Unicellular Ca^{2+} Signaling 'Toolkit' at the Origin of Metazoa

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Ca^{2+} signaling pathways control many physiological processes in almost all types of animal cells such as fertilization, muscle contraction, hormone release, and learning and memory. Each animal cell type expresses a unique group of molecules from the Ca^{2+} signaling 'toolkit' to control spatiotemporal patterns of Ca^{2+} signaling. It is generally believed that the complex Ca^{2+} signaling 'toolkit' has arisen from the ancestral multicellular organisms to fit unique physiological roles of specialized cell types. Here, we demonstrate for the first time the presence of an extensive Ca^{2+} signaling 'toolkit' in the unicellular choanoflagellate *Monosiga brevicollis*. Choanoflagellates possess homologues of various types of animal plasma membrane Ca^{2+} channels including the store-operated channel, ligand-operated channels, voltage-operated channels, second messenger-operated channels, and 5 out of 6 animal transient receptor potential channel families. Choanoflagellates also contain homologues of inositol 1,4,5-trisphosphate receptors. Furthermore, choanoflagellates master a complete set of Ca^{2+} removal systems including plasma membrane and sarco/endoplasmic reticulum Ca^{2+} ATPases and homologues of 3 animal cation/Ca^{2+} exchanger families. Therefore, a complex Ca^{2+} signaling 'toolkit' might have evolved before the emergence of multicellular animals.

Introduction

A fundamental question in evolutionary biology is to determine how animals (Metazoa) evolved from a unicellular ancestor by understanding the genetic basis of multicellularity (Ruiz-Trillo et al. 2007). Many signaling pathways critical for cell–cell communications and animal development probably predated, or occurred concurrently with, the origins of animals. Among these pathways, Ca^{2+} signaling pathway plays a key second messenger role in regulating many cellular processes in virtually all types of animal cells including fertilization, contraction, exocytosis, transcription, apoptosis, and learning and memory (Berridge 2005). Therefore, understanding the evolutionary origin of Ca^{2+} signaling pathway attains considerable significance.

In order to execute distinct physiological functions, each animal cell type selectively expresses a unique set of proteins from a comprehensive Ca^{2+} signaling 'toolkit' which allows them to transduce appropriate extracellular stimuli such as neurotransmitters, electrical signals, growth factors, and hormones into spatiotemporal Ca^{2+} signals (Berridge et al. 2003). The large repertoire of animal Ca^{2+} signaling machinery is generally believed to arise from ancient multicellular organisms in which diverse forms of cell–cell communication became essential for development and physiology (Case et al. 2007).

Choanoflagellates, a group of single-celled and colony-forming eukaryotes, resemble the collar cells (or "choanocytes") identified in sponges and similar collar cells in many other metazoans, including cnidarians, flatworms, and echinoderms (King 2005). In contrast, plants, fungi, and nonchoanoflagellate microbes do not have or resemble such collar cells (King 2005). Based on recent molecular phylogenetic studies, choanoflagellates are placed among unicellular common ancestors of animals (Ruiz-Trillo et al. 2007). One choanoflagellate species, *Monosiga brevicollis*, expresses homologues of metazoan cell surface adhesion molecules and receptor tyrosine kinases (King and Carroll 2001; King et al. 2003).

To further understand the role of Ca^{2+} signaling in the origin of animal multicellularity and gain mechanistic and evolutionary insights into molecular regulators of Ca^{2+} signaling (Cai and Lytton 2004; Cai 2007a, 2007b), we tested whether choanoflagellates *M. brevicollis* contain an extensive unicellular Ca^{2+} signaling 'toolkit' conserved in Metazoa and compared signature motifs of identified unicellular Ca^{2+} signaling molecules with their animal counterparts.

