Int. J. Dev. Biol. 51: 761-769 (2007)
doi: 10.1387/ijdb.072346jy

Developmental Expression Pattern

Developmental expression of *CagMdkb* during gibel carp embryogenesis

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ABSTRACT *Midkine (Mdk)* genes have been revealed to have different expression patterns in vertebrates and therefore, additional studies on *Mdk* expression patterns are required in more species. In this study, *CagMdkb* has been cloned and characterized from a SMART cDNA library of 10-somite stage embryos of *Carassius auratus gibelio*. Its full length cDNA is 1091 bp and encodes a sequence of 147 amino acids, which shows 97.3% identity to zebrafish Mdkb on the amino acid level. RT-PCR analysis reveals that *CagMdkb* is first transcribed in gastrula embryos and maintains a relatively stable expression level during subsequent embryogenesis. Western blot analysis reveals a 19 kDa maternal *Cag*Mdkb protein band and the zygotic *Cag*Mdkb protein is expressed from gastrula stage. At around 10 somite stage, the 19 kDa *Cag*Mdkb is processed to another protein band of about 17 kDa, which might be the secreted form with the 21-residue signal peptide removed. With immunofluorescence analysis, maternal *Cag*Mdkb protein was found to be localized in each blastamere cell of early embryos. The zygotic *Cag*Mdkb positive fluorescence signal was detected from a pair of large neurons at 18-somite stage. At the later stages, *Cag*Mdkb protein was also extended to numerous small neurons in the forebrain, midbrain and hindbrain, as well as to nerve fibers in the spinal cord. Co-localization with 3A10 antibody revealed *Cag*Mdkb immunoreactivity on developing Mauthner neurons, a member of reticulospinal neurons. In addition, ectopic expression of *CagMdkb* in early embryos of gibel carp and zebrafish suppressed head formation and *Cag*Mdkb function was found to depend on secretory activity. All these findings indicate that *Cag*Mdkb plays an important role in neural development during gibel carp embryogenesis and there is functional conservation of Mdkb in fish head formation.

KEY WORDS: *Mdkb, embryogenesis, Mauthner cell, nerve development, gibel carp*

Midkine (Mdk), originally identified as the product of a retinoic acid-responsive gene in embryonic carcinoma cells (Kadomatsu *et al.,* 1988; Tomomura *et al.,* 1990), had been suggested to have significant functions in neurogenesis, neuron outgrowth and survival, angiogenesis, wound healing and tumorigenesis because its widespread expression was observed in higher vertebrates (Kadomatsu and Muramatsu, 2004). In mouse, *Mdk* is widely expressed in tissues originating from all three germ layers (Kadomatsu *et al.,* 1990; Muramatsu *et al.,* 1993; Fan *et al.,* 2000). In chicken, *Mdk* is expressed throughout embryo but excluded from neural tube (Duprez *et al.,* 1993). Recently, two related *Mdk* genes, *Mdka* and *Mdkb*, have been isolated from zebrafish (Winkler *et al.,* 2003). Zebrafish *Mdkb* is expressed in the dorsal neural tube and is involved in posterior neural development (Winkler and Moon, 2001). Zebrafish *Mdka* is expressed in the paraxial mesoderm and functions in medial floor plate (MFP) formation (Schäfer *et al.,* 2005). A great deal of attention had been focused on *Mdk* mRNA expression patterns (Kadomatsu *et al.,* 1990; Sekiguchi *et al.,* 1995; Winkler *et al.,* 2001; 2003) and distinct expression patterns had been found in different vertebrates and fish species. The data implicated that fish *Mdk* might have different expression patterns from other vertebrate *Mdk* (Winkler *et al.,* 2003) and species-specific differences might exist in different vertebrates and even in different fish species. In protein level, Mdk localization in embryogenesis was only investigated in mouse (Muramatsu *et al.,* 1993), rat (Matsumoto *et al.,* 1994) and chicken (Duprez *et al.,* 1993) and no fish Mdk protein localization has not been studied up to the present. Therefore, additional studies on *Mdk* expression patterns, especially in

Abbreviations used in this paper: bp, base pairs; Cag, Carassius auratus gibelio; Mdk, midkine.

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0214-6282/2007/$30.00

Printed in Spain
www.intjdevbiol.com

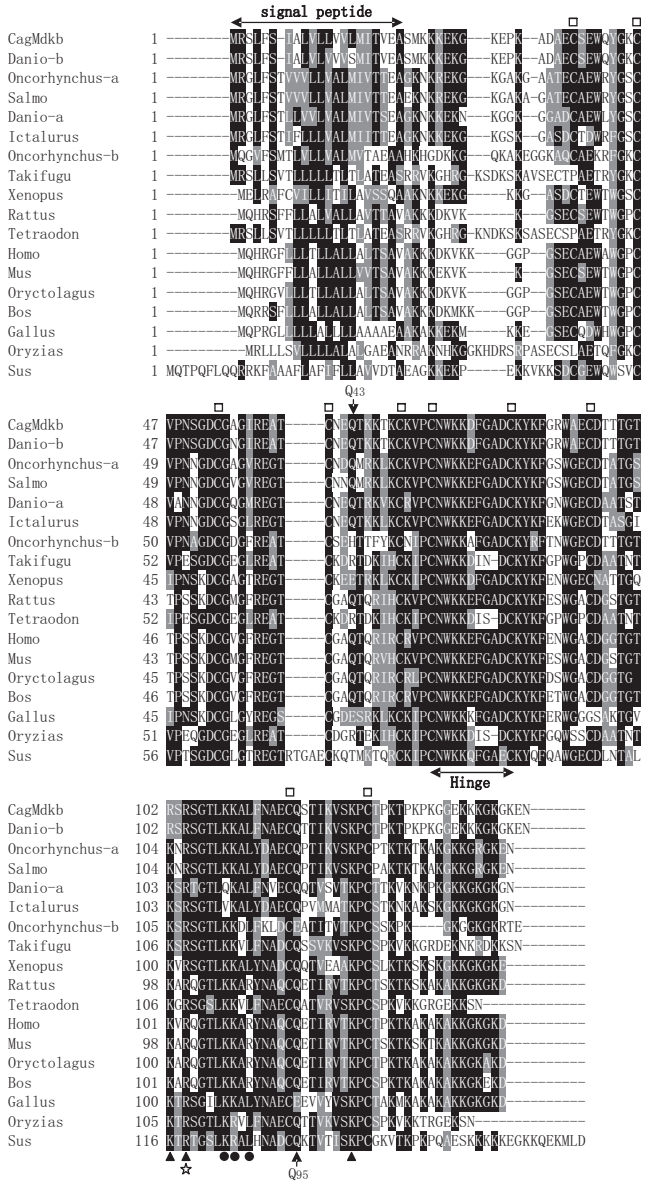
protein level, are required in more species of fish.

