Chapter 5
Structure, Function, and Phylogenetic Consideration of Calaxin

Kazuo Inaba, Katsutoshi Mizuno, and Kogiku Shiba

Abstract Sperm chemotaxis is widely seen both in animals and plants and is considered to be necessary for efficient success of fertilization. Although intracellular Ca^{2+} is known to play important roles in sperm chemotaxis, the molecular mechanism causing the change in flagellar waveform that drives sperm directed toward the egg is still unclear. Several Ca^{2+}-binding proteins, especially calmodulin, have been discussed as an important regulator of the molecular motor dynein in flagellar motility during chemotactic movement of sperm. However, there has been no experimental evidence to show the binding of calmodulin to dyneins. Recently, we found a novel Ca^{2+}-binding protein, termed calaxin, in the axonemes of sperm flagella in the ascidian *Ciona intestinalis*. Calaxin binds to the outer arm dynein in a Ca^{2+}-dependent manner and suppresses its activity to slide microtubules at high Ca^{2+} concentration. Inhibition of calaxin results in significant loss of chemotactic behavior of sperm, indicating that calaxin is essential for sperm chemotaxis. In this chapter, we describe the finding history, molecular nature, and the roles in sperm chemotaxis of calaxin, as well as its phylogenetic consideration.

Keywords Axonemal dynein • Calaxin • Opisthokont • Sperm chemotaxis

5.1 Ca^{2+} and Flagellar Motility

The flagellar wave is composed of a bend with larger angle called the principal bend (P-bend) and a bend with a smaller angle called the reverse bend (R-bend). Sperm showing the same extent of both bends show symmetrical waveform and swim

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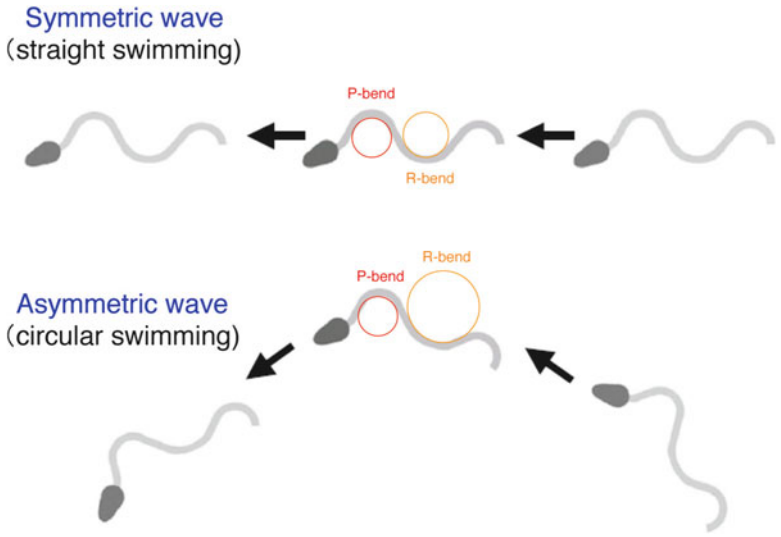

Fig. 5.1 Flagellar waveform and the direction of sperm movement. The flagellar wave is composed of a large principal bend (P-bend) and a smaller reverse bend (R-bend). Sperm with almost the same extent of P-bend and R-bend move straight, whereas those with larger R-bend move in circular fashion. Inverse of the radius of inscribed *circle* represents curvature, which is a parameter to express the extent of flagellar bending

straight, whereas a decrease in R-bend results in asymmetry of waveform and the circular swimming of sperm (Fig. 5.1). This waveform conversion is conducted by the regulation of dynein-driven axonemal motility. It has been well known that Ca^{2+} plays an important role in the regulation of the axonemal motility in eukaryotic flagella and cilia (Kamiya and Witman 1984; Gibbons and Gibbons 1980; Sale 1986). In fact, increasing the concentration of Ca^{2+} in Triton-demembranated sperm induces conversion of the flagellar waveform from symmetry to asymmetry (Brokaw 1979). An extremely asymmetrical waveform is induced at very high calcium concentrations (Gibbons and Gibbons 1980; Sale 1986). Sperm with this waveform show cane-shaped "quiescence," which is observed by electric or mechanical stimulation of sperm flagella in the sea urchin (Shingyoji and Takahashi 1995; Kambara et al. 2011). On the self-nonself recognition of sperm and egg in *Ciona*, sperm show quiescence with a straight flagellum in response to the increase in intracellular Ca^{2+} (Saito et al. 2012). The basic regulation of flagellar waveform by Ca^{2+} is thought to be performed by the specific activation of dynein arms, which results in the changes in flagellar waveforms (Brokaw 1979; Lindemann and Goltz 1988). Accumulating evidence indicates that the activity of inner arm dyneins is regulated by signals from the radial spoke/central pair in a Ca^{2+}-dependent manner (Smith 2002; Nakano et al. 2003). On the other hand, independent regulation of the outer arm dynein by Ca^{2+} is also pointed out (Mitchell and Rosenbaum 1985; Wakabayashi et al. 1997; Sakato and King 2003).