Materials and Methods

TBlastN and BlastP searches (Altschul et al. 1997) using protein sequences of selected *Homo sapiens* and/or *Caenorhabditis elegans* Ca^{2+} signaling molecules were conducted on the genomic and protein databases of the marine choanoflagellate *M. brevicollis* (King et al. 2008) at the DOE Joint Genome Institute (http://genome.jgi-psf.org/Monbr1/Monbr1.home.html) and at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov/blast/). Similar searches were also performed on the genomic and protein databases of Cnidarians *Nematostella vectensis* (Putnam et al. 2007). Sequence data sets were processed for sequence alignment, manual editing, and phylogenetic analysis, essentially as described previously (Cai and Lytton 2004; Cai and Zhang 2006).

Conserved protein domains/motifs were predicted using the simple modular architecture research tool (SMART) (Letunic et al. 2004) and conserved domain database (CDD) (Marchler-Bauer et al. 2005) servers. Putative transmembrane spanning regions of proteins were predicted using the ConPred II system (Arai et al. 2004).

Results and Discussion

An Extensive Ca^{2+} Signaling 'Toolkit' in the Unicellular Choanoflagellate *M. brevicollis*

Generation of Ca^{2+} Signals

Cytosolic Ca^{2+} signals triggered by various stimuli can come from 1) Ca^{2+} influx across the plasma membrane through Ca^{2+} channels and/or the "reverse mode" of exchangers; 2) Ca^{2+} release from intracellular Ca^{2+} stores—the endo/sarcoplasmic reticulum (ER/SR), through the

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intracellular Ca^{2+} release channels inositol 1,4,5-trisphosphate receptors (IP_3Rs) and/or ryanodine receptors (RyRs); or 3) the combination of both Ca^{2+} influx and Ca^{2+} release (Cai 2007b).

We found in *M. brevicollis* the presence of genes encoding homologues of various types of plasma membrane Ca^{2+} channels (fig. 1)—store-operated channel (Orai) and the ER Ca^{2+} sensor protein (STIM), ligand-operated channels (nicotinic acetylcholine receptor and P2X purinergic receptor), voltage-operated channel (similar to dihydropyridine-sensitive L-type Ca^{2+} channel), second messenger-operated channel (cyclic nucleotide-gated channel), and transient receptor potential (TRP) channels. *Monosiga brevicollis* appears to possess all 5 modes of regulated Ca^{2+} entry across the plasma membrane identified in animals (Parekh and Putney 2005). Although these channel homologues in *M. brevicollis* display high degree sequence similarities with their metazoan counterparts, it should be noted that further experimental data are needed to determine their physiological functions.

The presence of homologues of the Orai channel subunit and the STIM Ca^{2+} sensor in *M. brevicollis* provides the first evidence that store-operated Ca^{2+} entry could operate in unicellular organisms. This observation suggests that store-operated Ca^{2+} entry represents a primordial Ca^{2+} entry pathway. Choanoflagellate Orai possesses the intragenic repeat patterns and critical residues identified in multicellular animals (Cai 2007b). Similar to non-Chordata STIM molecules (Cai 2007a), choanoflagellate STIM contains a single N-terminal EF-hand domain and a sterile alpha motif (SAM) domain in the ER lumen, a single transmembrane segment (TMS), and a cytoplasmic coiled coil domain but lacks a C-terminal Lys-rich tail. Although store-operated Ca^{2+} entry has been implicated in few yeast studies (Parekh and Putney 2005), we did not find any homologues of Orai proteins in currently available yeast and plant genomes (data not shown). Therefore, store-operated Ca^{2+} entry mediated by Orai and STIM proteins likely belongs to evolutionary novelties that arose before the origin of Metazoa.

Six mammalian protein families of TRP channels regulate cellular responses to diverse stimuli such as mechanical stretch, temperature, osmolarity, chemical compounds, and second messengers (Nilius et al. 2007). We identified in *M. brevicollis* homologues of 5 mammalian TRP channel families, including TRPC, TRPV, TRPM, TRPML, and TRPA (fig. 1). Coexistence of such diverse TRP channel homologues may render *M. brevicollis* sensitive to changes in various environmental conditions.