Gibel carp (*Carassius auratus gibelio*), because of its unique triploid level and dual reproduction modes of gynogenesis and bisexuality (Zhou *et al.,* 2000; Zhou and Gui, 2002), has been used as a promising study model for developmental biology and evolutionary genetics (Dong *et al.,* 2004; Yang and Gui, 2004). Recently, we have initiated a systematic study to screen differentially expressed genes during oogenesis and early embryogenesis. Some important genes involved in oocyte maturation, egg fertilization and early embryogenesis, such as *cyclin A1*, *cyclin B*, *cyclin A2* (Xie *et al.,* 2001, 2003), *SNX* (Wen *et al.,* 2003), *hatching enzymes* (Liu *et al.,* 2003), *C-type lectin* (Dong *et al.,* 2004), *C1q-like factor* (Chen and Gui, 2004), *spindlin* (Wang *et al.,* 2005) and *CNBP* (Liu and Gui, 2005), have been identified and characterized in the model system.

To reveal differentially expressed genes at 10-somite embryos relative to gastrula embryos, we constructed the suppression subtractive hybridization (SSH) cDNA plasmid libraries between 10-somite embryos and gastrula embryos in the gynogenetic *Carassius auratus gibelio* (Liu *et al.,* 2005). A total of 98 positive clones were screened from 816 clones of the subtractive 10-somite embryo cDNA library. Sequencing analysis and database searches revealed a positive clone with a 318 bp cDNA fragment that is highly homologous to zebrafish *Mdkb* (Liu *et al.,* 2005). In this study, we clone the full length cDNA with RACE-PCR. Based on its molecular characterization, we analyze the *CagMdkb* expression pattern during embryogenesis and early larval development and generate a polyclonal antiserum against the *Cag*Mdkb, which for the first time allows an analysis of Mdk protein localization in fish embryos.

Results and Discussion

We have cloned full-length cDNA of *Carassius auratus gibelio Mdkb* (*CagMdkb*) by RACE-PCR from the SMART cDNA library prepared from 10-somite embryos. The *CagMdkb* cDNA is 1091



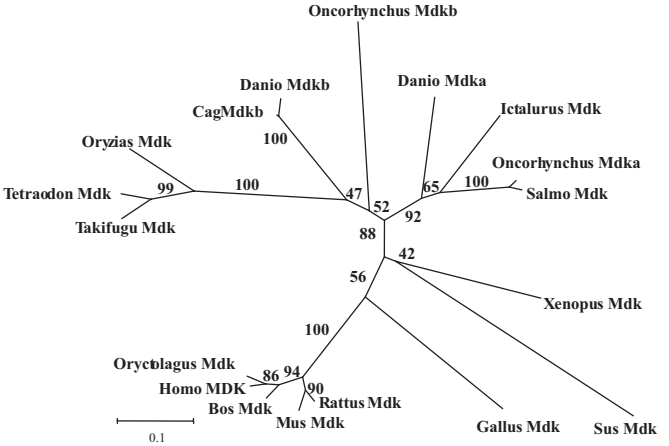


Fig. 1. Alignment and phylogenetic relationship of vertebrate Mdkb proteins. (A) *Amino acid alignment of vertebrate Mdk proteins with CLUSTALX. Identical residues are in black, conservative substitutions in gray. Conserved cysteins are indicated by squares and residues important for heparin binding are indicated by triangles (cluster I) or circles (cluster II). An arginine residue essential for receptor binding is marked by an asterisk and glutamine residues Q43 and Q95 important for dimerization are marked by arrows. The* CagMdkb *sequence has been deposited in GenBank under accession number DQ337174, other accession nos. are as follows:* Danio rerio *Mdkb AAG27035;* Oncorhynchus mykiss *Mdka CA346000 (EST);* Salmo salar *BG934205 (EST);* Danio rerio *Mdka AAM27446;* Ictalurus punctatus *BM495405 (EST);* Oncorhynchus mykiss *Mdkb CA365321 (EST);* Takifugu rubripes *scaffold S004868 (http://fugu.hgmp.mrc.ac.uk);* Xenopus laevis *AAH72776;* Rattus norvegicus *NP_110486;* Tetraodon nigroviridis *see Methods;* Homo sapiens *AAH11704;* Mus musculus *NP_001012336;* Oryctolagus cuniculus *AAS60202;* Bos Taurus *NP_776360;* Gallus gallus *CAA54020;* Oryzias latipes *BJ523610 (EST);* Sus scrofa *NP_999501*. **(B)** *Phylogenetic analysis of Mdk proteins with Mega 3.0 program. The phylogenetic tree (Minimum Evolution) is unrooted. Bootstrap (500 replicates; seed=64238). CNI (level=1) with initial tree=NJ MaxTrees=1*

