Rab11-FIP4 is predominantly expressed in neural tissues and involved in proliferation as well as in differentiation during zebrafish retinal development

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Abstract

Rab11 family interacting protein 4 (Rab11-FIP4) was initially identified in humans as an Rab11-binding protein, but its biological function has remained unknown. We cloned the zebrafish orthologue of Rab11-FIP4 (zRab11-FIP4) and analyzed its function in vivo by using antisense morpholino. zRab11-FIP4 was expressed as 2 alternative transcripts, i.e., the longer A-form predominantly expressed in neural tissues and the shorter B-form expressed ubiquitously; and in situ hybridization revealed that the A-form was the dominant form. In the developing retina, zRab11-FIP4 was expressed in progenitors throughout the retina at early stages; and then, along with the differentiation, the expression became gradually restricted to the ganglion cell layer and ciliary marginal zone. zRab11-FIP4A knockdown embryos exhibited eye phenotypes similar to those of the shh mutant, such as a small eye with impaired cell proliferation and the delay in cell-cycle exit and differentiation of retinal progenitors. The lack of induction of p57kip2 and enhanced expression of cyclin D1 were observed in the morphant retina. Importantly, the delay in cell-cycle exit was rescued by ectopic expression of either p57Kip2 or dominant-negative PKA, suggesting that Rab11-FIP4A plays pivotal roles in retinal development by regulating Shh signaling and a mechanism acting in parallel with Shh signaling in the control of cell-cycle exit. © 2006 Elsevier Inc. All rights reserved.

Keywords: Rab11-FIP4; Retina; Zebrafish; Proliferation; Differentiation

Introduction

In an effort to identify genes spatiotemporally regulated in the developing eye, we recently isolated a mouse homologue of human Rab11 family interacting protein 4 (Rab11-FIP4) by differential display as a gene whose expression was temporally controlled during eye development (manuscript in preparation). Rab11-FIP4 is known to be a Rab11/Arf5-binding protein (Wallace et al., 2002b) and was also isolated by other groups as KIAA1821 (Nagase et al., 2001) and Arfophilin2 (Hickson et al., 2003). Rab11 is a member of the Rab family of GTPases and has been implicated in regulating diverse pathways of vesicle trafficking including protein recycling from endosomes to plasma membrane and protein transport from endosomes to the trans-Golgi network (Ullrich et al., 1996; Wilcke et al., 2000). The involvement of Rab11 in cytokinesis was also demonstrated (Cheng et al., 2002). Arf5 is a member of the family of ADP-ribosylation factors (Arfs) and has been structurally categorized with Arf4 as class II Arfs (Shin et al., 1999; Shin et al., 2001). Although Arfs have also been demonstrated to play roles in regulating membrane trafficking, the function specific to these class II Arfs has been rarely investigated (Balch et al., 1992). The biological effects of activated (GTP-bound) proteins are thought to be mediated by their interaction with the downstream effector molecules specific to each GTPase (Meyers and Prekeris, 2002; Shin et al., 1999; Wallace et al., 2002a), and some Rab11- and Arf5-binding proteins were shown to be actually involved in vesicle trafficking (Cullis et al., 2002; Hickson et al., 2003; Mamamoto et al., 1999). Interestingly, recent advances in membrane trafficking and developmental biology fields suggest that molecules or signaling pathways involved in various steps of development interact with and are regulated by components of the membrane trafficking machinery (Seto et al., 2002; Stewart, 2002).

Although much knowledge has been accumulated regarding the mechanism of action of Rab11 and its interacting molecules, the physiological role of Rab11-FIP4, especially in the nervous
system, is not yet known. Therefore, in the present study, we analyzed the biological roles of Rab11-FIP4 in the zebrafish (Danio rerio). The zebrafish is a useful model animal to analyze vertebrate eye development since the structure and developmental mechanisms of the eye have been evolutionally conserved among the vertebrates. In addition, development is very rapid as the layer formation and differentiation of retinal cells are almost completed within 3 days after fertilization (Bilotta and Saszik, 2001). Furthermore, it has recently become practicable to perform gene knockdown experiments in developing zebrafish embryos by injecting morpholino-based antisense oligonucleotide (MO) into fertilized eggs (Nasevicius and Ekker, 2000).