The first protozoan IP_3R cloned from the ciliate *Paramecium* (GenBank accession number CAI39148.1) displays strongest sequence similarities to the rat type 3 IP_3R, about an overall identity of 19% and similarity of 34% (Ladenburger et al. 2006). *Monosiga brevicollis* contains 4 homologues of IP_3Rs, whereas *C. elegans* (Strange 2003) and humans (Mikoshiba 2007) possess 1 copy and 3 copies of IP_3Rs, respectively. Compared with the *Paramecium* IP_3R, *M. brevicollis* IP_3Rs show higher sequence similarity to metazoan IP_3Rs. For example, MbrIP3R1 (GenBank accession number XP_001747685.1) has an overall sequence identity of 35% and similarity of 53%

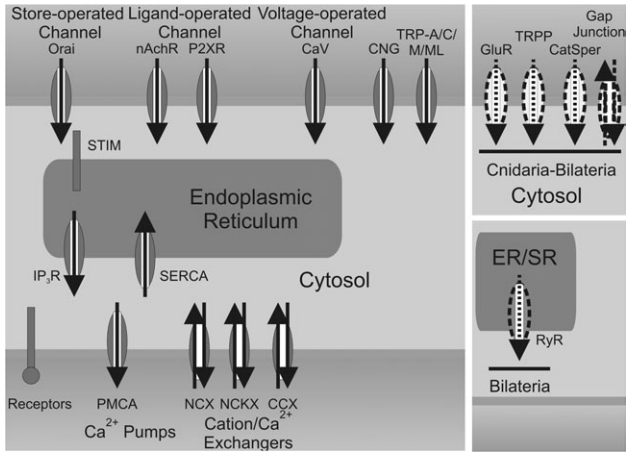

FIG. 1.—Evolution of the Ca^{2+} signaling 'toolkit' at the origin of animals. Left, Choanoflagellates, single-celled and colony-forming eukaryotes, appear to contain an extensive Ca^{2+} signaling 'toolkit' conserved in metazoans, including homologues of various types of Ca^{2+} channels, ATPases, and exchangers (blue). Right top, ionotropic glutamate receptors (GluR), TRPP, CatSper and gap junction channels (yellow) are found in the eumetazoan lineage (cnidarian–bilaterian). Right bottom, ryanodine receptors (RyRs) are present in bilaterians (purple). The black arrows indicate the directions of Ca^{2+} fluxes across the membranes. Exchangers not only primarily mediate Ca^{2+} efflux but can also induce Ca^{2+} influx under certain conditions. Gap junctions allow the rapid bidirectional passage of Ca^{2+} between adjacent cells. Color version of figure 1 is available as supplementary figure S1 (Supplementary Material online).

to its strongest BlastP (Altschul et al. 1997) hit, the human type 1 IP_3R (GenBank accession number BAA05065.1).

Monosiga brevicollis does not have homologues of RyRs. Furthermore, RyR is not present in the genome of early eumetazoan Cnidarians *N. vectensis*. Therefore, intracellular Ca^{2+} release induced by activation of voltage-operated Ca^{2+} channel might have evolved after the origin and early evolution of Metazoa. *Monosiga brevicollis* seems to utilize its cell surface receptors (King et al. 2003), multiple isoforms of phospholipase C (data not shown), and IP_3Rs to manipulate intracellular Ca^{2+} release, rather than through depolarization-dependent activation of its prototype voltage-operated Ca^{2+} channel.

Application of Ca^{2+} Signals

Monosiga brevicollis contains diverse Ca^{2+} signaling effectors such as calmodulin, Ca^{2+}/calmodulin-dependent protein kinases, calcineurin, calpain, and many C2 domain-containing proteins, and EF-hand domain-containing proteins as well as ER Ca^{2+} buffers/chaperones, calreticulin, and calnexin. We also identified homologues of synaptobrevin, syntaxins, and SNAP25 in *M. brevicollis*, all of which contain the classic protein domains/motifs involved in the docking and/or fusion of vesicles with the plasma membrane (Gurkan et al. 2007).