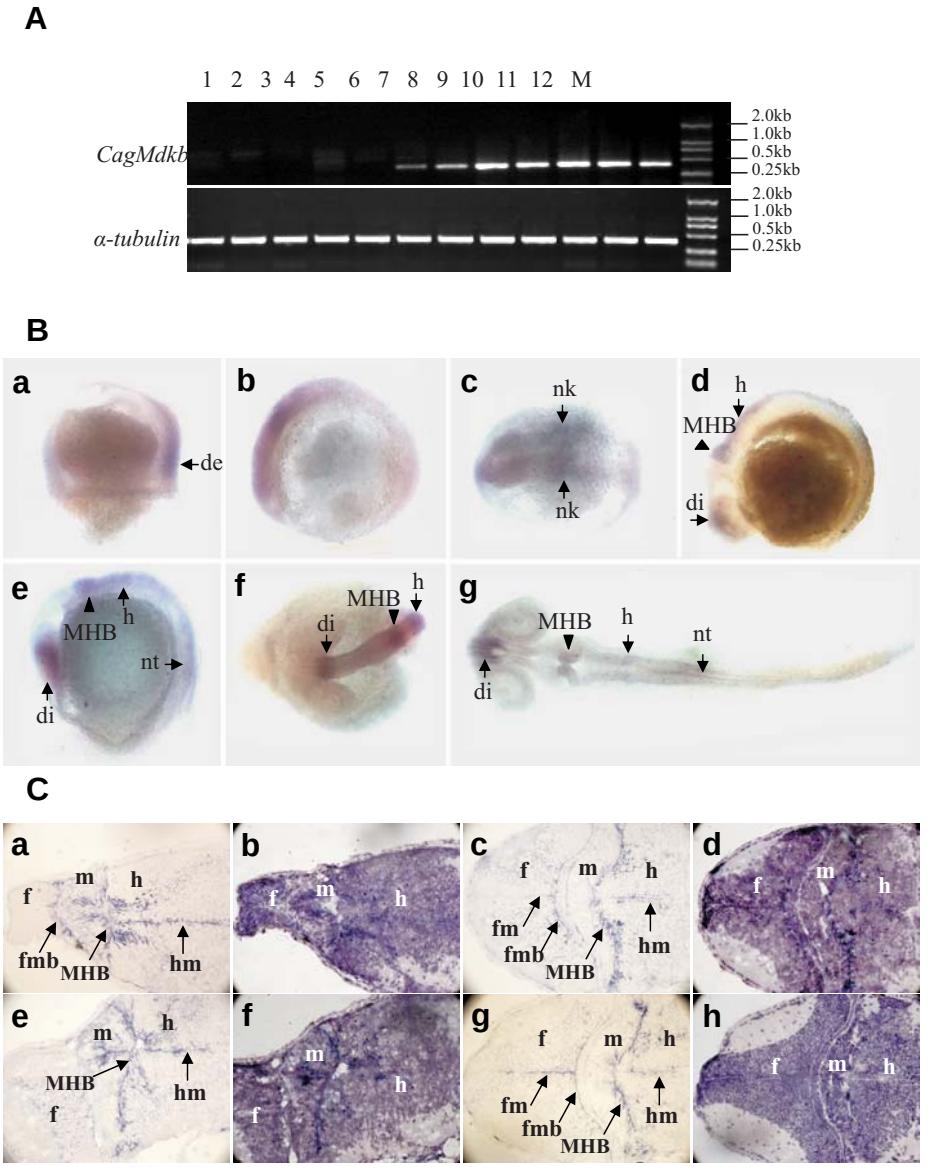


Fig. 2. *CagMdkb* expression pattern. (A) *Expression characterization of* CagMdkb *during embryogenesis revealed by RT-PCR. Ubiquitous expression of α-tubulin was used as the RT-PCR control. 1 to 12 respectively indicate 18 samples from (1) mature eggs, (2) fertilized eggs, (3) multicell stage embryos, (4) morula embryos, (5) blastula embryos, (6) early gastrula embryos, (7) neurula embryos, (8) 10-somite embryos, (9) muscle contract embryos, (10) heartbeat embryos, (11) prehatching embryos, (12) 1-day larvae. M, marker (DL2000).* **(B)** *Expression of* CagMdkb *during embryogenesis revealed by whole mount* in situ *hybridization.* **(a)** *60% epiboly, lateral view, dorsal to the right. Onset of* CagMdkb *expression in the dorsal epiblast (arrow).* **(b,c)** *1-somite stage, anterior to the left.* CagMdkb *expression at the edges of the forming neural keel (arrow). (b) lateral view; (c) dorsal view.* **(d)** *10-somite stage, lateral view.* CagMdkb *is expressed in the diencephalon, at the MHB (arrowhead) and in a gradient in the hindbrain, but excluded from the eye fields.* **(e,f)** *18-somite stage.* CagMdkb *is expressed in the diencephalon, at the MHB (arrowhead), in the hindbrain and in the neural tube. (e) lateral view; (f) dorsal view of head region.* **(g)** *Primula-5 stage, dorsal view.* CagMdkb *is expressed in the diencephalon, at the MHB (arrowhead), in the hindbrain and at the midline of the neural tube. de, dorsal epiblast; di, diencephalon; h, hindbrain; MHB, mid-hindbrain boundary; nk, neural keel; pt, neural tube.* **(C)** *Expression of* CagMdkb *at larva stage revealed by* in situ *hybridization. (a-d) 3-day larva, horizontal view, anterior to the left.* **(a)** *A horizontal section of 3-day larva brain.* **(b)** *Hematoxylin and eosin staining of (a).* **(c)** *Another section of 3-day larva.* **(d)** *Hematoxylin and eosin staining of (c). (e-h) 5-day larva, horizontal view, anterior to the left.* **(e)** *A horizontal section of 5-day larva.* **(f)** *Hematoxylin and eosin staining of (e).* **(g)** *Another section of 5-day larva.* **(h)** *Hematoxylin and eosin staining of (g). f, forebrain; fm, forebrain midline; fmb, fore-midbrain boundary; h, hindbrain; hm, hindbrain midline; m, midbrain; MHB, mid-hindbrain boundary.*

bp in length and consists of a 93 bp 5'-untranslated region (UTR), a coding sequence of 441 bp and a 557 bp 3'-UTR. The open reading frame encodes a sequence of 147 amino acids. A cleavage site for the putative signal peptide is located between amino acid position 21 and 22 by predicting with SignalP 3.0 program (Bendsten *et al.*, 2004).

We have compared amino acid alignments and their identities between the deduced *Cag*Mdkb and other vertebrate Mdk homologues. As shown in Fig. 1A, the amino acid identities between the *Cag*Mdkb and other Mdk range from 97.3% to 44.1%. The highest 97.3% identity exists between *Cag*Mdkb and zebrafish Mdkb and the lowest 44.1% identity is with *Sus scrofa* Mdk. As described previously by Winkler *et al.* (2003), 10 conserved cystein residues and a highly conserved hinge region that separates amino- and carboxy-terminal domains also include in the *Cag*Mdkb. Moreover, two clusters of basic residues that were demonstrated to be important for heparin binding in human Mdk (Iwasaki *et al.*, 1997) are found in the C-terminal of *Cag*Mdkb. Other significant residues, such as the arginine residue for its receptor binding and two glutamine residues for dimerization, are present in the *Cag*Mdkb (Fig. 1A). The data imply highly evolutionary conservation and functional significance of *Mdk* genes in vertebrates. Phylogenetic analysis revealed two distinct fish-specific groups of Mdk proteins and the *Cag*Mdkb belongs to the Mdkb group (Fig. 1B). Therefore, the newly cloned molecule was designated *CagMdkb*.

By semi-quantitative RT-PCR we analyzed the temporal expression pattern of *CagMdkb* during embryogenesis. As shown in Fig. 2A (top), *CagMdkb* is initially transcribed from early gastrula stage and then increases£gradually. Its transcripts reach the maximal level at 10-somite stage and maintain a relatively stable expression level during the following embryogenesis.

