTTGIVETQFEHKDLHFKMFVDVGGQ	204
Ci $\alpha_{11b}$	.....TEPR.....Q.....T.....VD.NF.G.T.....	204
Ci $\alpha_{11a}$	RSEKKWIHCFEGVTAIIFCVALSAYDLVLADEEMNRMHESMKLFDSICN	255
Ci $\alpha_{11b}$	.....	255
Ci $\alpha_{11a}$	NKWFTEYSIILFLNKKDLFEVKILKSPLSICFPEYPGQNTYAEAAAYIQLQ	306
Ci $\alpha_{11b}$	.....	306
Ci $\alpha_{11a}$	FEDLNKRKDSKEIYTHFTCATD'TTNIQFVFDVTDV IKNLKD CGLF	354
Ci $\alpha_{11b}$	.....	354

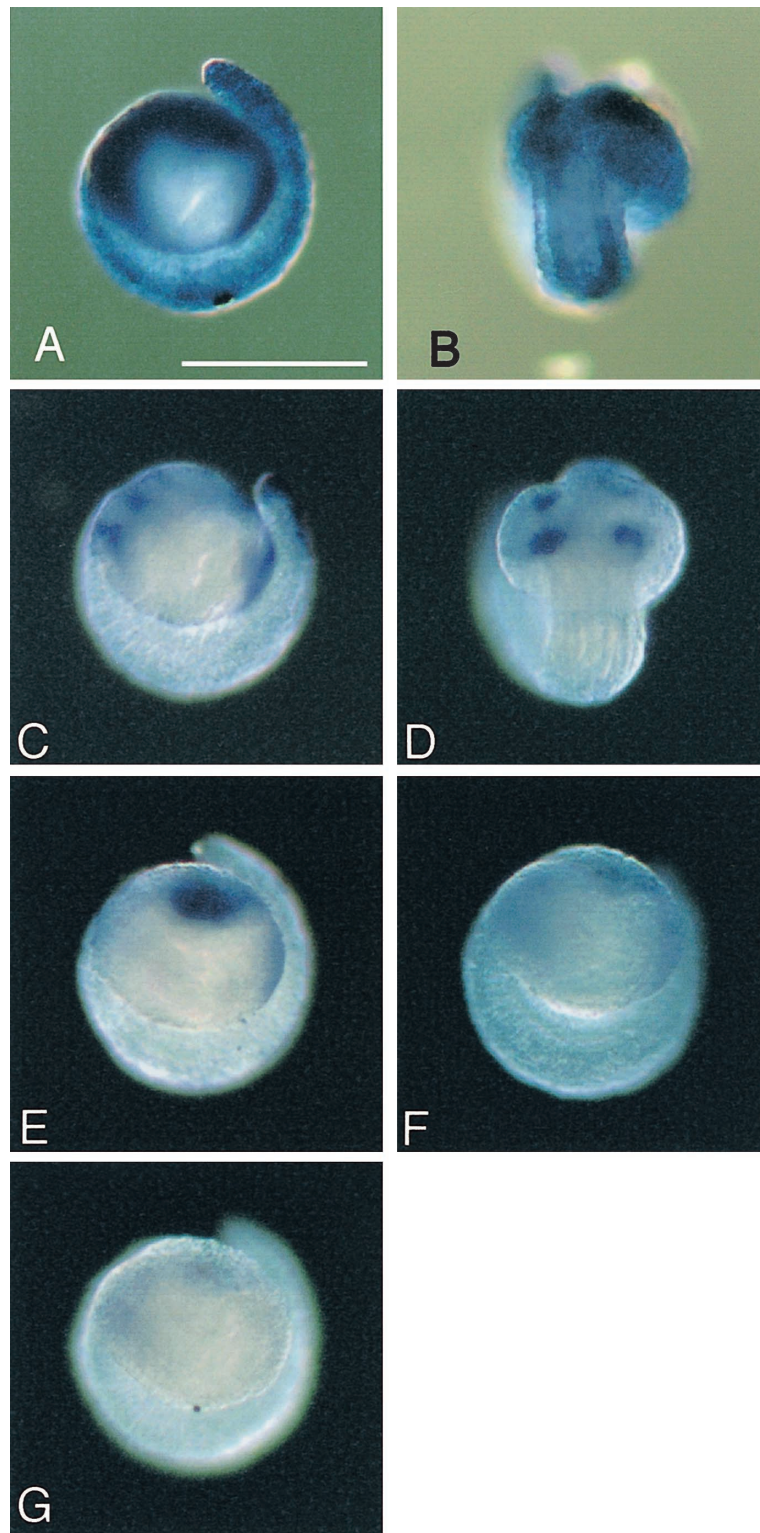
## B

common exon	TGGGAGACTGCATGAAACGAATGTGGGTGACAAAGGAGTGCAAGTCTGCTTCAACAGAT	60
	G D C M K R M W G D K G V Q V C F N R S	
common exon	CAAGGGAGTTTCAACTCAATGACTCTGCACAGTAGtgagtgcttagcttaaactttccatat	120
	R E F Q L N D S A Q Y	
common exon	ccattagaaaaactgagtcagggtctttttcatgtcactccactccctttattgctttatt	180
	tattaaatcccccttattactactattgtgtctgctccttgacactgctgctataaattg	240
common exon	aagttattttctattctactgataaacacacatcggttggtattgttattctttaaagg	300
	gtaaagttacaaagcaagcattgtgttctctatttaccatgatagttgctacaccaac	360
common exon	agtatttgtaattgctctttccaaaaaagaaagtttcaattaaagagttatgtctaacg	420
	tgtaaaacccgactagCTACCTGGATAGTCTTGACCGACTGGTTCATCGGACTACGTGCC	480
common exon	Y L D S L D R L V A S D Y V P	
	CACTGAGCAGGATGTGCTACGGTCTCGAGTGAAACACAGGAATTGTTGAGACGCAATT	540
common exon	T E Q D V L R S R V K T T G I V E T Q F	
	CGAACACAAAGATTACACTTTAAGtatgaataaccagagtcacctgttctcgtgttcag	600
common exon	E H K D L H F K	
	tttgcatgcgtatctgcattttttcttgggtgtcggttttgggttgacattgtttgttg	660
common exon	tttatgttggcagtggtgtgaaaggcttgcaattgatacgaatagtcagcagttgttga	720
	aagcagctttgtctgttttaagacagtagtatgtttatacactgcggttaggtcctact	780
common exon	cctaaaggcacacaaggggtttacaatttaaggcttttagacaaagttgatgaattgcct	840
	gaattccctaaagagtggtggttttaagtaaaacaaatagtagttccaccaggaagcttt	900
