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Phospholipase C cDNAs from sponge and hydra: antiquity of genes involved in the inositol phospholipid signaling pathway

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Abstract To know whether or not the set of genes involved in the inositol phospholipid signaling pathway already existed in the early evolution of animals, we carried out cloning of cDNAs encoding phospholipase Cs (PLCs) from *Ephydatia fluviatilis* (freshwater sponge) and *Hydra magnipapillata* strain 105 (hydra). We isolated two PLC cDNAs, PLC-βS and PLC-γS, from sponge and three cDNAs, PLC-βH1, PLC-βH2, and PLC-δH, from hydra. From the domain organization and the divergence pattern in the PLC family tree, the sponge PLC-βS and PLC-γS and the hydra PLC-δH are possibly homologous to the vertebrate PLC-β, PLC-γ and PLC-δ subtypes, respectively. A detailed phylogenetic analysis suggests that the hydra PLC-βH1 and PLC-βH2 are homologs of the vertebrate PLC-β1/2/3/*Drosophila* PLC21 and the vertebrate PLC-β4/*Drosophila norpA*, respectively. A phylogenetic analysis of the PLC family and the protein kinase C (PKC) family, together with that of the G protein α subunit (Gα) family, revealed that the origin of the set of genes Gαq, PLC, PKC involved in the inositol phospholipid signaling pathway is very old, going back to dates before the parazoan-eumetazoan split, the earliest branching among extant animal phyla.

Key words: Phospholipase C; Protein kinase C; Gene duplication; Phylogenetic tree; Evolution; Hydra; Sponge

1. Introduction

Hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate by inositol phospholipid-specific phospholipase C (IP-PLC or simply PLC) is a key event in the inositol phospholipid signaling pathway which regulates a large array of cellular processes. The binding of extracellular signaling molecules to G protein-linked receptors stimulates a trimeric G protein called Gq, which in turn activates a PLC called PLC-β. One of the products, diacylglycerol (DAG), generated by the activated PLC-β elicits intracellular responses by activating protein kinase Cs (PKCs) (e.g. [1,2] for review). The PLCs comprise a small protein family which can be classified into at least three subtypes or subfamilies, PLC-β, PLC-γ, and PLC-δ. These subtypes are distinguished from each other by domain structure and function. Each subtype contains more than one isoforms which bear close resemblance to each other in overall structure and function [1,2].

In animal gene families involved in signal transduction and developmental control, different subtypes originated from one or a few ancestral genes by gene duplication and domain shuffling during the early evolution of animals (hereafter we will to refer gene duplication that gave rise to different subtypes as subtype duplication). Later, vertebrates underwent further gene duplications (isoform duplications) which generated multiple isoform genes that are virtually identical in structure and function in the same subtype, but tend to differ in tissue distribution (tissue specific isoforms) [3–5]. From a phylogenetic analysis of the G protein α subunit (Gα) family, the protein tyrosine kinase (PTK) family (Suga et al., submitted), the phosphodiesterase (PDE) family [6], and the *Pax* family [7], we recently showed extensive subtype duplications in these animal specific genes in the very early evolution of animals before the parazoan-eumetazoan split, the earliest branching among extant animal phyla.

To know whether or not the set of genes involved in the inositol phospholipid signaling pathway already existed in the early evolution of animals, we carried out cloning of cDNAs encoding PLCs from the freshwater sponge and the hydra. From a phylogenetic analysis of the PLC family and the PKC family, we report here that the origin of the set of genes involved in the inositol phospholipid signaling pathway is very old, going back to dates before the parazoan-eumetazoan split.