Calmodulin has been a strong candidate to regulate the conversion of flagellar and ciliary waveform. In fact, several studies have discussed the presence and potential roles of calmodulin in *Tetrahymena* cilia (Jamieson et al. 1979; Blum et al. 1980), *Chlamydomonas* flagella (Gitelman and Witman 1980), and sperm flagella (Tash and Means 1983; Brokaw and Nagayama 1985; Lindemann et al. 1991). It is well demonstrated that calmodulin is present in radial spokes and central pair and regulates the function of these structures in the modulation of axonemal dyneins in *Chlamydomonas* (Smith and Yang 2004). Another Ca^{2+}-binding protein, centrin, is known to be a component of inner arm dynein (Piperno et al. 1992).

In contrast, analysis of *Chlamydomonas* mutants indicates that outer arm dyneins are essential for conversion of waveform asymmetry in response to changes in Ca^{2+} concentration (Kamiya and Okamoto 1985; Wakabayashi et al. 1997; Sakato and King 2003). In fact, a Ca^{2+}-binding protein is contained in the outer arm dynein as a light chain (LC4) (King and Patel-King 1995). Another Ca^{2+}-binding protein associated with the outer arm dynein in *Chlamydomonas* flagella is DC3, a component of outer arm dynein docking complex (ODA-DC) (Casey et al. 2003). DC3 protein is structurally distinct from other Ca^{2+}-binding proteins in *Chlamydomonas* flagella, such as calmodulin, centrin, and LC4. Intriguingly this protein shows sequence similarity to a protein predicted in the Apicomplexa *Plasmodium yoelii* and *Plasmodium falciparum* (Casey et al. 2003), but its orthologue has not been found in the genome of the ascidian *Ciona intestinalis* (Hozumi et al. 2006). Thus, the Ca^{2+}-binding protein that regulates axonemal dyneins had not been fully characterized in sperm flagella. Calmodulin was reported to regulate flagellar motility and could be extracted from axonemes with outer arm dynein, but it has not been clarified whether calmodulin can bind directly to outer arm dynein (Tash et al. 1988). In fact, isolated outer arm dynein does not contain calmodulin as a subunit in *Ciona* and sea urchin (Inaba 2007).

5.2 Finding Calaxin

During the course of immunoscreening-based cDNA screening for axonemal proteins in *C. intestinalis*, we isolated multiple clones from testis cDNA library encoding a protein with sequence similarity to calcineurin B (Padma et al. 2003). Phylogenetic analysis revealed that this protein is grouped not into calcineurin B but into a family of neuronal calcium sensor (NCS). We named this novel Ca^{2+}-binding NCS family protein in *Ciona* as "calaxin," for calcium-binding axonemal protein (Mizuno et al. 2009).

NCS proteins have been identified in many organisms ranging from yeast to human (Burgoyne and Weiss 2001). Major five classes of NCS have been well studied in human: NCS-1 (frequenin), neurocalcin and its related proteins (visinin-like protein VILIP and hippocalcin), recoverin, GCAP (guanylyl cyclase-activating protein), and KChIP (Kv channel-interacting protein) (Burgoyne 2004). In mammals, recoverin and GCAPs are expressed only in the retina and regulate phototransduction and others

are expressed in neuronal tissues. NCS-1 is also expressed in many nonneuronal cell types, and its orthologue is present in yeast. The NCS proteins contain four EF hand motifs but only three (or two in the case of recoverin and KChIP1) are able to bind Ca^{2+}. Eleven of 15 mammalian NCS proteins are N-terminally myristoylated, which are important in Ca^{2+}-dependent interaction with the plasma membrane. In contrast to these NCS proteins, calaxin does not possess the N-terminal consensus motif for myristoylaion and belongs to a class distinct from these five NCS classes (Mizuno et al. 2009).