In the work presented here, we isolated the Rab11-FIP4 gene from zebrafish and showed that it was predominantly expressed in neural tissues including the retina. This gene was found to be highly conserved among species with respect to its structure and expression pattern, suggesting that Rab11-FIP4 plays an important role in retinal development. This possibility was assessed by conducting a loss-of-function experiment using zebrafish embryos and a morpholino antisense oligo (MO) specific to zebrafish (z) Rab11-FIP4. Our results indicate that this gene is involved in regulating the proliferation and differentiation of the retinal cells during development.

Materials and methods

Animals

Zebrafish (D. rerio) originally purchased from a local pet shop were maintained in the laboratory on a 13-h light/11-h dark cycle. Fertilized eggs were maintained in the laboratory on a 13-h light/11-h dark cycle. Fifty hpf embryos were used for microinjection (IM-300, Narishige) as described elsewhere (Kurita et al., 2003). Induction of the heat shock promoter was done by incubating the DNA-injected embryos at 39°C for 1 h from 50 hpf. For the proliferation assay, the embryos were injected with BrdU at 50 hpf and fixed at 56 hpf. For the differentiation analysis, embryos were fixed at 60 hpf. Embryos were observed under a fluorescence dissecting microscope (MZFL III, Leica).

In situ hybridization

For in situ hybridization, digoxigenin (DIG)-labeled RNA probes were used. All cDNAs used as templates to prepare the RNA probes were isolated by RT-PCR and subcloned into the PGEM-T easy vector (Promega). DIG-labeled sense or antisense RNA probes were synthesized by using a DIG RNA labeling kit (Roche Diagnostics GmbH). Whole-mount in situ hybridization of zebrafish was performed as described previously (Kurita et al., 2003). In situ hybridization of frozen sections was performed according to the standard method. Briefly, cryosections rehydrated with PBS were sequentially treated with HCl and Proteinase K and then acetylated in acetic anhydrate solution. The pretreated samples were next hybridized at 60°C with DIG-labeled RNA probe, and the probe was detected with an anti-DIG antibody-conjugated to alkaline phosphatase and visualized by using a BCIP/NBT solution kit (Nacalai Tesque Inc.).

BrdU, apoptosis assays and immunohistochemistry

BrdU solution was microinjected into zebrafish embryos as described previously (Kurita et al., 2003). After incubation for 2–20 h (refer to each figure legend for actual incubation time), these embryos were fixed, sectioned and stained with the anti-BrdU antibody (Roche Diagnostics GmbH). Apoptotic cells were examined by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay by using an in situ apoptosis detection kit (TAKARA BIO Inc, Shiga, Japan). Frozen sections were used for immunohistochemistry as described previously (Kurita et al., 2003). Primary antibodies used in this study were as follows: anti-Hu (Molecular Probes, Inc., 500-fold dilution), anti-BrdU (Roche Diagnostics GmbH, 15-fold dilution), zn-5 (Developmental Studies Hybridoma Bank [DSHB], 200-fold dilution), zpr-1 (DSHB, 200-fold dilution) and anti-phospho-histone H3 (Upstate Biotechnology Inc., 1000-fold dilution). The immunoreactivity was visualized by using anti-mouse or anti-rabbit secondary antibodies conjugated to either Alexa488 or Alexa546 (Molecular Probes, Inc.).

Cell count

The growth of retina and the ratios of S- and M-phase cells to total retinal cells were scored by counting the number of cells in transverse retinal sections cut through the lens. At least 15 sections from 3 embryos were examined for each sample.

Results

Isolation of zebrafish orthologue of Rab11-FIP4

Rab11-FIP4 was originally identified in humans as a binding partner of Rab11 (Wallace et al., 2002b). By differential display

was used as a control-MO. MOs were prepared at a concentration of approximately 3 μg/μl in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, and 5.0 mM HEPEs [pH 7.6]) for microinjection. pBS[hsntPKA/EGFP] (a kind gift from Dr. J. Masai and Dr. R. T. Moon) and pGFP[HSE:p57kip2] (a kind gift from Dr. C. Neumann) were used to express dominant-negative PKA and p57kip2, respectively, under the control of a heat shock promoter. Synthesized mRNA, plasmid DNA and MOs were injected into the yolk of 1- to 8-cell stage fertilized zebrafish eggs by using a microinjector (IM-300, Narishige) as described elsewhere (Kurita et al., 2003). Induction of the heat shock promoter was done by incubating the DNA-injected embryos at 39°C for 1 h from 24 hpf. For the proliferation assay, the embryos were injected with BrdU at 50 hpf and fixed at 56 hpf. For the differentiation analysis, embryos were fixed at 60 hpf. Embryos were observed under a fluorescence dissecting microscope (MZFL III, Leica).