2. Materials and methods

2.1. Isolation and sequencing of sponge PLC cDNAs

Poly(A)$^+$ RNA of *Ephydatia fluviatilis* (freshwater sponge) was extracted from the cells hatched from the gemmules [8] using the Quick Prep mRNA isolation kit (Pharmacia). Total RNA of *Hydra magnipapillata* (strain 105) was extracted from the whole tissue by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method. The poly(A)$^+$ selected mRNAs of *E. fluviatilis* and the total RNA of *H. magnipapillata* were reverse transcribed to cDNAs using oligo(dT) primer with reverse transcriptase (SuperScript II, Gibco). These cDNAs were used as templates for PCR amplification with the Expand High Fidelity PCR System (Boehringer Mannheim). The sense and antisense degenerate primers were designed from conserved amino acid residues within the PLC X-Y regions as follows: (1) 5′-GTGGATCC(A/T)(G/C)I(A/T)(G/C)ICA(T/C)AA-(T/C)ACNTA-3′, corresponding to the amino acid sequence SSHNTY, and (2) 5′-GAGGATCC(A/T)(G/C)ICA(T/C)AA(T/C)ACNTA(T/C)(T/C)T-3′, corresponding to SHNTYL for sense primers; (3) 5′-GTGAATTCTG(G/A)CANCCN(G/A/C)(A/C)(G/A)-TTCCA-3′, corresponding to WN(G/A/V/C)GCQ, and (4) 5′-GC-GAATTCNN(G/A)CA(G/A)TG(G/A)TT(T/C)TC(G/A)AA-3′, corresponding to FENHCX for antisense primers. Each primer contains a *Bam*HI or *Eco*RI restriction site at the 5′ end (underlined). PCR amplification was conducted as follows: 2 min denaturation at 94°C; then 5 cycles of 94°C (1 min), 46°C (2 min), and 72°C

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Abbreviations: PLC, phospholipase C; PKC, protein kinase C; Gα, G protein α subunit; DAG, diacylglycerol; Myr, million years

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases.

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(5 min); followed by 30 cycles of 94°C (30 s), 60°C (1 min), and 72°C (2 min); and finally 1 cycle of 60°C (5 min) and 72°C (10 min). For cloning of *E. fluviatilis* PLCs, PCR was carried out with primers 1 and 3. For cloning of *H. magnipapillata* PLCs, the first round of PCR was carried out with primers 1 and 4. The second round of PCR was performed with nested primers 2 and 4 with the primary amplification products. The PCR products were separated in a 1.5% agarose gel containing ethidium bromide. Products of expected size were isolated as gel slices, purified using GeneCleanII (BIO101) and cloned into the pT7Blue vector (Novagen). Then, *Escherichia coli* strain DH5α (Toyobo) was transformed with the ligated vector. More than three independent clones were isolated for sponge PLC genes and sequenced by the dideoxy chain termination method [9] using synthetic oligonucleotide as primers. The full-length sponge PLC coding sequences were obtained by 5′- and 3′-rapid amplification of cDNA ends (Gibco-BRL) [10]. *E. fluviatilis* and *H. magnipapillata* genomic DNA fragments containing the PLC sequences were identified by Southern blot analysis with specific probes.