common exon	ttggtttcttggctagaagttctgatgcttgcctcagtagtggtgcctttctgtttgtg	960
	gtctgatttggagcaaaaactctaagcgggaaaatttgccttttttttaattcttgatga	1020
common exon	aatttattgaattttgtctcaacaaaggcagtgagttttacaaactaatgtcgttcgat	1080
	gcagccaccacggtgttttaaccttgaaaaatcaatacttgcgagcctaaattacattgg	1140
common exon	tttttgggtgtaacatcatcaacaggattatgttttttcatctactgctcatccag	1200
	tgatgcagattacaaactcagaaacttggtagtaactttaattatgcacaccatta	1260
common exon	aaaaagaaagaaatcaataaatttaagtggtatcattcaacccttttagtttcaagttgc	1320
	attttaaatcatagtcgccaatatattttgtcaagatcatctcatccagcttttca	1380
common exon	tttggtaggggtgtatatctatgtggaatgtctcctaagatgagactaaatcgcttgc	1440
	cagcatttaaaataaagttttatcttcaaaagaaatcatcatttatattgatgcgat	1500
common exon	ttatacagatataccaaaaatgtatatattttctaccctgctagttttttgtaccctg	1560
	catgtttgtattgcattgtttatgtttgttcagTACTTGGATTCCCTTGATCGATTGAC	1620
common exon	Y L D S L D R L T	
	TGAACCTCGGTATGTCCCAACCCAGCAGGATGTGCTTAGAACAGAGTTAAACTACAGG	1680
common exon	E P R Y V P T Q Q D V L R T R V K T T G	
	CATCGTCGAAGTAGACTTTAACTTTAAAGTCTCACCTTCAAGtgagtagacagagatca	1740
common exon	I V E V D F N F K G L T F K	
	tctaaccacccacccccctataataatgcacattttataattaccaccaatccagctgc	1800
common exon	agggttttaattttttatgaatcaccaattgggtcgtaggacttttaagtaagattctg	1860
	tttggttgggtttcatgtccgtggtgattagcatgagtataactctgttttgcagatga	1920
common exon	ttttatgtgtatacatattacatgtgatgtattagatttttaattgctgacatgctga	1980
	tggtttattataaaattatcagcccttttaattattatgcatgatcaattaccgaac	2040
common exon	actgccaaatttcaatgacccctgcgacgtccataaattaacaaaacctcaattcttga	2100
	catagGATGTTTGTGTTGGTGGTCAGCGTTCAGAAAGAAAGAAATGGATTGATTGTTT	2160
common exon	M F D V G G Q R S E R K K W I H C F	
	GAAGGTGTGACTGCTATCATCTTCTGTGTAGC	2192
common exon	E G V T A I I F C V	