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Cell count

The growth of retina and the ratios of S- and M-phase cells to total retinal cells were scored by counting the number of cells in transverse retinal sections cut through the lens. At least 15 sections from 3 embryos were examined for each sample.

Results

Isolation of zebrafish orthologue of Rab11-FIP4

Rab11-FIP4 was originally identified in humans as a binding partner of Rab11 (Wallace et al., 2002b). By differential display
using mouse neural tissues, we recently cloned mouse Rab11-FIP4 as a gene predominantly expressed in embryonic neural tissues (manuscript in preparation), but its function is still unknown. To examine the function of Rab11-FIP4 by using zebrafish (D. rerio) as a model system, we isolated the zebrafish orthologue of this gene. We first searched zebrafish EST databases (GenBank, NCBI) for sequences homologous with the mouse and human Rab11-FIP4 sequences, but we did not
find any candidate gene. We therefore isolated the zebrafish orthologue of Rab11-FIP4 (zRab11-FIP4) cDNA by RT-PCR by using degenerate primers in combination with 5′- and 3′-RACE. The isolated zebrafish cDNA contained a single long ORF of 1863 bp encoding a deduced protein of 621 amino acid residues. Overall identities of this protein to mouse (m) and human (h) Rab11-FIP4 proteins were 69% and 68%, respectively, at the amino acid level (Fig. 1A). A motif search identified a single EF-hand motif (a Ca²⁺-binding domain) and an Rab11-binding domain (RBD) in regions proximal to the N- and C-termini, respectively (Fig. 1A). C-terminal half region was predicted to form a coiled-coil structure, and 2 leucine-zipper-like motifs were identified within this region. All these characteristic features are conserved in human Rab11-FIP4. Chromosomal localization determined by radiation hybrid mapping indicated this gene to be located in a region on LG3 syntenic to human and mouse chromosomal regions where the Rab11-FIP4 gene was mapped (data not shown), further suggesting the isolated gene to be the zebrafish orthologue of Rab11-FIP4.

In the process of full-length cDNA cloning, we also identified another type of 5′-RACE product, revealing an alternative form of zRab11-FIP4 transcript. This alternative transcript, designated as B, had 295 bp of unique 5′-sequence instead of 464 bp of that of the originally isolated form, designated as A. A putative initiation codon was not found in this gene to be located in a region on LG3 syntenic to human and mouse chromosomal regions where the Rab11-FIP4 gene was mapped (data not shown), further suggesting the isolated gene to be the zebrafish orthologue of Rab11-FIP4.

The expression pattern of zRab11-FIP4 in developing zebrafish embryos

The expression pattern of zRab11-FIP4 in adult zebrafish examined by RT-PCR indicated that the A-form was expressed specifically in neural tissues, whereas the B-form was detected in them as well as in others (Fig. 1B). These patterns appeared to be comparable to those found in the adult mouse (manuscript in preparation). The spatial and temporal expression of zRab11-FIP4 in developing zebrafish embryos was then examined by whole-mount and tissue section in situ hybridization using a DIG-labeled antisense RNA probe prepared from the full-length cDNA (Figs. 1C, E). zRab11-FIP4 expression was readily detected at the bud stage (10 hpf) in the most anterior region of the neural plate, from which the neural retina is derived; and faint signals were observed in the entire neural keel (Fig. 1C). In the following few hours, optic primordia are formed by bilateral evagination of a part of the diencephalon. Along with this evagination, the expression of zRab11-FIP4 also bilaterally extended into the optic primordia at the 8-somite stage (13 hpf; Fig. 1C). At this stage, the zRab11-FIP4 transcript was detected throughout the optic primordia, with a relatively higher level on the outer side. The expression of zRab11-FIP4 in the midline region of the presumptive hypothalamus was still retained as well. From 18 to 26 hpf, zRab11-FIP4 was specifically expressed in most neural tissues, but the expression level was low (Fig. 1C and data not shown). However, by 30 hpf, just after the onset of differentiation of the retinal ganglion cells (RGCs), the expression of zRab11-FIP4 became more evident in the region surrounding the ventricles in the midbrain and diencephalon as well as in the retina (Fig. 1C). To determine whether the neural-tissue-specific expression pattern observed by in situ hybridization was due to the A-form transcript or the B-form one, we also conducted in situ hybridization using probes specific for either A- or B-form. Only the A-form was detected with the pattern comparable to that shown by using the common probe (Fig. 1D).