Immunolocalization reveals that calaxin is localized at the vicinity of the outer arm dyneins (Mizuno et al. 2009). Sucrose density gradient centrifugation clearly indicates that calaxin directly interacts with the outer arm dynein in a Ca^{2+}-dependent manner. Far Western blotting and a cross-linking experiment show that calaxin binds to the β-heavy chain in the presence of Ca^{2+}, whereas it binds to β-tubulin in both the presence and absence of Ca^{2+}. Preliminary experiments showed that calaxin binds to the N-terminal stem region of β-heavy chain (Mizuno, unpublished observation).

Although a phylogenetic analysis shows that *Chlamydomonas* LC4 and *Ciona* calaxin are grouped into different classes of Ca^{2+}-binding protein, there are many similarities between them (Sakato and King 2003; Sakato et al. 2007; Mizuno et al. 2009; 2012). First, they appear to undergo dynamic conformational change in response to Ca^{2+} binding. Second, their binding sites are the stems of specific dynein heavy chains: γ-heavy chain of *Chlamydomonas* outer arm dynein for LC4 and its orthologue in *Ciona*, β-heavy chain for calaxin. Third, they mediate binding between dynein and microtubules. Regardless of these common properties, calaxin exhibits characteristic features: Ca^{2+}-dependent binding to dynein heavy chain and Ca^{2+}-independent binding to β-tubulin (and possibly to intermediate chain 2 [IC2], orthologue of *Chlamydomonas* IC1). Because *Ciona* lacks both LC4 and DC3, calaxin might be evolved to play double roles of LC4/DC3 in Ca^{2+}-dependent regulation of outer arm dynein (also see next section).

5.3 Mechanism of Calaxin-Mediated Modulation of Flagellar Movements During Sperm Chemotaxis

During chemotaxis in *Ciona*, sperm repeat straight and turn movements to come toward the egg (Fig. 5.2). The turn movement accompanies transient increase in intracellular Ca^{2+} concentration and asymmetry of flagellar waveform (Shiba et al. 2008). It was not been elucidated how the increase in intracellular Ca^{2+} concentration induced the modulation of flagellar bending during sperm chemotaxis. As we previously showed that calaxin was directly bound to the outer arm dynein in a Ca^{2+}-dependent manner, it was a strong candidate for direct Ca^{2+}-dependent modulator for flagellar waveform of sperm (Mizuno et al. 2009). Localization of calaxin to epithelial cilia also suggests a possibility that calaxin is a general Ca^{2+} sensor to modulate ciliary and flagellar motility (Mizuno et al. 2009).

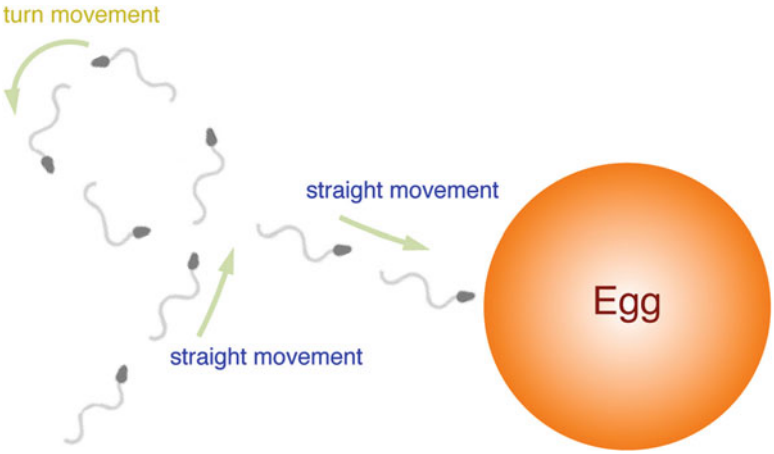

Fig. 5.2 Sperm trajectory during chemotaxis to the egg. During chemotactic movements, *Ciona* sperm show a unique turning movement associated with a flagellar change to an asymmetrical waveform, followed by a straight movement with symmetrical waveform

An antidiabetic compound, repaglinide, is a specific inhibitor for NCS and is also effective on calaxin (Okada et al. 2003; Mizuno et al. 2012). In the presence of repaglinide, sperm do not show the unique turn movement, resulting in less effective chemotaxis. Flagellar bending with strong asymmetry continues for ~0.1 ms during one turn in normal sperm. Repaglinide-treated sperm exhibit transient flagellar asymmetry, but the asymmetry is not sustained for long, and sperm exhibit only incomplete turning (Mizuno et al. 2012). Thus, it is suggested that calaxin plays a key role in sustaining the asymmetrical waveform, not in its formation (Mizuno et al. 2012).