**Fig. 3.** Two splicing variants of the *Ci $\alpha_{11}$*  gene: *Ci $\alpha_{11a}$*  and *Ci $\alpha_{11b}$* . (A) The deduced amino acid sequences of *Ci $\alpha_{11a}$*  and *Ci $\alpha_{11b}$* . The entire sequence of *Ci $\alpha_{11a}$*  is shown. Dots represent the amino acid residues of *Ci $\alpha_{11b}$*  identical to those of *Ci $\alpha_{11a}$* , and the letters represent the variable positions in *Ci $\alpha_{11b}$* . The variable region encoded by the alternatively spliced exons is boxed. (B) Genomic structure of a part of the *Ci $\alpha_{11}$*  gene containing alternatively spliced exons for *Ci $\alpha_{11a}$*  and *Ci $\alpha_{11b}$* . 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  $CiG\alpha_{i1a}$ , and the downstream exon



**Fig. 4.** Spatial expression patterns of mRNAs for five  $G\alpha$  isoforms in *C. intestinalis* tailbud embryos. Transcripts of each  $G\alpha$  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  $CiG\alpha_x$  probe. (C, D) Lateral (C) or dorsal (D) view of a tailbud embryo hybridized with a  $CiG\alpha_q$  probe. (E–G) Lateral view of tailbud embryos hybridized with a  $CiG\alpha_{i1a}$  (E),  $CiG\alpha_{i1b}$  (F), or  $CiG\alpha_{i2}$  (G) probe. See text for details of the expression patterns. Scale bar, 100  $\mu$ m.