Spatial expression pattern of *CagMdkb* during embryogenesis was further examined by whole mount *in situ* hybridization. As shown in Fig. 2B, the embryonic distribution of *CagMdkb* tran-

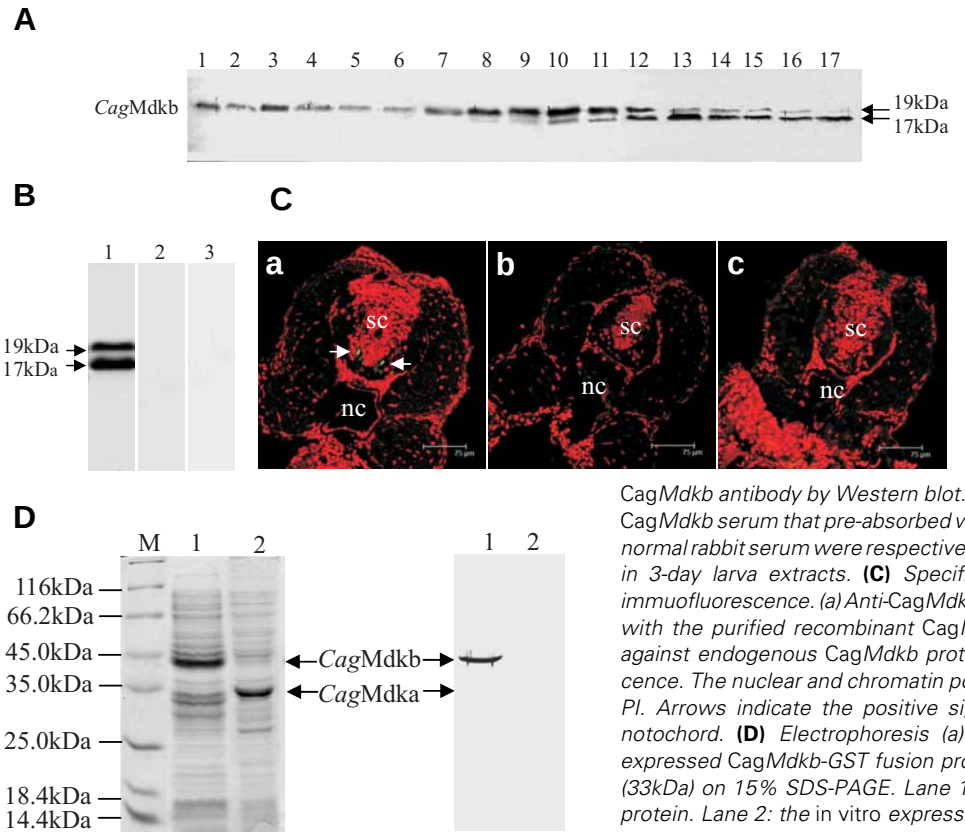


Fig. 3. *Cag*Mdkb protein detection and speficicity of *Cag*Mdkb antibody. **(A)** Cag*Mdkb protein detection during embryogenesis revealed by Western blot. 1 to 17 respectively indicate 17 samples from (1) mature eggs, (2) fertilized eggs, (3) multi-cell stage embryos, (4) morula embryos, (5) blastula embryos, (6) early gastrula embryos, (7) neurula embryos, (8) 10-somite embryos, (9) muscle contract embryos, (10) heartbeat embryos, (11) pre-hatching embryos, (12) 1-day larvae, (13) 3-day larvae, (14) 6-day larvae, (15) 9 day-larvae, (16) 12-day larvae, (17) 15-day larvae. The 17 kDa and 19 kDa protein bands of* Cag*Mdkb are indicated by arrows.* **(B)** *Specificity detection of* Cag*Mdkb antibody by Western blot. Lane 1, 2, 3 reveal anti-*Cag*Mdkb serum, anti-*Cag*Mdkb serum that pre-absorbed with the purified recombinant* Cag*Mdkb protein, normal rabbit serum were respectively tested against endogenous* Cag*Mdkb protein in 3-day larva extracts.* **(C)** *Specificity detection of anti-*Cag*Mdkb antibody by immuofluorescence. (a) Anti-*Cag*Mdkb serum, (b) anti-*Cag*Mdkb serum, pre-adsorbed with the purified recombinant* Cag*Mdkb protein, (c) normal rabbit serum tested against endogenous* Cag*Mdkb protein in 3-day larva extracts by immunofluorescence. The nuclear and chromatin positions were stained with red fluorescence by PI. Arrows indicate the positive signals in the spinal cord. sc, spinal cord; nc, notochord.* **(D)** *Electrophoresis (a) and Western blot (b) detection of* in vitro *expressed* Cag*Mdkb-GST fusion protein (41kDa) and* Cag*Mdka-His fusion protein (33kDa) on 15% SDS-PAGE. Lane 1: the* in vitro *expressed* Cag*Mdkb-GST fusion protein. Lane 2: the* in vitro *expressed* Cag*Mdka-His fusion protein.*

scripts is basically similar to that of zebrafish *mdkb* during embryogenesis (Winkler and Moon, 2001). *CagMdkb* transcripts are firstly expressed in dorsal epiblast cells of early gastrula embryos (Fig. 2Ba). At 1-somite stage, *CagMdkb* transcripts are located in the two lateral stripes at the forming brain and neural keel (Fig. 2Bb, 2Bc). At 10-somite stage, *CagMdkb* is highly expressed in the future diencephalon, mid-hindbrain boundary (MHB) and hindbrain (Fig. 2Bd). At 18-somite stage (Fig. 2Be, 2Bf) and hatching stage (Fig. 2Bg), *CagMdkb* transcripts are obviously restricted to the diencephalon, MHB, hindbrain and dorsal neural tube. Moreover, the *in situ* hybridization on horizontal sections of 3-day (Fig. 2Ca-d) and 5-day (Fig. 2Ce-h) larvae revealed cellular distribution of *CagMdkb* transcripts in the developmental brain. As shown in Fig. 2C, the *CagMdkb* transcripts are mainly distributed in the nerve cells of forebrain midline, fore-midbrain boundary, MHB and hindbrain midline.

We also generated a polyclonal antibody against the *Cag*Mdkb from rabbit. With the polyclonal antibody, Western blot analysis reveals a 19 kDa maternal *Cag*Mdkb protein band from eggs and early embryos and the zygotic *Cag*Mdkb protein is expressed from gastrula stage. At around 10 somite stage, the 19 kDa *Cag*Mdkb is processed to another protein band of about 17 kDa, which might be the secreted form with removal of the 21-residue signal peptide (Fig. 3A). In contrast to gradual decrease of the 19 kDa *Cag*Mdkb content, the 17 kDa *Cag*Mdkb protein content increases gradually along with the following development of embryos and larvae. The data suggest that the maternal *Cag*Mdkb should be intact protein, while the zygotic *Cag*Mdkb might exert its functional roles through the secreted form without the signal peptide.