2.2. Sequence data

Accession numbers of sequence data from GenBank release 107.0 and PIR(*) database release 56.0 are as follows (#, this work): **PLC**: sponge PLC-βS (#); hydra PLC-βH2 (#); *Drosophila melanogaster norpA* (J03138); rat PLC-β4 (A48047*); hydra PLC-βH1 (#); *Drosophila melanogaster* plc21 (M60453); *Xenopus laevis* PLC1 (L20816); human PLC-β3 (U26425); human PLC-β2 (M95678); *Meleagris gallopavo* PLC-β (U49431); rat PLC-β1 (M20636); sponge PLC-γS (#); *Drosophila melanogaster* PLC-γD (D29806); human PLC-γ1 (M34667); human PLC-γ2 (M37238); hydra PLC-δH (#); *Caenorhabditis elegans* R05G6.8 (U58746); *Caenorhabditis elegans* K10F12.3 (AF025462); human PLC-L (D42108); bovine PLC-δ2 (S14113*); rat PLC-δ4 (U16655); human PLC-δ1 (U09117); *Caenorhabditis elegans* PLC210 (AF044576). The following three sequences were used as an outgroup of the PLC family tree: *Schizosaccharomyces pombe* plc1 (D38309); *Botryotinia fuckeliana* BCPLC1 (U65685); *Saccharomyces cerevisiae* PLC1 (L13036). **PKC**: human PKCζ (Z15108); human PKCι (L33881); *Xenopus laevis* PKCλ/ι (U12588); *Caenorhabditis elegans* F09E5.1 (U37429); human PKCθ (L07032); human PKCδ (L07861); *Geodia cydonium* GCPKC1 (X87684); *Caenorhabditis elegans* TPA 1A (D49525); human PKC-L (M55284); human PKCε (X65293); *Aplysia californica* PRKC (M94884); *Caenorhabditis elegans* PKC1B (U00181); *Drosophila melanogaster* dPKC98F (J04848); *Geodia cydonium* GCPKC2 (X87683); *Drosophila melanogaster* dPKC53E(ey) (J04845); *Drosophila melanogaster* dPKC53E(br) (X05076); *Caenorhabditis elegans* kin-11 (U29376); *Aplysia californica* PRKC (M94883); painted urchin PKC1 (U02967); human PKCγ (Z15114); *Xenopus laevis* PKC I (A37237*); human PKCα (X52479); *Xenopus laevis* PKC II (B37237*); human PKCβ I (X06318). The following sequences were used as an outgroup of the PKC family tree: human AKT2 (M95936); human rac protein kinase α (M63167); rat RAC-PKγ (JC4345*); *Drosophila melanogaster* DRAC-PK85 (X83510); *Caenorhabditis elegans* C12D8.10b (Z73969); *Xenopus laevis* PKN (D43890); human PRK1 (U33053); human PRK2 (U33052); *Caenorhabditis elegans* F46F6.2 (Z50028); *Aspergillus niger* pkcA (U10549); *Trichoderma reesei* pkc1 (U10016); *Schizosaccharomyces pombe* pkc1+ (L07637); *Schizosaccharomyces pombe* pck1+ (D14337); *Candida albicans* PKC1 (X81142); *Saccharomyces cerevisiae* PKC1 (Z35866).

2.3. Alignment and phylogenetic tree inference

Optimal alignment of sequences was obtained by the methods of Needleman and Wunsch [11] and Berger and Munson [12], together with manual inspections. The number of amino acid substitutions per site or evolutionary distance was calculated by the method of Jukes and Cantor [13] for regions where unambiguous alignment was possible; amino acid sites where gaps existed in the alignment were excluded from the calculation. The evolutionary distance was applied to phylogenetic inference by the neighbor-joining (NJ) method [14]. Bootstrap analysis was carried out by the method of Felsenstein [15]. The phylogenetic tree of the PLC family inferred by the NJ method was reexamined by the maximum likelihood (ML) method of protein phylogeny [16,17] based on the JTT model (PROTML version 2.2 in Adachi and Hasegawa's program package MOLPHY).

3. Results and discussion

All three subtypes of PLCs have two regions X and Y containing the catalytic site. They also have a pleckstrin homology (PH) domain near the N-terminal end, EF hands, and a C2 domain near the C-terminal end. The PLC-β subtype has an extra sequence at the C-terminal end, called P-G region rich in basic amino acids [18]. The X and Y regions of PLC-γ