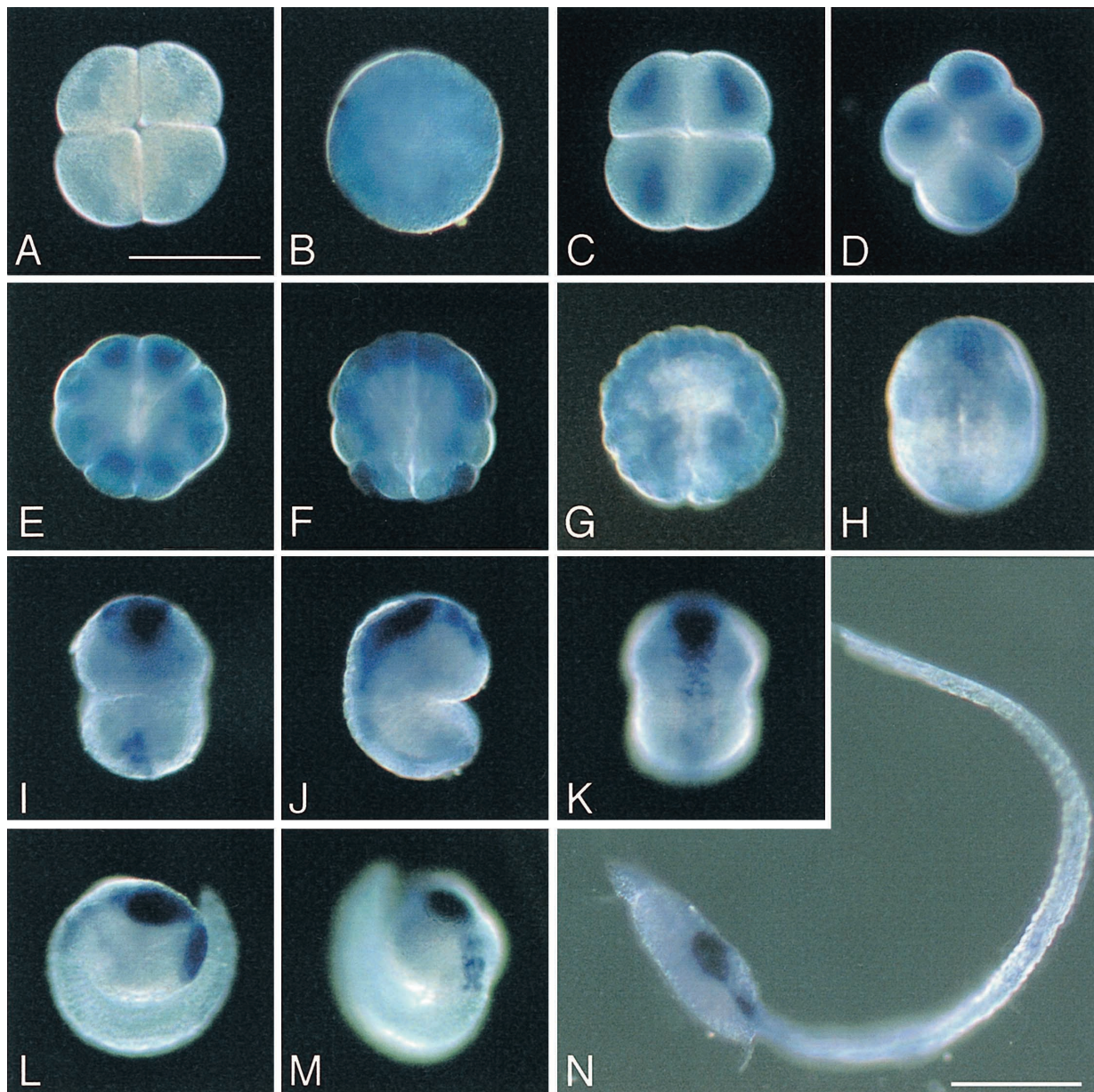


encodes that of  $CiG\alpha_{i1b}$ . The positions of the introns adjacent to the isoform-specific exons of  $CiG\alpha_{i1a/b}$  are conserved with respect to the  $G\alpha$  sequences in mammals and insects (Fig. 3A,B). The genomic organization of the  $CiG\alpha_{i1}$  gene strongly suggests that the  $CiG\alpha_{i1a}$  and  $CiG\alpha_{i1b}$  mRNAs are produced by alternative splicing.

#### Expression patterns of $G\alpha$ mRNAs in *C. intestinalis* embryos

The expression patterns of mRNAs for *C. intestinalis*  $G\alpha$  isoforms were examined in tailbud embryos by whole-mount *in situ* hybridization (Fig. 4). The  $CiG\alpha_x$  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  $CiG\alpha_q$  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  $CiG\alpha_q$ -expressing regions seem to contain the developing adhesive organ. On the dorsal trunk epidermis, the  $CiG\alpha_q$ -expressing regions were bilaterally located as two pairs of patches (Fig. 4D). Both of the two splicing variants of  $CiG\alpha_{i1}$ ,  $CiG\alpha_{i1a}$ , and  $CiG\alpha_{i1b}$ , were specifically expressed in the brain and adhesive organ (Fig. 4E, F). A difference in expression patterns was not clear between  $CiG\alpha_{i1a}$  and  $CiG\alpha_{i1b}$ , although the hybridization signals were much weaker for  $CiG\alpha_{i1b}$ . We failed to detect clear hybrid-



**Fig. 5.** Whole-mount *in situ* hybridization using a  $CiG\alpha_{i1}$  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  $\mu$ m.



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

The distinct expression of the *CiG $\alpha_{i1}$*  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 *CiG $\alpha_{i1b}$*  (Fig. 4E,F). Therefore, we used a longer probe that can be hybridized with both *CiG $\alpha_{i1a}$*  and *CiG $\alpha_{i1b}$*  mRNAs (see Materials and Methods). The *CiG $\alpha_{i1}$*  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 *CiG $\alpha_{i1}$*  mRNA was observed until the gastrula stage (Fig. 5G). At the neurula stage, the *CiG $\alpha_{i1}$*  expression was restricted to the anterior ectoderm, especially the presumptive brain vesicle, although the hybridization signal was ambiguous (Fig. 5H). The *CiG $\alpha_{i1}$*  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 G $\alpha$ isoforms in ascidians