In a demembranated sperm model treated with repaglinide, flagellar bending becomes attenuated at a high concentration of Ca^{2+}. This attenuation is not observed at low concentration of Ca^{2+}, suggesting that calaxin regulates dynein-driven microtubule sliding for asymmetrical bending at higher Ca^{2+} concentration ($<10^{-6}$ M). By using an in vitro assay system with purified dynein, microtubules, and calaxin, the roles of calaxin in the regulation of dynein-driven microtubule sliding can be directly examined (Mizuno et al. 2012). Increasing the concentration of Ca^{2+} has a small effect on the velocity of microtubule sliding by *Ciona* outer arm dynein. Addition of calaxin gives no significant change in the sliding. On the other hand, at higher Ca^{2+} concentrations ($<10^{-6}$ M), addition of calaxin significantly reduced the velocity of microtubule translocation. Thus, calaxin is thought to bind and suppress outer arm dynein at high concentrations of Ca^{2+}. This suppression is thought necessary for the propagation of asymmetrical bending.

The mechanism of calaxin-mediated chemotactic turn is summarized in Fig. 5.3. Before the chemotactic turn, Ca^{2+} concentration in sperm is low and the calaxin is dissociated from dynein. Symmetric P- and R-bends are properly propagated, resulting in straight swimming of sperm. When intracellular Ca^{2+} concentration is

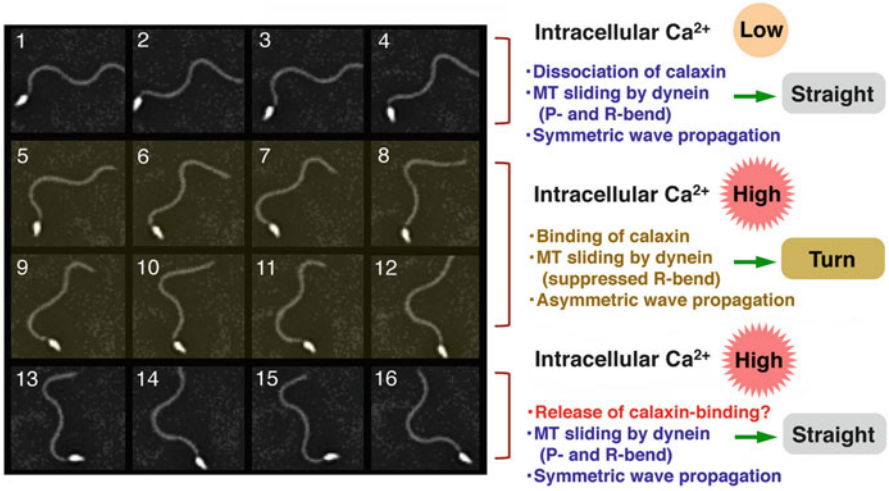

Fig. 5.3 Molecular events during chemotactic turn movement of *Ciona* sperm shown by 16 sequential images of sperm waveform during chemotactic turn. The 8 images highlighted in the *center* represent turn with asymmetrical waveform. The molecular events during this process are driven by the changes of intracellular Ca^{2+} and interaction between calaxin and dynein (see text for more detail)

raised by Ca^{2+} influx, calaxin suppresses dynein-driven microtubule sliding, resulting in propagation of asymmetrical bending and turn movement of sperm. After the chemotactic turn, sperm show straight movement. Therefore, calaxin is thought to be again dissociated from dynein and sperm swim straight with a symmetrical flagellar waveform. Ca^{2+} imaging of live *Ciona* sperm, however, demonstrates that intracellular Ca^{2+} concentration is still high just after the chemotactic turn (Shiba et al. 2008). It is possible that binding of calaxin to dynein is controlled not by absolute Ca^{2+} concentration but by the difference in Ca^{2+} concentration. Alternatively, calaxin may be downregulated by some factors after the chemotactic turn. The mechanism of calaxin after the chemotactic turn is still to be elucidated.