Removal of Ca^{2+} Signals

Cytosolic Ca^{2+} signals must also be rapidly eliminated in order to maintain Ca^{2+} homeostasis. *Monosiga brevicollis* contains a complete set of Ca^{2+} removal 'toolkit'

FIG. 2.—Sequence alignment of the transmembrane segment (TMS) 1 region of the Ca^{2+} release-activated Ca^{2+} channel subunit Orai. Shown are sequences from human (HsaOrai 1–3), *Ciona intestinalis* (CinOrai), *Caenorhabditis elegans* (CelOrai), Cnidaria *Nematostella vectensis* (NveOrai), and choanoflagellates *Monosiga brevicollis* (MbrOrai). The putative TMS1 region of Orai proteins is overlined, and the key acidic residue in TMS1 (E106 in HsaOrai1) is indicated by a filled circle. The asterisk symbol indicates the location of R91 (HsaOrai1), whose mutation can cause a severe human genetic disease.

(fig. 1): 3 copies of plasma membrane Ca^{2+} ATPases (PMCAs), 1 copy of SR/ER Ca^{2+} ATPase, 2 copies of Na^+/Ca^{2+} exchangers (NCXs), and 1 copy each for K^+-dependent Na^+/Ca^{2+} exchanger (NCKX) and cation/Ca^{2+} exchanger (CCX).

NCXs and NCKXs were previously found only in multicellular animals (Cai and Lytton 2004). CCXs (Cai and Lytton 2004) and PMCAs (Okamura et al. 2003) are expressed in multicellular organisms—fungi, plants, and animals. PMCA-type Ca^{2+} ATPases have also been characterized in protozoan parasites, but they also localize to acidocalcisomes and do not have a regulatory calmodulin-binding domain conserved in animal PMCAs (Moreno and Docampo 2003). Motif scan searches indicate that the PMCA homologue in *M. brevicollis* exhibits a calmodulin-binding domain at the equivalent position in the C-terminal tail of animal PMCAs. Thus, even though some aspects of Ca^{2+} signaling mechanisms have been observed in lower eukaryotes (Shemarova and Nesterov 2005), *M. brevicollis* appears to have evolved a primordial Ca^{2+} signaling system more closely related to that in multicellular animals, at least at the level of sequence similarity and functional domain.

In summary, our findings suggest that the unicellular choanoflagellates *M. brevicollis* has developed an extensive Ca^{2+} signaling 'toolkit' before the emergence of multicellular animals.

Conservation and Divergence of Molecular Regulators of Ca^{2+} Signaling between Choanoflagellates and Multicellular Animals

Sequence comparison of prototype choanoflagellate proteins in the Ca^{2+} signaling 'toolkit' with their animal counterparts has given us an unprecedented opportunity to understand the ancient structure–function relationship of these channels. For instance, R91 at the TMS1 of human Orai1 is highly conserved in bilaterian Orai proteins (Cai 2007b). An R91W mutation can cause hereditary severe combined immune deficiency syndrome in humans (Feske et al. 2006). As shown in figure 2, R91 is conserved from early eumetazoan Cnidaria *N. vectensis* to humans. *Monosiga brevicollis* Orai contains an uncharged Gln at the equivalent location, suggesting that the positive charge

