Int. J. Dev. Biol. 49: 891-894 (2005)
doi: 10.1387/ijdb.052031tt

Expression of ADP-ribosylation factor (ARF)-like protein 6 during mouse embryonic development

TATSUYUKI TAKADA*[,1,2], KEIKO IIDA[1,2], HIROSHI SASAKI[3], MASANORI TAIRA[2,4] and HIROSHI KIMURA[1,2]

[1]Department of Experimental Radiology, Shiga University of Medical Science, Ohtsu, [2]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, [3]Laboratory for Embryonic Induction, RIKEN, Center for Developmental Biology, Chuo-ku, Kobe and [4]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo, Japan

ABSTRACT ADP-ribosylation factor (ARF)-like protein 6 (ARL6) is a member of the ARF-like protein (ARL) subfamily of small GTPases (Moss, 1995; Chavrier, 1999). ARLs are highly conserved through evolution and most of them possess the consensus sequence required for GTP binding and hydrolysis (Pasquallato, 2002). Among ARLs, ARL6 which was initially isolated from a J2E erythroleukemic cell line is divergent in its consensus sequences and its expression has been shown to be limited to the brain and kidney in adult mouse (Ingley, 1999). Recently, it was reported that mutations of the *ARL6* gene cause type 3 Bardet-Biedl syndrome in humans and that ARL6 is involved in ciliary transport in *C. elegans* (Chiang, 2004; Fan, 2004). Here, we investigated the expression pattern of *ARL6* during early mouse development by whole-mount *in situ* hybridization and found that interestingly, *ARL6* mRNA was localized around the node at 7.0-7.5 days post coitum (dpc) embryos, while weak expression was also found in the ectoderm. At the later stage (8.5 dpc) *ARL6* was expressed in the neural plate and probably in the somites. Based on these results, a possible role of ARL6 in early development is discussed in relation to the findings in human and *C. elegans* (Chiang, 2004; Fan, 2004).

KEY WORDS: *ADP-ribosylation factor (ARF)-like protein 6 (ARL6), small GTPase, embryonic development, node, organizer*

ADP-ribosylation factors (ARFs) have been reported to play an important role in intracellular membrane trafficking (Moss, 1995; Chavrier, 1999). Although ARF-like proteins (ARLs) are very similar to ARFs in amino acid sequences, their biological function remains unclear. ARL1 and ARL3 have recently been shown to be required for localization of GRIP-domain proteins to Golgi membranes (Lowe, 1996; Lu, 2001; Panic, 2003; Setty, 2003). In addition, the expression of *ARL4* and *ARL5* was found to be developmentally regulated (Lin, 2000; Lin, 2002; Schurmann, 2002). These observations suggest the possibility that the ARL proteins function in membrane traffic which might mediate some developmental processes. *ARL6* was first identified in a J2E erythroleukemic cell line. The *ARL6* transcript is up-regulated during erythropoietin-induced differentiation of erythroid cells and down-regulated during interleukin-6-induced macrophage differentiation, suggesting a possible role in hemopoietic development (Ingley, 1999). In adult mice, *ARL6* shows a tissue-specific expression pattern with the highest expression observed in the brain and kidney. In addition, yeast two-hybrid screening and co-immunoprecipitation reportedly show that ARL6 interacts with the protein-conducting channel subunit SEC61β (Ingley, 1999; Pettersson, 2000). However, its biological functions remain unclear. Recently, *ARL6* was identified as the gene that causes Bardet-Biedl syndrome type 3 (BBS3) (Chiang, 2004; Fan, 2004). Four different homozygous substitutions in the regions including the GTP binding domain in ARL6 were found to be involved in BBS3. BBS3 is a multisystemic disorder characterized by obesity, blindness, polydactyly, renal abnormalities and cognitive impairment. Similar to other BBS's, BBS3 is also thought to result from ciliary dysfunction because loss-of-function mutations of *ARL6* in *C. elegans* impair cilia structure and function (Blacque, 2004). The observations that *ARL6* is specifically expressed in ciliated cells including sensory neurons and involved in intraflagellar transport in *C. elegans* (Chiang, 2004), are good agreement with its involvement in BBS3.

We isolated *ARL6* cDNA during a screen for genes which show localized expression patterns in the early mouse embryo and found by RT-PCR that *ARL6* mRNA is expressed in the brain of 11.5 dpc embryos. To elucidate the developmental aspect of

Abbreviations used in this paper: ARF, ADP-ribosylation factor; ARL, ARF-like; dpc, days post coitum.