5.4 A Phylogenetic Consideration of Calaxin

Homology search against databases of other organisms demonstrates that calaxin orthologues are present in vertebrates, such as human, mouse and *Xenopus*. Calaxin is also found in invertebrates, both deuterostome (*Ciona*, lancet, and sea urchin) and protostome (*Drosophila*). Search against genome databases of the sea anemone *Nematostella vectensis* and the choanoflagellate *Monosiga brevicollis* also identifies calaxin orthologues in these organisms (Mizuno et al. 2009). However, calaxin has not been found in yeast, *Volvox*, *Trypanosoma*, or *Arabidopsis*, implying that calaxin is metazoan specific (Mizuno et al. 2009). Recently, high-throughput next-generation sequencing enables us to determine draft sequences from a number of other organisms.

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Hs      MNRKKLQKLTDTLTKN--CKHFNKFEVNCLIKLFYDLVGGVERQGLVVGLDRNAFRNILH
Ci      -MSKKNQKLAEELYKTSCQKHFTKTEVESLIICYKNLLEGLK-------MDRNLFRDILH
Bd      ---------------MLAKTCVSRAEIDILGRSFTLTAQTSDK------IDRSRFRDMLA
                              . ..: *:: *   :        .       :**. **::*

Hs      VTFGMTDDMIMDRVFRGFDKDNDGCVNVLEWIHGLSLFLRGSLEEKMKYCFEVFDLNGDG
Ci      QKFNMTEDLLMDRVFRAFDKDSDSYISLTEWVEGLSVFLRGTLDEKMEYTFTVFDLNGDG
Bd      DTFGVDDSLIMD-------RDADNYISFDEYIKGMSVFLNGRYEERLKFCFRVYDLNGDR
         .*.: :.:::**        :* *. :.. *:::.*:*:**.*  :*:::: * *:*****

Hs      FISKEEMFHMLKNSLLKQPSEEDPDEGIKDLVEITLKKMDHDHDGKLSFADYELAVREET
Ci      YISREEMFQMLKTCLVKQPTEEDPDEGIKDLVEIALKKMDHDHDSRLSKKDFKDAVLIEP
Bd      YISKEEMFQMLKNCLVKGAVEEDED-GVKDLVDLVLKKLDEDRDGRVSEADWAGAIAKET
        :**:****:***..*:* . *** * *:****::.***:*.*:*.::*  *:  *:  *.

Hs      LLLEAFGPCLPDPKSQMEFEAQVFKDPNEFNDM
Ci      LLLEAFGKCLPDEKSSEIFEYHVLGVKQCRG--
Bd      LLMEAFGHCLPDAKVDEYD--------------
        **:**** **** * .
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Fig. 5.4 Multiple alignment of calaxin. Sequences of calaxin from *Homo sapiens* (Hs), *Ciona intestinalis* (Ci), and *Batrachochytrium dendrobatidis* (Bd) are aligned by ClustalW. *Asterisks*, *colons*, or *dots* indicate identical residues in all sequences in the alignment, conserved substitutions, or semi-conserved substitutions, respectively

We have recently found a calaxin orthologue in the chytrid fungus *Batrachochytrium dendrobatidis* (Fig. 5.4). Further search against genome databases of other organisms supports the idea that calaxin is an opisthokont-specific protein to regulate axonemal dyneins (Inaba et al., manuscript in preparation).

Ca^{2+}-dependent regulation of flagellar waveform is important for responses of organisms to several stimuli. For example, *Chlamydomonas* exhibits several light-induced behavioral responses, including phototaxis, photophobic response, and photokinesis (Witman 1993; Wakabayashi and King 2006). *Paramecium* swims both backward and forward according to the changes in intracellular Ca^{2+} (Naitoh and Kaneko 1972). The outer arm dynein of *Paramecium* cilia has not been well characterized, but a gene for the orthologue of *Chlamydomonas* LC4 or DC3 is found in the *Paramecium* genome. On the other hand, neither LC4 nor DC3 is found in *Ciona*, as already described. Considering the regulation of the outer arm dynein by Ca^{2+} commonly seen in *Chlamydomonas*, *Paramecium*, and *Ciona*, it is possible to consider that calaxin is an opisthokont-specific innovation for Ca^{2+}-dependent regulation of axonemal dyneins.

5.5 Perspectives

Calaxin was first identified in *Ciona* sperm but was found to be distributed all through opisthokonts. Considering the presence of Ca^{2+}-dependent regulator for dynein, LC4, in *Chlamydomonas* and other bikont species, what does this "innovation of calaxin" in opisthokonts mean? It is possible that an unknown mechanism for motility

regulation might have been innovated in the supergroup of opisthokonts as well as structural diversification in the axonemes at the base of bikonts and opisthokonts. Further phylogenetic or structural evidence is necessary to conclude the evolutional and reproductive significance of calaxin.

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