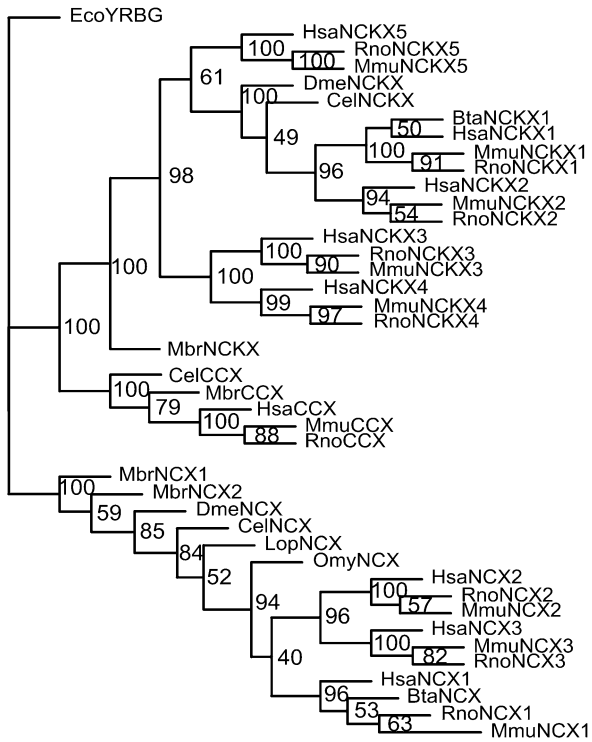

FIG. 3.—Phylogenetic tree of the animal and choanoflagellate CCXs. The bootstrapped phylogenetic tree, showing the presence of choanoflagellate homologues of 3 animal CCX families, was constructed using the maximum likelihood method with a putative *E. coli* Ca^{2+}/H^+ exchanger (EcoYRBG) as an outgroup. The tree model was further confirmed by the Neighbor-Joining analysis and the maximum parsimony approach (data not shown). Bta, *Bos taurus*; Cel, *Caenorhabditis elegans*; Dme, *Drosophila melanogaster*; Hsa, *Homo sapiens*; Lop, *Loligo opalescens*; Mbr, *Monosiga brevicollis*; Mmu, *Mus musculus*; Omy, *Oncorhynchus mykiss*; and Rno, *Rattus norvegicus*.

R91 might reflect a metazoan adaptation. In addition, 2 acidic residues, E106 and E190 in human Orai1, are also highly conserved at symmetric positions of the intragenic repeat regions in all metazoan Orai proteins (Cai 2007b). However, the second acidic residue, E190, is replaced by an Asp residue in *M. brevicollis* Orai (data not shown). E190Q mutation, but not E190D mutation, disrupts the normal function of Orai1 in human T cells and fibroblasts (Prakriya et al. 2006). Nevertheless, whether the second acidic residue Asp in *M. brevicollis* Orai is important for its function and/or regulation remains to be established.

As shown in figure 3, *M. brevicollis* contains homologues of all 3 animal CCX families (Cai and Lytton 2004; Lytton 2007). Animal CCXs are defined by the presence of 2 internal α-repeat regions (Schwarz and Benzer 1997) and signature motifs (Cai and Lytton 2004). Sequence comparison of human and choanoflagellate exchangers indicates the high degree conservation of the 2 α-repeats, especially the signature motifs, from the unicellular ancestral organism to human (fig. 4). Notably, 2 key acidic residues in the 2 α-repeats proposed to neutralize 2 positive charges on Ca^{2+} are identical within each exchanger family—Glu and Asp for NCXs and NCKXs and Asp and Asp for CCXs (fig. 4).

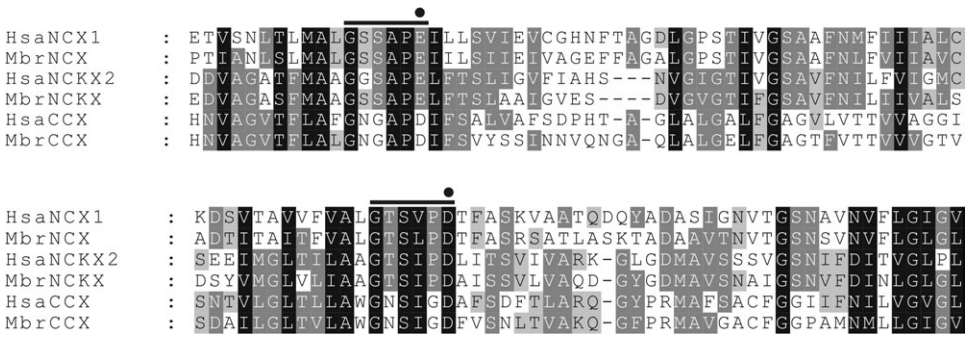

FIG. 4.—Conservation of the 2 α-repeats and signature motifs between human and choanoflagellate CCXs. Sequences of the α1-repeat (top) and α2-repeat (bottom) regions of selected exchanger molecules were aligned and manually edited to refine alignments. The signature motifs of each exchange family (Cai and Lytton 2004) are overlined. The key acidic residue in each α-repeat is indicated by a filled circle. Hsa, *Homo sapiens* and Mbr, *Monosiga brevicollis*.