A more precise examination of the expression pattern at later stages in the developing eyes was done by using tissue sections (Fig. 1E). Consistent with the results described above, the expression of zRab11-FIP4 was detected throughout the retina by 30 hpf (Fig. 1C). However, as development progressed, the expression of zRab11-FIP4 became gradually decreased on the outer side of the central retina and was restricted by 48 hpf to the RGC layer (GCL) as well as to the inner half of the inner nuclear layer (INL), where amacrine cells reside (Fig. 1E). It is notable that the expression of zRab11-FIP4 was also observed even at 104 hpf in the peripheral region (the ciliary marginal zone [CMZ]) of the zebrafish retina (Fig. 1E), a region in which proliferation and subsequent differentiation of retinal cells occur throughout life (Perron and Harris, 2000).

Functional analysis of zRab11-FIP4 in developing embryos

To analyze the function of zRab11-FIP4, we first examined the effect of ectopic expression of zRab11-FIP4 by mRNA injection. Control-EGFP-injected embryos grew normally up to 3 dpf (Table 1 and data not shown). Embryos coinjected with zRab11-FIP4A mRNA (300 pg) and EGFP mRNA as a tracer developed without any defects before the onset of epiboly, whereas at later stages these embryos failed to undergo epiboly, and the migration of deep cells of the blastoderm toward the...
Table 1
Effect of Rab11-FIP4 expression in zebrafish embryos

<table>
<thead>
<tr>
<th>RNA injected (pg)</th>
<th>Percentage (no.) of surviving embryos</th>
<th>6 hpf</th>
<th>12 hpf</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>zRab11-FIP4A (300)</td>
<td>100.0 (41)</td>
<td>4.9 (2) (^a)</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>zRab11-FIP4A (75)</td>
<td>n.t.</td>
<td>73.9 (17) (^b)</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>zRab11-FIP4A (25)</td>
<td>n.t.</td>
<td>98.4 (61)</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>zRab11-FIP4B (300)</td>
<td>100.0 (41)</td>
<td>97.5 (40)</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>zRab11-FIP4AΔRBD (300)</td>
<td>100.0 (21)</td>
<td>0.0 (0)</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>No injection (300)</td>
<td>100.0 (41)</td>
<td>100.0 (41)</td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>

Note. Two- to 8-cell stage embryos were injected with the indicated mRNA. “n” indicates the total number of analyzed embryos.

\(^a\) The embryos exhibited abnormal morphology (Supplementary Fig. 1) and did not survive beyond 24 hpf.

\(^b\) Eleven among 17 embryos developed abnormally. Among the 11 embryos, 5 embryos died before 24 hpf and the rest showed abnormal morphology including tail truncation and thickness of the yolk sac extension at later stage.

Effect of Rab11-FIP4 expression in zebrafish embryos

- zRab11-FIP4A was fused in frame with the EGFP-coding region. mRNA synthesized in vitro from the fusion gene was injected into fertilized eggs with control-MO or either of the zRab11-FIP4A-MOs. Standard control-MO available from the manufacturer was used as a negative control. EGFP fluorescence observed in the control-MO-injected embryos was abolished in embryos injected with either one of the zRab11-FIP4-MOs (Fig. 2B), indicating that these MOs were able to repress the expression of the target gene effectively.

Next, we examined the biological effects of zRab11-FIP4A knockdown by the MO injection on the development of zebrafish embryos. Until 20 hpf, no apparent defect in the morphology of the embryos injected with zRab11-FIP4-MO1 was observed (data not shown). At 25 hpf, however, the zRab11-FIP4-MO1-injected embryos displayed a head of slightly smaller size (Fig. 2C). There was no apparent difference in the number of somites, size or morphology of the body or onset of heart beats, indicating that the phenotype would not be due to a simple delay of development caused by some non-specific effect of the zRab11-FIP4-MO1 injection (data not shown). At 36 hpf, 88.7% (n = 159) of the embryos injected with zRab11-FIP4-MO1 exhibited the apparently small eye and head phenotypes (Fig. 2C). The severity of the phenotypes became more significant at later stages; and, in addition, small pectoral fins and jaw defects were also found in these embryos at 60 hpf, but the size of the trunk or tail was not notably affected (Fig. 2C and data not shown). To confirm the specificity of these effects, we used the 2nd MO designed to be complementary to a distinct region of the zRab11-FIP4A and observed that the phenotypes of zRab11-FIP4-MO2-injected embryos were similar to those induced by zRab11-FIP4-MO1 injection (73%, n = 259, data not shown).