To further test the specificity of *Cag*Mdkb antibody, the antiserum was pre-absorbed with the purified recombinant *Cag*Mdkb protein for 16 h at 4 ºC and set as negative control for both Western blot detection and immunofluorescence localization. Indeed, both 17 kDa protein band and 19 kDa protein band (Fig. 3B, lane 1) can not be recognized by the pre-adsorbed antiserum (Fig. 3B, lane 2) and no positive green immunofluorescence (FITC) is stained by the pre-adsorbed antiserum (Fig. 3Cb) in comparison with Fig. 3Ca. Control is also done with normal rabbit serum and no signal can be detected in Western blot analysis (Fig. 3B, lane 3) and immunofluorescence localization (Fig. 3C-c). Moreover, the *Cag*Mdka-His fusion protein is also expressed *in vitro* (Fig. 3Da) and can not be recognized by the anti-*Cag*Mdkb antibody (Fig. 3Db). The data demonstrate the specificity of anti-*Cag*Mdkb antibody.

To investigate whether CagMdkb function depends on the complete form with signal peptide, we injected RNAs encoding wild type full-length *CagMdkb* and N-terminally truncated *Mdkb* without signal peptide into 1-cell stage gibel carp embryos respectively. In comparison with the control embryos (Fig. 4A, lane 1), both 19 kDa protein and the 17 kDa protein increase in the wild type *CagMdkb* RNAs injected embryos (Fig. 4A, lane 2), whereas only 17 kDa protein increases in the N-terminally truncated *Mdkb* RNAs injected embryos (Fig. 4A, lane 3), which confirms that the 19 kDa protein is the complete *Cag*Mdkb with signal peptide and the 17 kDa protein is the secreted form without signal peptide.

Additionally, in comparison with control embryos (Fig. 4Ba, Bb), 54.4% (31/57) of embryos injected with wild type *CagMdkb* RNAs show severe deficiencies including reduction of forebrain region (Fig. 4Bc) and eye structure (Fig. 4Bd), whereas no any deficiencies are observed in the N-terminally truncated *Mdkb* RNAs injected embryos (Fig. 4Be, Bf). Moreover, we further injected the two types of RNAs into zebrafish early embryos. Western detection (Fig. 4C) shows identical results to that in gibel carp embryos. Similarly, in comparison with control embryos (Fig. 4Da, Db), 65.9% (58/88) of zebrafish embryos injected with wild type *CagMdkb* RNAs also show forebrain and eye structure deficiencies (Fig. 4Dc, Dd), whereas no any deficiencies are observed in the N-terminally truncated *Mdkb* RNAs injected embryos (Fig. 4De, Df). The data indicate that ectopic expression of *CagMdkb* suppresses head formation, which is consistent with that reported previously in zebrafish (Winkler and Moon, 2001) and *Cag*Mdkb function depends on the secretory activity. Furthermore, the interesting finding suggests the functional conservation of Mdkb in head formation of fish.

Then we use *Cag*Mdkb antibody to trace the immunofluorescence localization and distribution during embryogenesis. Fig. 5 shows its dynamic distribution characterization during embryogenesis. In one-cell fertilized eggs, the majority of *Cag*Mdkb is localized at the animal pole and cytoplasmic movement from the central toward the animal pole is also observed in the yolk cell (Fig. 5A). In blastula and gastrula embryos, uniform immunofluorescence is ubiquitously distributed in cytoplasm of blastomere cells (Fig. 5B, 5C). It is until 18-somite stage (Fig. 5D) a pair of large neurons with *Cag*Mdkb immunofluorescence signal is firstly detected in the transverse section through the head, while the ubiquitous fluorescence of the maternal protein is weakened. In the hatching stage embryo (Fig. 5E, 5F) and 3-day larva (Fig. 5G, 5H), the *Cag*Mdkb positive soma becomes very large in the hindbrain and the axon of the large neuron with strong *Cag*Mdkb immunofluorescence is clearly observed from the transverse sections through the spinal cord. In 5-day larva (Fig. 5I, 5J), *Cag*Mdkb protein is not only located in this pair of large neurons, but also in other small neurons in the forebrain, midbrain and hindbrain (Fig. 5I) and in small nerve fibers in the spinal cord (Fig. 5J). As the picture shown (Fig. 5J), the diameter of the large axon is at less ten times larger than other small axons. We then slice the 5-day larva horizontally to get the full appearance of this pair of large neurons. As shown in Fig. 6 (A, D, G, J, M), it has two large somata in the hindbrain and long axons extending caudally in the spinal cord. Although the emergence of new signal of *Cag*Mdkb protein is later than its mRNA expression in the brain and neural tube, the CagMdkb protein distribution pattern is approximately consistent with the spatial expression pattern of *CagMdkb* mRNA.

Since the new immunofluorescent signal of *Cag*Mdkb is first detected in this pair of large neurons, we are very curious about what kind of neuron it is. According to the characterization, we proposed that the *Cag*Mdkb positive large neuron might be Mauthner neuron, which has the largest axon in spinal cord and begins to extend in 18-somite stage (Kimmel *et al.,* 1990). To confirm this proposition, we used a monoclonal antibody 3A10 that can recognize exclusively the Mauthner neuron in zebrafish embryogenesis (Hill *et al.,* 1995), to perform co-localization of double immunofluorescences in combination with anti-*Cag*Mdkb antibody. As shown in Fig. 6, the anti-*Cag*Mdkb antibody presents green fluorescence (FITC) on the axon and cell soma from