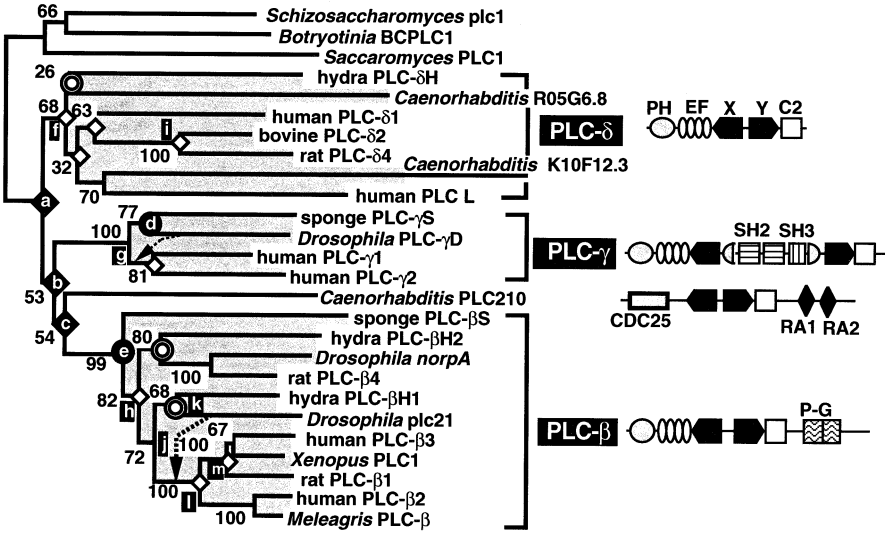

Fig. 1. Phylogenetic tree of the PLC family. On the basis of the alignment of the highly conserved X and Y regions, the tree was inferred by the NJ method [14] using fungal PLC sequences as an outgroup. The number at each branch point represents the bootstrap probability that two lineages join together to form a cluster. Filled circles, parazoan-eumetazoan split; double circles, diploblast (hydra)-triploblast split; filled rhombi, gene duplications that gave rise to different subtypes; open rhombi, gene duplications in the same subtype. Bovine PLC-δ2 and rat PLC-δ4 are possibly paralogous, judging from the number of synonymous substitution [21,22]. The branch length is proportional to the number of accumulated amino acid substitutions. The domain structures of four subtypes are schematically shown [1,2,18,19]. PH, pleckstrin homology (PH) domain; EF, EF hands; X and Y, X and Y regions, respectively; C2, C2 domain; SH2 and SH3, SH2 and SH3 domains, respectively; CDC25, CDC25-like domain; RA1 and RA2, upstream and downstream Ras-associating domains, respectively; P-G, P-G region. For nodes a–l and arrows, see text.

are interrupted by a large insert that has an additional PH domain, two SH2 domains and an SH3 domain [1,2,19]. We have isolated two PLC cDNAs, PLC-βS and PLC-γS, from sponge and three cDNAs, PLC-βH1, PLC-βH2, and PLC-δH, from hydra. The domain organization of the cloned PLC-βS, PLC-βH1 and PLC-βH2 is identical to that of the vertebrate PLC-β subtype, suggesting that they belong to the PLC-β subtype. Also the sponge PLC-γS and the hydra PLC-δH are identical to the vertebrate PLC-γ and PLC-δ in domain organization, respectively. Thus the five cDNAs cloned here are possibly sponge and hydra homologs of respective PLC subtypes.

Recently a new subtype PLC210 has been identified from *C. elegans*, which additionally possesses a CDC25-like domain and two Ras-associating domains (RA1 and RA2) in the N- and C-terminal ends, respectively, but lacks the PH domain and the EF hands commonly found in all vertebrate PLCs identified to date [20]. From a comparison of the amino acid sequences of the X and Y regions of the sponge and hydra PLCs with those of known PLCs including the *C. elegans* PLC210, a phylogenetic tree of the PLC family was inferred using fungal PLCs as an outgroup (Fig. 1). As Fig. 1 shows, members in the same subtype form a cluster, and the sponge and hydra PLCs belong to the corresponding clusters of subtypes. The *C. elegans* PLC210 does not belong to any clusters of known PLC subtypes and comprises an independent cluster.