Early retinal and region-specific brain markers expressed normally in zRab11-FIP4-MO-injected embryos

To address whether the defects of retinal development observed in zRab11-FIP4 morphants at later stages were caused by earlier patterning defects, we examined the expression of several retinal and brain markers (Fig. 2D). Zebrafish embryos injected with either zRab11-FIP4-MO1 or cont-MO were fixed at the 18-somite stage (18 hpf) and then subjected to whole-mount in situ hybridization using DIG-labeled antisense RNA probes. Rx1, six6, pax6, vsx2 and mab21L2 are all expressed in early-stage optic vesicles and involved in retinal development (Kawahara et al., 2002; Levine and Green, 2004; Stenkamp et al., 2002). Mab21L2 is also expressed in the midbrain. Eng2, wnt1, krox20 and pax2.1 are region-specific brain markers; and patterning defects in the neural keel have been shown to lead to the perturbed expression of these genes (Wurst and Bally-Cuif, 2001). In control embryos, wnt1 and eng2 were expressed at the midbrain–hindbrain boundary (MHB). Pax2.1 was expressed not only at the MHB but also in the optic vesicles and optic stalks. Krox20 is a specific marker for rhombomeres 3 and 5. We found that the expression patterns of all genes examined were comparable in both zRab11-FIP4 morphants and control embryos, indicating that zRab11-FIP4A was not necessary for
the regionalization of the central nervous system or optic vesicle formation (Fig. 2D).

**zRab11-FIP4 plays a role in proliferation of early retinal progenitors**

A decrease in the size of eyes in the zRab11-FIP4-MO1-injected embryos (zRab11-FIP4 morphants) suggested that zRab11-FIP4A was involved in proliferation and/or cell death of retinal cells. We first examined whether the viability of the retinal cells was impaired by zRab11-FIP4-MO1 injection. Since the small eye and head phenotypes in the morphants became apparent between 24 and 36 hpf, we conducted the TUNEL assay using embryos at 27, 30 and 34 hpf (Fig. 3A). An excess number of TUNEL-positive cells was observed in the hindbrain and spinal cord of the morphant but not in the retina.
Fig. 3. Impaired proliferation of retinal progenitors in zRab11-FIP4 morphants. (A) Cell death in the MO-injected embryos. Embryos injected with either control-MO (left column) or zRab11-FIP4-MO1 (right column) were subjected to the TUNEL assay using whole embryos (left panel) and tissue sections (right panel). (Left panel) Embryos fixed at 27, 30 and 34 hpf were analyzed. Excess cell death in the hindbrain region of the morphants is marked with brackets. (Right panel) Transverse sections prepared from control and the morphants at 30 hpf were analyzed. Sections at the levels of the eye (Ey) and hindbrain (HB) were used, and the positions are indicated in the left panel. (B) Increase in the retinal cell number during development as estimated by counting the cells in tissue sections. The cell number/retinal section at each time was calculated as the average of numbers scored from at least 17 (control, closed circles) or 23 (zRab11-FIP4 morphants, open squares) sections prepared from 4 to 5 embryos. Standard deviations are indicated as error bars. The differences are statistically significant at 30 and 36 hpf ($P < 0.001$ in each case) but not at 24 hpf ($P > 0.1$). (C) Proliferating cells in the retina were examined by a long-term incorporation of BrdU from 28 to 48 hpf. (D) Retinal cells in the S-phase were labeled by short-term (2 h) BrdU incorporation starting at either 24 or 30 hpf. (Upper panel) Transverse sections were immunostained with the anti-BrdU antibody. (Lower panel) The number of BrdU+ cells was counted, and the ratio to total cells was scored. Standard deviations are indicated as error bars. (E) Mitotic cells in the retina were immunostained with an anti-phospho-histone H3 antibody. (Upper panel) Immunostained mitotic cells in transverse sections are seen. (Lower panel) Ratios of mitotic cells (pHisH3+) to total cells in retinal sections of 24- and 30-hpf embryos were scored. In panels D and E, the differences between control and zRab11-FIP4 morphants are statistically significant at 30 hpf (BrdU; $P = 0.01$, pHisH3; $P = 0.008$) but not at 24 hpf (BrdU; $P = 0.86$, pHisH3; $P = 0.75$). Scale bars indicate 50 μm.
during this period, thereby suggesting that cell death may have contributed to the small head but not to the small eye phenotype induced by the injection of zRab11-FIP4-MO1. However, at 52 hpf, an increased level of apoptosis was observed in the morphant retina (Supplementary Fig. 2).