***Address correspondence to:** Dr. Tatsuyuki Takada. Research Center for Animal Life Science, Shiga University of Medical Science, Ohtsu, Shiga, 520-2192, Japan. Fax: +81-77-548-2414. email: ttakada@belle.shiga-med.ac.jp

0214-6282/2005/$25.00

Printed in Spain
www.intjdevbiol.com

ARL6 function further, we herein report the expression pattern of *ARL6* mRNA during early mouse embryonic development.

There are three domains conserved among ARL family members. These domains are thought to be involved in guanine nucleotide binding and hydrolysis (Moss, 1995). Comparison with other ARL members showed that ARL6 lacks some consensus amino acids in these domains; for instance, tryptophan and glycine in domain II and glutamine in domain III (Fig. 1A). These were substituted to phenylalanine, serine and methionine, respectively. We also cloned *Xenopus ARL6* from cDNA library and found that *Xenopus* ARL6 shows 88% identity with mouse ARL6 in deduced amino acid sequence. Importantly, the amino acids of these domains were completely conserved among the mouse, human and *Xenopus* (Fig. 1B). Therefore it is possible that these substitutions may relate to a specific function of ARL6 with regard to the GTP binding and hydrolysis. Furthermore, it is noted that mutations resulting in a nonconservative amino acid change in threonine 31 of domain I and other mutations in invariable glycine 169 and leucine 170 (Fig. 1B), have been found in families affected with BBS3, indicating the importance of these residues for its normal functions.

We performed whole mount *in situ* hybridization to characterize the expression pattern of *ARL6* during early mouse embryogenesis. Whereas no obvious expression of *ARL6* was detected at early to mid-streak stage (Fig. 2A), at the late streak stage, when the primitive streak reached to its distal end, we observed localized *ARL6* expression at the node, which is located at the anterior tip of the primitive streak and functionally corresponds to the gastrula organizer (Fig. 2B). This localized expression was still observed at early bud stage and weak signal was also detected throughout the embryonic portion (Fig. 2C). At this stage, the node consists of two germ layers: a dorsal layer that is continuous with the epiblast or ectoderm and a ventral layer that is continuous with the endoderm. Sagittal sectioning of this embryo revealed that the strong signal was localized to the ventral layer of the organizer and the weak signal was observed throughout the ectoderm (Fig. 2E). The similar expression pattern was maintained at early head fold stage, when the node became morphologically evident (Fig. 2D). At late head fold stage, strong expression of *ARL6* was not restricted to the node but was observed throughout embryonic portion (Fig 3 A,B). At later stage (somite stage, 8.5 dpc), *ARL6* expression was mainly observed in the neural plate and probably in the somites (Fig. 3 C,D). No obvious signal was observed in the extraembryonic tissues throughout the stages analyzed (6.5-8.5 dpc, Figs. 2 and 3). Because *ARL4* is reportedly expressed in the somites and at the junction of forebrain and midbrain at the 10-12 somite stage (8.5 dpc) (Lin, 2000), the localization of transcript in the neural plate is characteristic to *ARL6* at this stage.

Our data showed that *ARL6* displays a dynamic pattern of expression during early mouse development. Its localized expression at the node that plays a central role in establishing the basic body plan as the gastrula organizer implies possible involvement of this small GTPase in early mouse embryonic development. Interestingly, our whole mount *in situ* hybridization analysis revealed that the strong signal of *ARL6* expression was localized to the ventral layer of the node, which is distinguished by the presence of a single, motile, central cilium, showing good agreement with the observation that *ARL6* is expressed specifically in ciliated cells and mediate intraflagellar transport in *C. elegans*. However, the mutations of *ARL6* found in BBS3 did not seem to affect the body axis formation and left-

A

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domain I                domain III
ARL1   24 GLDGAGKT 31    ARL1   126 NKQD 129
ARL2   24 GLDAAGKT 31    ARL2   126 NKQD 129
ARL3   24 GLDNAGKT 31    ARL3   126 NKQD 129
ARL4   27 GLDCAGKT 34    ARL4   134 NKQD 137
ARL5   28 GLDSAGKT 35    ARL5   135 NKQD 138
ARL6   24 GLDNSGKT 31    ARL6   130 NKMD 133

domain II
ARL1   66 WDLGGQ 71
ARL2   66 WDVGGQ 71
ARL3   66 WDIGGQ 71
ARL4   74 WDVGGQ 79
ARL5   75 WDVGGQ 80
ARL6   68 FDMSGQ 73
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B