The tree clearly shows that the three subtype duplications (the branch points a, b, and c) that gave rise to four different subtypes antedate the divergence (the branch points d and e) of sponge (parazoan) and eumetazoans, the earliest branching among extant animal phyla; there is a possibility for the close association of *Drosophila* PLC-γD and human PLC-γ1/2 lineages (indicated by a dotted arrow). In this case, the parazoan-eumetazoan split corresponds to the branch point g. The number of subtype duplications was reexamined statistically by the method described previously [5]. The numbers of subtype du-

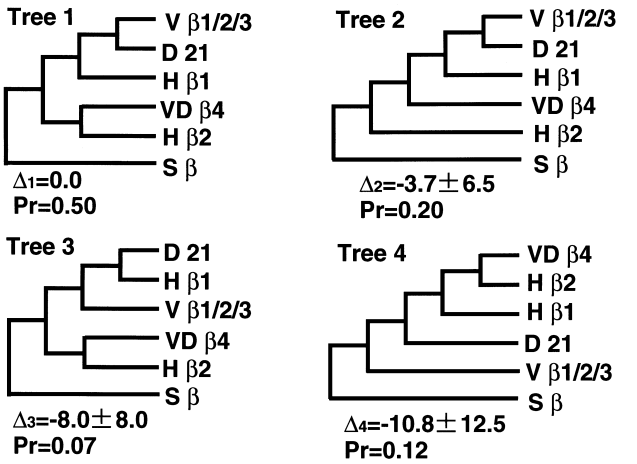

Fig. 2. Phylogenetic trees of members in the PLC-β subtype inferred by the ML method. Using the sponge PLC-βS (Sβ) as an outgroup, a ML analysis [16,17] was carried out for five groups. Vβ1/2/3, vertebrate PLC-β1, -β2, and -β3 group; D21, *Drosophila* plc21; Hβ1, hydra PLC-βH1; VDβ4, rat PLC-β4 and *Drosophila norpA* group; Hβ2, hydra PLC-βH2. Δ_i (i = Tree 1 − Tree 4) = $L_i - L_{Max}$, where L_i and L_{Max} represent the log-likelihood values of Tree i and the maximum likelihood tree, respectively, and Pr represents the probability that Tree i is realized during bootstrap resampling. All trees except for four trees shown here are excluded with a significance level of ±1 S.E.M.