Next, we counted the number of retinal cells in transverse sections cut through the lens. At 24 hpf, comparable numbers of cells were scored in both control and the zRab11-FIP4 morphant retinas; whereas the rate of increase in retinal cell number during the following 12 h was lower in the morphants than in the control embryos (Fig. 3B). In spite of the slower increase in cell number, when examined by the BrdU incorporation assay using a long labeling protocol (20 h from 28 to 48 hpf) to detect all the proliferating cells, most retinal cells were found to be proliferating even in the morphant retina; and the proportion of proliferating cells to the total retinal cells in the morphant was equivalent to that in the control (Fig. 3C). These results imply that such a slower increase in the cell number of the morphant retina may have been due to a slower progression of the cell cycle rather than to a smaller proportion of proliferating cells.

We next examined the relative length of S- and M-phases by a combination of short-term (2 h) labeling with BrdU and immunostaining for an M-phase marker, phospho-histone H3 (pHisH3). Labeling with BrdU for 2 h from either 24 hpf or 30 hpf in control embryos showed a decrease in BrdU-positive cell population by about 25% during this period (Fig. 3D). In contrast, the population of M-phase (pHisH3+) cells was increased in the control retina during the same period (Fig. 3E). Consistent with these observations, it has been reported that the proliferation rate of retinal cells in normal zebrafish embryos accelerates during early development (22–27 hpf), conceivably due to a shortening of the length of the S-phase (Li et al., 2000). However, such changes in the relative numbers of S- and M-phase cells were not observed in the morphant retina (Figs. 3D and E), suggesting that zRab11-FIP4A is likely to play a role in cell-cycle progression by regulating the length of the S-phase during an early stage of retinal development.

**Cell-cycle exit and differentiation of retinal cells delayed in zRab11-FIP4-MO-injected embryos**

During normal development of the zebrafish retina, all cells except those in CMZ completely cease proliferation by 60 hpf (Fig. 4A). However, cells in the zRab11-FIP4 morphant retinas continued proliferation for an extended period; and a significant number of proliferating cells (BrdU+ or pHisH3+) were still present in the central region of the retina at 60 hpf (Fig. 4A), suggesting that the timing of cell-cycle exit was also affected in the zRab11-FIP4 morphant retina. To explore the molecular mechanisms involved, we examined the expression of cyclin D1 (Masai et al., 2005) and p57kip2 cdk inhibitor (Shkumatava and Neumann, 2005), which are positive and negative regulators of proliferation, respectively (Fig. 4B). In the control embryos, the expression of cyclin D1 was detected throughout the retinal epithelium at 37 hpf, but only in the CMZ at 45 hpf. In contrast, in the morphant retina, the transcript was still expressed throughout the retina at 45 hpf. p57kip2 was detected at the central part of the retina in the control embryos, where the newly differentiated neurons were located, at 37 hpf. As differentiation proceeded, the domain of p57kip2 expression moved peripherally and the transcript was detected only in the region immediately adjacent to CMZ. However, p57kip2 expression was significantly impaired in the morphant retina; and low level of expression was barely detected only in the central region at 45 hpf. These results suggest that zRab11-FIP4A may regulate the timing of cell-cycle exit through the expression of these cell-cycle regulators.

Next, we analyzed the differentiation of retinal cells in zRab11-FIP4-MO-injected embryos by immunostaining with antibodies specific for retinal subpopulations. Staining with monoclonal antibody 16A11 (an anti-Hu C/D antibody) showed a small number of RGCs to be present in the innermost layer of the central retina in both control embryos and zRab11-FIP4 morphants at 36 hpf (Fig. 5A). Although the differentiation of RGC gradually increased as development progressed, the number of RGC was always lower in the zRab11-FIP4 morphant retinas than in the control retinas (Fig. 5A).