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                                        I
Mouse   MGLLDRLSGLLGLKKKEVHVLCLGLDNSGKTTIINKLKPSNAQSQDIVPTIGFSIEKFKS    60
Human   MGLLDRLSVLLGLKKKEVHVLCLGLDNSGKTTIINKLKPSNAQSQNILPTIGFSIEKFKS    60
Xenopus MGLFDKLAGWLGLKKKEVHVLCLGLDNSGKTTIINKLKPANAQTHDIVPTIGFSIEKFKT    60
        ***.*.*...*****************************.***...*.***********.

                II
SSLSFTVFDMSGQGRYRNLWEHYYKDGQAIIFVIDSSDKLRMVVAKEELDTLLNHPDIKH   120
SSLSFTVFDMSGQGRYRNLWEHYYKEGQAIIFVIDSSDRLRMVVAKEELDTLLNHPDIKH   120
SSLSFTVFDMSGQGRYRNLWEHYYKEGQAIIFVIDSSDKLRMVVAKEELETLLNHADIKH   120
**********************.***********.**********.*****.****

               III
RRIPILFFANKMDLRDSVTSVKVSQLLCLESIKDKPWHICASDAIKGEGLQEGVDWLQDQ   180
RRIPILFFANKMDLRDAVTSVKVSQLLCLENIKDKPWHICASDAIKGEGLQEGVDWLQDQ   180
RRMPVLFFANKMDLRDSLSSVKVSQLLSLENIKDKPWHICASDGLTGEGLQEGVDWLQDQ   180
**.*.***********...********.**.************...**************

IQAVKT                                                          186
IQTVKT                                                          186
IQTMKT                                                          186
**..**
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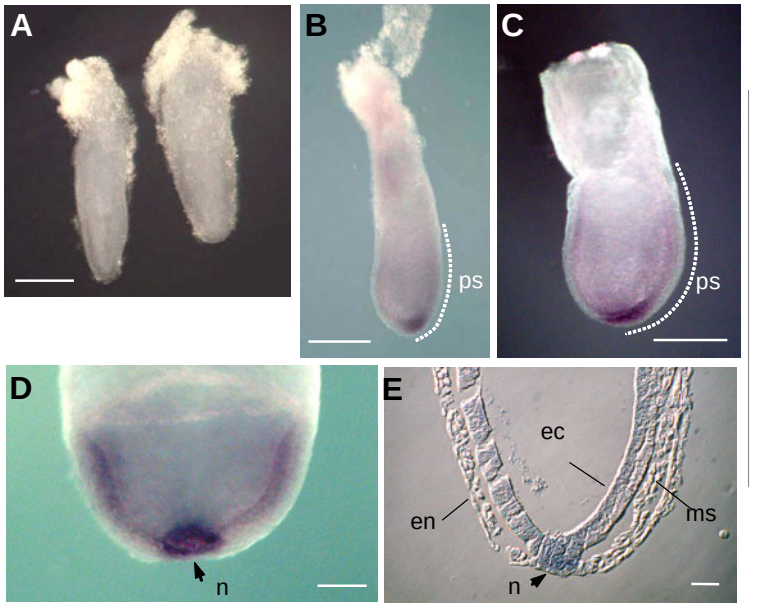
Fig. 1. Alignment of deduced amino acid sequence of ARL6. (A) *Amino acid alignment of conserved domains (I, II and III) supposedly involved in guanine nucleotide binding and hydrolysis in the mouse ARL family. Substituted amino acid residues are indicated by boldface.* **(B)** *Alignment of the deduced amino acid sequences of mouse, human and* Xenopus *ARL6. Conserved domains (I, II and III) are boxed. Arrowheads indicated mutated amino acid residues found in BBS3 patients. Accession numbers: AB232697 (mouse), Q9H0F7 (human) and AB232698* (Xenopus).