Metazoan Novelties of the Ca^{2+} Signaling 'Toolkit'

A few components of the Ca^{2+} signaling 'toolkit' are likely to be unique to metazoans: ionotropic glutamate receptors (GluRs), TRPP and CatSper channels, and gap junctions (pannexin/innexin) were found in the Cnidaria *N. vectensis* genome (Putnam et al. 2007), not in the currently available choanoflagellate genome. A putative metabotropic GluR homologue with a 7-TMS structure, but none of the ionotropic homologues, was found in *M. brevicollis*. CatSper channels are sperm-specific proteins essential for the hyperactivation of sperm cell motility (Clapham and Garbers 2005). Choanoflagellates in the single cells state are already structurally similar to sperms (King 2005). It is conceivable that CatSper channels might have evolved to promote motility of spermatozoa from Cnidaria and other animals.

Mutations or deletion of mammalian TRPP channels can cause abnormal kidney functions. It is not clear why TRPP channel is the only mammlian TRP channel family absent in choanoflagellates. On the other hand, gap junctions mediate rapid passage of ions and small molecules between the cytoplasm of 2 adjacent cells. Its unique function might require gap junction to emerge concurrently or after the establishment of animal multicellularity. We did identify multiple copies of putative 2-pore cation channel protein homologues in *M. brevicollis*; however, neither of the animal counterparts of 2-pore cation channels have been functionally characterized so far (Clapham and Garbers 2005).

Interestingly, RyRs are identified only in bilaterians, perhaps reflecting an adaptation to advanced bilaterian physiology, particularly for muscle and neuron tissues. Intracellular Ca^{2+} release through the opening of RyRs, induced by activation of voltage-operated Ca^{2+} channels, plays a critical role in cardiac and skeletal muscle contraction and neuronal function (Zalk et al. 2007). Compared with early emergence of intracellular Ca^{2+} release by activation of receptors and generation of IP_3, RyRs in bilaterians may represent a more rapid way of inducing Ca^{2+} release and effecting Ca^{2+} signals by membrane depolarization.

Nevertheless, comparative genomics of sponges, other cnidarians and unicellular ancestral organisms of metazoans may be required to exclude the possibility of lineage-specific gene loss in choanoflagellates. Alternatively, in *M. brevicollis*, these channel homologues might be distantly related to their metazoan counterparts so that their sequences are too divergent to be identified by currently available database search programs. In addition, the completion, assembly, and annotation of the draft genomic sequences are also necessary for further analysis of the evolution of the Ca^{2+} signaling 'toolkit'.

Conclusion

We conclude that an extensive Ca^{2+} signaling 'toolkit' exists in the unicellular choanoflagellates, preceding the origins of animals (Metazoa). The current hypothesis of Ca^{2+} signaling acquires new dimensions in light of this novel discovery. Why does such an apparently simple unicellular organism need a complex Ca^{2+} signaling machinery? Little is known about the biology of choanoflagellates. Presumably, as a single-celled ancestral organism of animals (Ruiz-Trillo et al. 2007), choanoflagellates might have developed critical signaling molecules that would be further diversified in individual cell types in metazoans. Thus, choanoflagellates might form distinct Ca^{2+} microdomains to mediate coordinated action of different Ca^{2+} regulators in response to various stimuli such as nutrition (King et al. 2003). Moreover, expression and functional regulation of the Ca^{2+} signaling 'toolkit' might be important for the transition from the single-celled state to the colonial status. Understanding the nature of biological properties and regulation of choanoflagellates will no doubt provide novel evolutionary and mechanistic insights into Ca^{2+} biology and many other signaling pathways in animals.

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