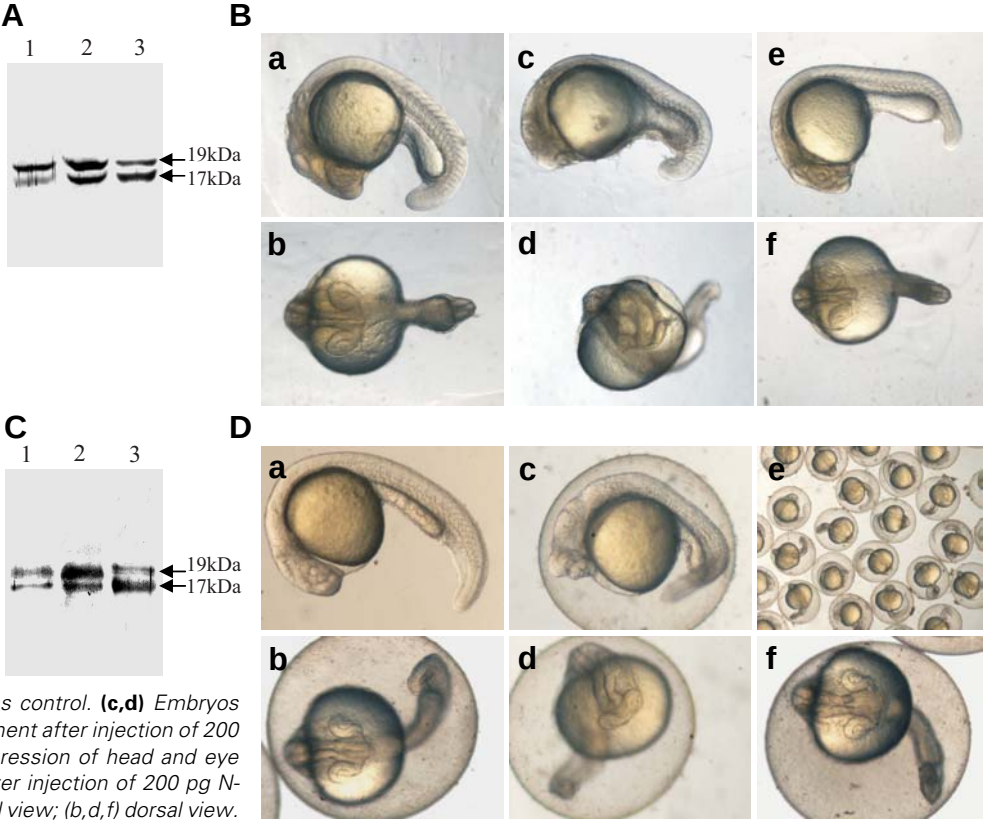


Fig. 4. Ectopic expression of *CagMdkb* and N-terminally truncated *Mdkb*. **(A)** *Western blot detection of* Cag*Mdkb protein in 36 hpf gibel carp embryos. Lane 1, normal embryos; lane 2, embryos injected of 200 pg* CagMdkb *RNAs; lane 3, embryos injected of 200 pg N-terminally truncated* Mdkb *RNAs.* **(B)** *Optic observation of gibel carp embryos at 36hpf.* **(a,b)** *Normal embryos as control.* **(c,d)** *Embryos exhibit severe deficiencies in head development after injection of 200 pg* CagMdkb *RNAs. It leads to specific repression of head and eye structures.* **(e,f)** *Embryos appear normal after injection of 200 pg N-terminally truncated* Mdkb *RNAs. (a,c,e) Lateral view; (b,d,f) dorsal view.* **(C)** *Western blot detection of* Cag*Mdkb protein in 24 hpf zebrafish embryos. Lane 1, normal embryos; 2, embryos injected of 200 pg* CagMdkb *RNAs; lane 3, embryos injected of 200 pg N-terminally truncated* Mdkb *RNAs.* **(D)** *Optic observation of zebrafish embryos at 24hpf.* **(a,b)** *Normal embryos as control.* **(c,d)** *Embryos exhibit severe deficiencies in head development after injection of 200 pg* CagMdkb *RNAs. It leads to specific repression of head and eye structures.* **(e,f)** *Embryos appear normal after injection of 200 pg N-terminally truncated* Mdkb *RNAs. (a,c) lateral view; (b,d,f) dorsal view.*

horizontal sections through spinal cord (Fig. 6A) and brain (Fig. 6D). The antibody 3A10 shows deep blue fluorescence (Rhodamine) on the same regions (Fig. 6 B,E). Lapping over the two fluorescence pictures indicates that the staining regions of *Cag*Mdkb and Mauthner neuron component are completely in superposition because the color changes to their syncretic color grey (Fig. 6 C,F). Higher amplifications of the stained axon (Fig. 6 G,H,I) and cell soma (Fig. 6 J,K,L) more clearly reveal the overlapping co-localization of *Cag*Mdkb and the Mauthner neuron component. Furthermore, in combination with anti-*Cag*Mdkb antibody (Fig. 6M) and 3A10 antibody (Fig. 6N), the PI for staining nucleic acids to show red fluorescence was also used to localize the Mauthner neuron nucleus. As shown in Fig. 6O, the triplicate fluorescence reveals detailed morphological characterization of the Mauthner neuron.

In the current study, *Cag*Mdkb protein is ubiquitously distributed in all blastomere cells of early embryos. The newly synthesized *Cag*Mdkb protein starts to appear in Mauthner cells of the hindbrain at 18-somite stage and later it is detected in the outgrowth axons of Mauthner cells. In 5-day larva, *Cag*Mdkb protein extents to a large number of neurons in the forebrain, midbrain and hindbrain as well as many nerve fibers in the spinal cord. This indicates that gibel carp Mdkb protein distribution is different from mouse, rat and chicken Mdk, which might lead to diverse functional implications among these animals.

Although Mdk has diverse activities among species, there is a common function in most of animals. It is that Mdk plays important roles in neurogenesis and it is located along the route through which neurons migrate. For example, Mdk protein is present in the radial glial processes in the cerebral cortex of 17-day rat embryos (Matsumoto *et al.,* 1994). When human fetal brain cells are separated into astrocytes and neurons, the astrocytes have been shown to synthesize Mdk (Satoh *et al.,* 1993). Mouse Mdk protein is detected in the brain cells and it promotes neurite outgrowth (Muramatsu *et al.,* 1993). Zebrafish *Mdkb* transcripts are detected in the diencephalon, the dorsal roof of mesencephalon, the rhombencephalon and the dorsal neural tube and it functions in posterior neural development (Winkler and Moon, 2001). *Xenopus Mdk* mRNA is also detected in brain and spinal cord of the larvae (Sekiguchi *et al.,* 1995; Yokota *et al.,* 1998). In the current study, *Cag*Mdkb protein is definitely localized on the developing Mauthner neurons in the brain and spinal cord. Therefore, the data provide protein level support for the Mdk function in nervous system of lower vertebrates.