In the present study, we showed that mRNAs encoding at least five different G $\alpha$  isoforms are present in ascidian embryos. Mammals have at least 17 functional G $\alpha$  genes, several of which are spliced alternatively, that encode 23 distinct protein products (Nürnberg *et al.*, 1995). The presence of multiple G $\alpha$  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 G $\alpha$  subunits can be classified into G $\alpha_s$ , G $\alpha_i$ , G $\alpha_q$ , and G $\alpha_{12}$  classes (Simon *et al.*, 1991; Suga *et al.*, 1999). The present analysis identified two members of the G $\alpha_q$  class and three of the G $\alpha_i$  class in *C. intestinalis* embryos. Although we have not found G $\alpha_s$  isoforms in *Ciona*, we recently identified a cDNA clone encoding G $\alpha$  closely related to vertebrate G $\alpha_s$  isoforms in another ascidian species, *Halocynthia roretzi* (Iwasa *et al.*, 2001). To date, however, no G $\alpha$  isoforms have been assigned to the G $\alpha_{12}$  class in ascidians.

Among the G $\alpha_q$  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 $\alpha_q$ -class isoforms are further classified into G $\alpha_q$  and G $\alpha_{15/16}$  subfamilies. Another *Ciona* G $\alpha_q$ -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 G $\alpha$  subfamily. It will be interesting to see whether G $\alpha$  closely related to *CiG $\alpha_x$* , and is present in other animals, including vertebrates.

Based on their primary structure, G $\alpha_i$ -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 $\alpha_i$  class members of *Ciona* belong to the G $\alpha_i$  subfamily, and so far no ascidian G $\alpha$  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 $\alpha_o$  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 *CiG $\alpha_{i1}$* transcripts

A comparison of the *CiG $\alpha_{i1a}$*  and *CiG $\alpha_{i1b}$*  cDNA sequences and the genomic structure of the *CiG $\alpha_{i1}$*  gene strongly suggest that *CiG $\alpha_{i1a}$*  and *CiG $\alpha_{i1b}$*  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 G $\alpha$  (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, G $\alpha_{i1}$ , G $\alpha_{i2}$ , and G $\alpha_{i3}$ , which are closely-related to each other (Itoh *et al.*, 1988). Transcripts of the mammalian G $\alpha_{i2}$  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 G $\alpha$  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 G $\alpha$  isoforms in a wide variety of animals. Interestingly, the way alternative

splicing occurs differs between *CiG $\alpha_{i1}$*  and vertebrate *G $\alpha$*  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 $\alpha_{i1a}$*  and *CiG $\alpha_{i1b}$*  in the regulation of signal transduction during ascidian development.

### Spatially restricted expression of *Ciona* *G $\alpha$* genes and roles of G-protein signaling in ascidian embryos and larvae

The present study demonstrated that multiple *G $\alpha$*  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* *G $\alpha$*  isoforms may participate in these cellular activities.

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

From fertilization to the gastrula stage, *CiG $\alpha_{i1}$*  mRNA is present ubiquitously as maternal messages. Therefore, *CiG $\alpha_{i1}$*  may mediate signaling between blastomeres during the cleavage stages. Later in embryogenesis, the *CiG $\alpha_{i1}$*  expression is restricted to the CNS and the adhesive organ. This expression pattern suggests that *CiG $\alpha_{i1}$*  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<sub>t</sub>* (transducin) are responsible for signal transduction in the photoreceptor cells of the retina by coupling with rhodopsin, while invertebrate opsins activate *G<sub>q</sub>* and *G<sub>o</sub>* (Tsuda and Tsuda, 1990). However, our extensive search has failed to find *G $\alpha_t$*  in the *Ciona* EST and genome sequence databases. Interestingly, both *G<sub>t</sub>* and *G<sub>o</sub>* belong to the *G<sub>i</sub>* class and the vertebrate rhodopsin can activate *G<sub>i</sub>* *in vitro* (Terakita *et al.*, 2002). We have shown that *CiG $\alpha_{i1}$*  is expressed in the brain vesicle including photoreceptor cells. Therefore, *CiG $\alpha_{i1}$*  may interact with *Ci-opsin1* in phototransduction of

the ascidian larval ocellus. Since no *G $\alpha_t$*  has been reported in invertebrates to date, *G $\alpha_t$*  may have appeared during early vertebrate evolution after the separation between vertebrates and urochordates.

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