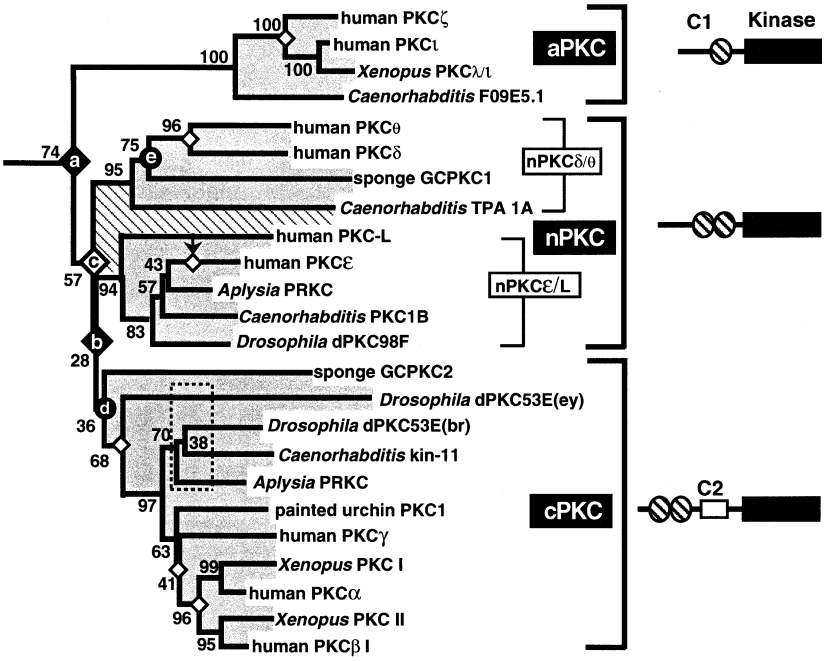

Fig. 3. Phylogenetic tree of the PKC family. On the basis of the alignment of the kinase domain, the tree was inferred by the NJ method using the PKB, PRK and fungal PKC sequences as an outgroup. The number at each branch point represents the bootstrap probability that two lineages join together to form a cluster. Filled circles, parazoan-eumetazoan split; filled rhombi, gene duplications that gave rise to different subtypes; open rhombi, gene duplications in the same subtype. A detailed analysis suggests close association of human PKC-L and human PKCε. The branching order of *Drosophila* dPKC53E(ey), *Drosophila* dPKC53E(br), *Caenorhabditis* kin-11, and *Aplysia* PRKC is obscure. The branch length is proportional to the number of accumulated amino acid substitutions. The domain structures of three subtypes are schematically shown [23,24]. C1, C2, and Kinase, C1, C2 and kinase domains, respectively.

plications before and after the parazoan-eumetazoan split were 3.0 ± 0.3 and 0.01 ± 0.1, respectively. This implies that, because animals separated from fungi or plants about 1070 million years (Myr) ago and the parazoan-eumetazoan split occurred about 940 Myr ago [23], the set of four PLC subtypes with diverse function already existed in the very early evolution of animals before the parazoan-eumetazoan split. The explosive subtype duplication in the early evolution of animals was also observed in animal gene families involved in signal transduction and developmental control, including the Gα family, the PTK family (Suga et al., submitted), the PDE family [6], the protein tyrosine phosphatase (PTP) family (Ono et al., manuscript in preparation), and the *Pax* family [7].

The phylogenetic tree of Fig. 1 also revealed that the evolutionary rate v_I of amino acid substitution in the first 130 Myr (First period) before the parazoan-eumetazoan split in animal evolution is at least 3–4-fold higher than that, v_{II}, in the remaining 940 Myr (Latter period): $v_I = 0.88 \times 10^{-9}$/site/year and $v_{II} = 0.30 \times 10^{-9}$ for PLC-β subtype, and $v_I = 0.94 \times 10^{-9}$ and $v_{II} = 0.22 \times 10^{-9}$ for PLC-γ subtype; the v_I of the PLC-β was estimated by calculating the branch length between the branch points a and e; for v_{II}, the mean branch length of the PLC-β subtype was calculated by successively averaging the branch lengths of paired lineages from extant species to the branch point e, based on the tree topology of Fig. 1; for PLC-γ subtype, the branch point g was used as the boundary of the First and Latter periods. This method gives an underestimate for v_I and an overestimate for v_{II}. The rapid evolutionary rate in the First period was also observed in the Gα, PTK, PDE, PTP, and *Pax* families.

On the basis of the alignment of regions including the C2 domain, PH domain and EF hands in addition to the X and Y regions, a ML analysis [16,17] of the PLC-β subtype has been carried out for the phylogenetic relationship of five groups, hydra PLC-βH1, hydra PLC-βH2, *Drosophila* plc21, rat PLC-β4/*Drosophila norpA* group, and vertebrate PLC-β1/2/3 group, using sponge PLC-βS as an outgroup. All tree topologies except for four (Trees 1–4) shown in Fig. 2 were excluded at the significance level of ±1 S.E.M. Tree 1 in Fig. 2 is the ML tree with the largest log-likelihood value, in which *Drosophila* plc21 and vertebrate PLC-β1/2/3 group form a cluster (indicated by arrow in Fig. 1). Judging from the bootstrap probability and log-likelihood value, Tree 1 is highly likely, although Trees 2–4 are not excluded statistically. According to Tree 1, the branching order of vertebrate PLC-β4, *Drosophila norpA*, and hydra PLC-βH2 corresponds to species order in evolution, suggesting that they are homologous. Similarly, vertebrate PLC-β1/2/3, *Drosophila* plc21, and hydra PLC-βH1 are possibly homologous.