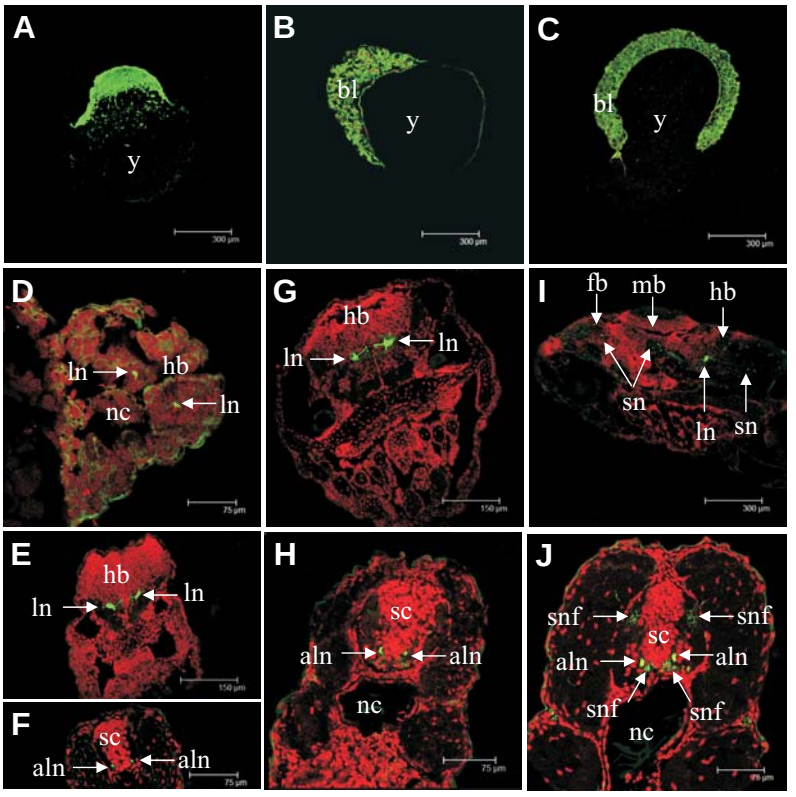


Fig. 5. Localization and distribution of *Cag*Mdkb protein during embryogenesis detected by immunofluorescence. (A) *One-cell stage embryo, lateral view.* **(B)** *Blastula embryo, lateral view.* **(C)** *Gastrula embryo, lateral view.* **(D)** *18-somite stage embryo, transverse view through hindbrain.* **(E,F)** *Pre-hatching stage embryo, transverse view through handbrain and spinal cord.* **(G,H)** *3-day larva, transverse view through hindbrain and spinal cord.* **(I)** *5-day larva, lateral view of brain.* **(J)** *5-day larva, transverse view through spinal cord. aln, axon of large neuron; b, brain; bl, blastomere cell; fb, forebrain; hb, hindbrain; ln, large neuron; md, midbrain; nc, notochord; sc, spinal cord; sn, small neuron; snf, small nerve fiber; t, tail; y, yolk. Green fluorescence is immunostained by* Cag*Mdkb antibody, while red fluorescence stained by PI shows the nuclear and chromatin position.*

Experimental Procedures

Sampling of brood fish and embryos

The brood fish of gynogenetic gibel carp (*Carassius autatus gibelio*), belonging to clone D, was selected in Guanqiao Experimental Station, Institute of Hydrobiology, Chinese Academy of Sciences. During the reproduction season, the selected brood fish were artificially induced into spawning by two intraperitoneal injections with a mixture of acetone-dried carp pituitary, HCG and LRH-A as described previously (Gui, 1999). Ovulated eggs were inseminated with sperms from red common carp (*Cyprinus carpio*) to stimulate gynogenesis (Jiang *et al.,* 1983). The inseminated eggs were incubated in about 20ºC until the embryos developed into larvae.

RACE-PCR and RT-PCR

Total RNAs were extracted from gibel carp embryos and tissues using SV RNA isolation reagent (Promega). cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Gibco). SMART cDNA was synthesized using a Clontech SMART PCR synthesis kit (Clontech). Primers used for RACE-PCR were:

5' end	5'-CTTGTGCTGCTGGTGGTGT-3',
	5'-AACAACGCAGAGTACTTTTTTTT-3';
3' end	5'-CCTTCCCTTTGCCTTTCTTC-3',
	5'- AACGCAGAGTACGCGGG-3'.

Primers used for RT-PCR were:

CagMdkb	5'-CTTGTGCTGCTGGTGGTGT-3',
	5'-CCTTCCCTTTGCCTTTCTTC-3';
α-*tubulin*	5'-GTGCACTGGTCTTCAGGGGTT-3',
	5'-GGGAAGTGGATGCGTGGGTAT-3'.

In situ *hybridization*

Whole mount *in situ* hybridization was performed as described previously (Nusslein-Volhard and Dahm, 2002). An antisense probe for the *in*

situ hybridization was transcribed with SP6 from the full-length cDNA in the original pGEM-T vector digested with NcoI (sense: NotI and T7). For sections, the gibel carp brain was embedded in OCT and 9 micrometers sections were made for *in situ* hybridization. Sections were hydrated and hybridized with a digoxigeninlabeled probe. After the chromogenic reaction, the sections were counterstained with hematoxylin and eosin.

RNA injections

The fragment containing the coding sequence for wild type full-length *CagMdkb* was amplified from the SMART cDNA library using the following primers, in-frame stop codon and upstream BamHI and downstream XhoI restriction sites:
mb-1(5' ->3'):
GGATCCATGAGTATGAAGAAAAAAGAAAAG and
mb-2 (5' ->3'):
CTCGAGTTAGTTTTCCTTCCCTTT. In order to investigate if *Cag* Mdkb function depends on its secretory activity, we designed an N-terminally truncated version of *CagMdkb* (aa 22-147). Primers with upstream BamHI and downstream XhoI restriction sites were designed:
mb-3 (5' ->3')
GGATCCGCCACCATGGTGAGTATGAAGAAAAAAG- and
mb-4 (5' ->3')
CTCGAGTTAGTTTTCCTTCCCTTTGCCTTT. The amplified wild type *CagMdkb* fragment and N-terminally truncated *Mdkb* fragment were respectively cloned into the BamHI and XhoI sites of pCS21+. Both the plasmids were linearized with NotI for synthesis of capped RNAs. About 200 pg of RNA was injected into one-cell stage gibel carp embryos and zebrafish embryos. Effects of ectopic expression were observed at 36 h postfertilization (hpf) in gibel carp embryos and at 24 hpf in zebrafish embryos.

CagMdkb antiserum preparation and specificity analysis

A cDNA fragment coding for mdk protein of 139 aa (from 9 to 147) was subcloned between the EcoRI and HindIII sites of pGEX-KG expression vector (Guan and Dixon, 1991) and transformed into the expression cell, *E.coli* BL21 (DE3). Protein expression was induced with IPTG (final concentration 1 mM) and was separated by SDS-PAGE electrophoresis. The GST fusion protein was excised from the gel and used to prepare polyclonal antiserum by immunizing white rabbit as described previously (Dong *et al.*, 2004). The acquired antiserum was deposited with ammonium persulfate and then was dissolved with ultrapure water. IgG was purified with Protein-A Agarose Kit (Oncogene). This purified antiserum was used for the following Western blot and Immunofluorescence analysis.

Monoclonal antibody 3A10 against Mauthner cell was purchased from developmental studies hybridoma bank (DSHB) at the University of Iowa.