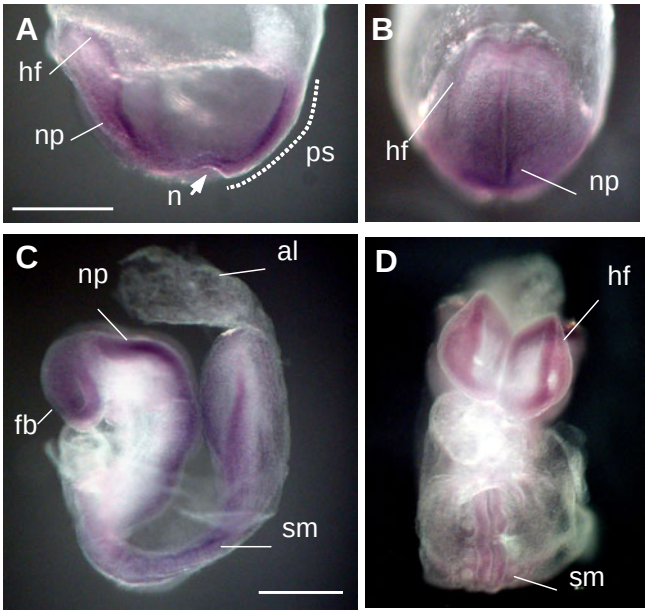


Fig. 2 (Left). Localization of *ARL6* mRNA in 6.0-7.5 dpc mouse embryos. (A) *Early to mid-streak stage embryos. Lateral view of late streak stage* **(B)**, *early bud stage* **(C)**. *Anterior view of early head fold stage embryo* **(D)**. **(E)** *Sagittal section of early bud stage embryo stained for* ARL6. *Scale bars indicate 150μm (A), 200μm (B,C), 100μm (D) and 50μm (E). Abbreviations: ec, ectoderm; en, endoderm; ms, mesoderm; n, node; ps, primitive streak.*

Fig. 3 (Right). Localization of *ARL6* mRNA in 8.0-8.5 dpc mouse embryos. *Late head fold stage embryo, lateral view (anterior to the left)* **(A)**, *anterior view* **(B)**. *Somite stage embryo (8.5 dpc), lateral view (anterior to the left)* **(C)**, *anterior view* **(D)**. *Scale bars indicate 200μm (A,B). Abbreviations: al, allantois; fb, fore brain; hf, head fold; n, node; np, neural plate; ps, primitive streak; sm, somite.*

right patterning, which are deeply related to the organizer activity and the function of nodal cilia, respectively (Essner, 2002; Nonaka, 2002; Watanabe, 2003). Therefore, it would be worthwhile to investigate the function of ARL6 in the ciliary transport in relation to the nodal cilia and left-right asymmetry and also to tissue differentiation of the CNS and somites by generating *ARL6* knockout mice.

Experimental Procedures

Mouse *ARL6* cDNA was amplified with RNA from 11.5 dpc embryos by RT-PCR using primers, 5'-cctttggattggcgtcaaagatcag-3' and 5'-cactgaggtctccagggactatctc-3' and cloned into pBluescript KS(+) plasmid. Thirty cycles of PCR were carried out at 94ºC for 30 sec, 55ºC for 1 min and 72ºC for 1 min. *Xenopus ARL6* cDNA was also amplified from a cDNA library (stage 17/18) by PCR using primers 5'-cgggatccaccatgggattgtttgacaag-3' and 5'-ccgctcgagttactgcagggtgtcttcatc -3' and cloned into pCS107 plasmid (a gift from R Harland). Inserts were sequenced on both strands. Mouse embryos were collected from ICR (CLEA, Japan). Noon of the day on which a vaginal plug was observed was considered 0.5 dpc. Staging of mouse embryos were performed by their morphology (Downs, 1993). Embryos were fixed overnight at 4ºC in 4 % paraformaldehyde, dehydrated through a graded ethanol series then stored at -20ºC until use. Whole mount *in situ* hybridization was performed as described (Wilkinson, 1992). The full-length *ARL6* cDNA (KS+ARL6) was used to generate digoxygenin-labeled sense or antisense riboprobe by transcribing using T7 or T3 RNA polymerase, respectively and digoxygenin-labeling RNA mix (Roche) according to the manufacturer's protocol. All probes were run on a 2% agarose/formaldehyde gel before use to verify yield and length. After whole mount staining, some late streak stage embryos were subsequently embedded in paraffin and sectioned at 7 μm to further characterize the expression pattern. Images were captured with a digital camera and imported into Adobe Photoshop for assembly of the final figures.

Acknowledgements

We thank H. Takano, T. Yamamoto and M. Suzaki for helpful suggestion and technical assistance. This work was supported in part by grants from the Ministry of Education, Science, Sports and culture (14580798).

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Received: March 2005
Reviewed by Referees: April 2005
Modified by Authors and Accepted for Publication: May 2005
Edited by: Makoto Asashima