A similar phylogenetic analysis is also possible for the PKC family, a subgroup of the Ser/Thr kinase superfamily. The PKC family consists of three categories, conventional PKCs (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs), on the basis of cofactor requirements and structure. The cPKCs require DAG and calcium as cofactors and have separate domains (C1 and C2 domains) for binding of the respective cofactors. The nPKCs require DAG and lack the C2 domain. In the aPKCs the entire C2 domain and half of the C1 domain are lacking (e.g. [24,25] for review). Thus the three PKC subtypes are likely to differ from each other in basic function.

The sequences of two PKCs, GCPKC1 and GCPKC2, from the sponge *Geodia cydonium* have already been reported [26]. The GCPKC1 and GCPKC2 are virtually identical in domain organization to nPKC and cPKC, respectively. Including the two sponge PKCs, a phylogenetic tree of the PKC family has been inferred by the NJ method [14], using PKBs, PRKs, and fungal PKCs as an outgroup (Fig. 3). The tree revealed three (or four) separate clusters corresponding to cPKC, nPKC, and aPKC subtypes; the nPKCs are likely to be further subdivided into two categories, and thus we tentatively assigned them as nPKCε/L and nPKCδ/θ subtypes, although they are identical in domain organization. It remains possible that the basic functions of nPKCε/Ls and nPKCδ/θs differ from each other. Judging from the phylogenetic position and the similarity of domain organization, the sponge GCPKC1 and GCPKC2 are possibly homologs of vertebrate nPKCs and cPKCs, respectively. Although the bootstrap probability that the GCPKC2 belongs to cPKC subtype is not high enough (36%), the ML analysis [16,17] for five groups, GCPKC2, cPKCs, nPKCε/Ls, nPKCδ/θs, aPKCs, gives a considerably high probability of 72%.

According to the phylogenetic tree of Fig. 3, the three subtype duplications (branch points a, b, and c) that gave rise to four different subtypes antedate the parazoan-eumetazoan split (branch points d and e). A bootstrap analysis by the method described previously [5] showed that the numbers of subtype duplications before and after the parazoan-eumetazoan split are 2.8 ± 0.7 and 0.1 ± 0.4, respectively. It is therefore likely that the set of four PKC subtypes was established in the very early evolution of animals before the parazoan-eumetazoan split, as in the case of the PLC family. The rapid evolutionary rate in the First period was also observed in the PKC family; the evolutionary rates v_I and v_{II} were estimated to be 0.57×10^{-9}/site/year and 0.27×10^{-9}, respectively for the cPKC subtype, and 0.30×10^{-9} and 0.29×10^{-9}, respectively for the nPKCδ/θ subtype.

In conclusion, we have isolated the sponge PLC-βS and PLC-γS cDNAs, which are likely to be homologs of vertebrate PLC-β and PLC-γ, respectively. Also possible homologs of vertebrate cPKC and nPKC have already been isolated from sponge [26]. Furthermore we recently isolated the Gαq related cDNAs (spGα-3 and hyGα-3) from sponge and hydra (Suga et al., submitted). Thus the set of Gαq, PLC, and PKC genes involved in the inositol phospholipid signaling pathway exists in sponge, the most primitive multicellular animal. It may be interesting to note that the sponge, which lacks the cell cohesiveness and coordination typical of eumetazoans [27], has the set of genes involved in the signaling pathway. Phylogenetic analyses of the Gα, PLC and PKC families revealed that in each family, an almost complete set of subtypes with distinct functions had been established in the very early stage of animal evolution before the parazoan-eumetazoan split about 940 Myr ago, long before the Cambrian explosion [28]. It seems likely, therefore, that there is no direct link between the Cambrian explosion and the creation of new genes with novel functions. The molecular mechanisms of the Cambrian explosion should be reexamined by factors which could generate organismal diversity without further gene duplications (Suga et al., submitted).

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