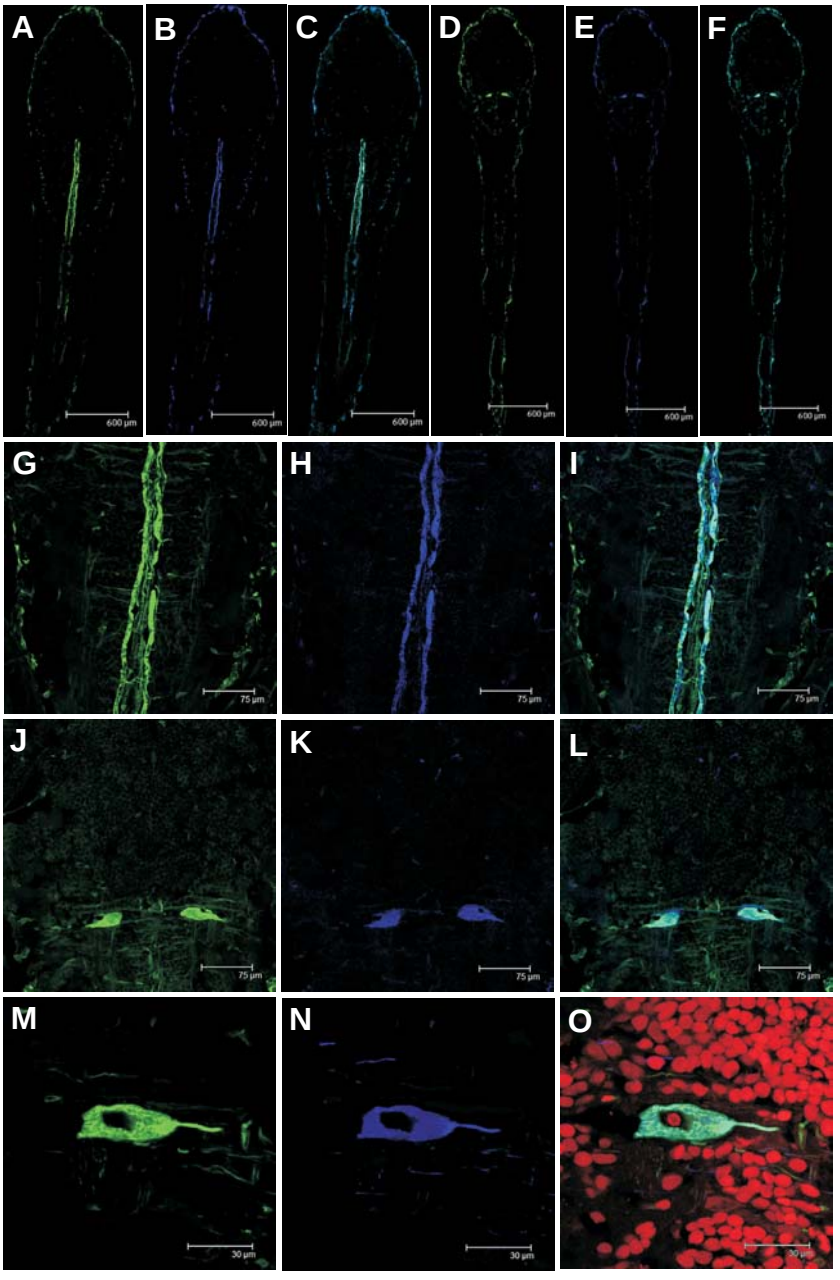


Fig. 6. Double immunofluorescence co-localization of *Cag*Mdkb and Mauthner cell component in 5 day larva. (A,D,G,J,M) Cag*Mdkb protein was stained by anti-*Cag*Mdkb antibody (FITC, green fluorescence).* **(B,E,H,K,N)** *Mauthner cell component was stained by monoclonal antibody 3A10 (Rhodamine, blue fluorescence) on the axon and cell soma.* **(C)** *The overlapping image of (A) and (B).* **(F)** *The overlapping image of (D) and (E).* **(I)** *The overlapping image of (G) and (H).* **(L)** *The overlapping image of (J) and (K).* **(O)** *The triplicate fluorescence image of (M, N) and red fluorescence stained by PI for nucleic acids. A-O, dorsal view through spinal cord.*

Western blot analysis

100 embryos were taken respectively from several development stages. The protein extract was prepared in a homogenizer with 1 ml Extraction Buffer (50 mM Hepes, 25 mM β-glycerol phosphate, 5 mM EGTA, 10% NP-40, 100 mM PMSF, 1 mM DTT, 0.1 M Na-Vanadate, 1 mg/ml Leupeptin, 1 mg/ml Aprotinin, 1 mg/ml Pepstatin, pH7.4) on ice. The extracts were centrifuged 15 min at 15,000g. Clear supernatants were added to methanol-chloroform (3:1) admixture in order to get rid of the yolk ingredient. These treated extracts were separated on 15% SDS-PAGE gel. Proteins were electrophoretically blotted to PVDF membranes (Millipore). These membranes were blocked with 5% nonfat dry milk in TBST buffer (100 mM NaCl, 100 mM Tris–HCl and 0.05% Tween 20, pH 7.5). Blocked membranes were incubated with the rabbit antiserum at a dilution of 1:500 in TBS buffer containing 1.0% milk at room temperature for 1 h. The membranes were washed three times for 15 min each in TBST buffer and then incubated with 1:2000 diluted alkaline phosphatase-conjugated goat anti-rabbit IgG (Vector). After three washes of 10 min

each in TBST buffer, detection was performed using BCIP/NBT staining.

Immunofluorescence localization

Embryos and larvae were freshly fixed by 4% paraformaldehyde in PBS (pH 7.0) at 4! overnight. After washing with PBS (pH 7.0) three times, the samples were immersed in 30% saccharose - PBS buffer at 4ºC overnight. They were then embedded in Tissue-TekR optimal cutting temperature (O.C.T.) compound (10.24% w/w polyvinyl alcohol, 4.26% w/w polyethylene glycol, 85.50% w/w nonreactive ingredients, Sakura, California, U.S.A.) and sectioned at 7 µm in thickness with frozen microtomy (Leica). The cryostat sections were rehydrated in PBS for 30 min and incubated for 1 h with 5% dry milk in PBS at room temperature to prevent nonspecific binding of antibodies. The sections were then incubated with the rabbit anti-*Ca*gMdkb serum (1:100 dilution) or 3A10 mouse monoclonal antibody for about 12 h at 4ºC. After 5 rinses in PBST(10 min each), they were exposed to 10% goat serum for 1 h and subsequently incubated for 1 h with fluorescein isothiolyanate (FITC)-conjugated secondary antibody (goat anti-rabbit IgG (H+L),FITC, 1:50 dilution, Pierce) or Rhodamine-conjugated secondary antibody (goat anti-rabbit IgG (H+L), Rhodamine, 1:50 dilution, Pierce) in the dark. After 5 time washes in PBS (10 min each), the sections were incubated with PI (1:1000, CalBiochem) for half an hour at room temperature. Then the sections were washed with PBS for 5 times (5 min each). Images were acquired using a confocal microscope (Leica).

Acknowledgements

This work was supported by grants from the National Major Basic Research Program (2004CB117401) and the Innovation Project of Chinese Academy of Sciences (KSCX2-YW-N-020).

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Edited by Makoto Asashima
Published Online: 3rd October 2007