Using the same antibody, we also found that amacrine cells, which were readily detected at 48 hpf in the control retina, first...
appeared at 60 hpf in the morphant retina (Fig. 5B). The delayed differentiation of amacrine cells was further confirmed by using an anti-syntaxin antibody (Kurita et al., 2003), which also recognizes amacrine cells (data not shown). Moreover, the differentiation of photoreceptors was significantly retarded by the depletion of zRab11-FIP4A, which was shown by staining...
with antibodies zpr-1 (Fig. 5B) and zpr-3 (data not shown), which recognize the red/green double cone photoreceptors and rod photoreceptors, respectively.

To analyze the retarded differentiation of RGCs in more detail, we examined by the whole-mount in situ hybridization the expression of ath5, which is the earliest RGC marker and known to be prerequisite for RGC differentiation, and islet1, which is an early neuronal marker known to be expressed in RGC (Masai et al., 2000). Ath5 expression in the control retina was initially detected in the ventronasal region adjacent to the choroidal fissure at 26 hpf and then spread to the dorsal and temporal areas by 30 hpf (Fig. 5C). At 34 hpf, the expression level became higher; and ath5 transcripts were detected throughout the retina except in the marginal region (Fig. 5C). In the zRab11-FIP4 morphant retina, ath5 was expressed in the ventronasal region at 26 hpf, indicating that the onset of the ath5 expression was not affected by downregulation of zRab11-FIP4A (Fig. 5C). Nevertheless, the spread of ath5 expression from the ventral to dorsal region was significantly repressed even at 34 hpf (Fig. 5C). The expression of islet1 was earlier shown to start in the ventronasal region a little after the ath5 expression and to spread with a fan-shaped pattern similar to that of ath5 (Masai et al., 2000). In zRab11-FIP4 morphants, the islet1 expression was severely affected and first detected at 34 hpf (Fig. 5C). Thus, zRab11-FIP4A is likely to play a role in the progression but not initiation of the neural differentiation of the RGC. Despite the significant delay in differentiation, immunostaining with the monoclonal antibody zn-5, which specifically recognizes cell bodies and axons of RGCs, demonstrated that the RGCs showed normal maturation in the zRab11-FIP4 morphants with respect to the extension of axons and the formation of the optic chiasm (Fig. 5D).

Rescue of the morphant phenotypes by expression of either p57Kip2 or dnPKA

As demonstrated above, cell-cycle exit as well as differentiation was significantly retarded in the zRab11-FIP4 morphant retina. The expression of cell-cycle inhibitor p57Kip2 was also impaired, raising the question as to whether these phenotypes were rescued by the expression of p57Kip2. To address this question, we overexpressed p57Kip2 by using a plasmid containing heat-shock-inducible promoter (hsp) (Fig. 6A). The plasmid used (GFP:HSE:p57Kip2) had a bidirectional hsp, which drives the expression of both GFP and p57Kip2 (Shkumatava and Neumann, 2005), and so we could detect the p57Kip2-expressing cells by GFP expression. Either this plasmid or a plasmid containing hsp-GFP alone, as a control, was coinjected with zRab11-FIP4-MO into 1- to 4-cell embryos. Differentiation of photoreceptors was examined by immunostaining with zpr-1 at 60 hpf. Zpr-1/GFP double-positive cells are pointed out by arrowheads. Scale bars indicate 50 μm.

We also found that p57Kip2-expressing cells formed radially extended clusters of EGFP-positive cells, but the proportion of cells exiting cell cycle (BrdU-negative cells) were varied among clusters (Fig. 6A). These results therefore suggest that the defects in the cell-cycle exit in the zRab11-FIP4 morphant retina were mediated at least in part by the impaired expression of p57Kip2.

Since the phenotypes of the zRab11-FIP4 morphant retinas were similar to those of the sonic hedgehog (shh) mutant zebrafish (Neumann and Nuesslein-Volhard, 2000; Shkumatava et al., 2004; Stenkamp et al., 2002), we surmised the possibility of involvement of Shh signaling modification in the zRab11-FIP4A morphant. Shh signaling is known to be negatively regulated by protein kinase A (PKA) activity (Hammerschmidt Fig. 6. Co-expression of p57kip2 or dnPKA with zRab11-FIP4 morpholino. (A, B) The cell cycle was analyzed by BrdU incorporation. Zebrafish embryos were injected with zRab11-FIP4-MO1 in combination with a plasmid to express p57Kip2/EGFP, dnPKA-EGFP or EGFP alone (control) under the regulation of the heat shock promoter. These genes were induced by incubating at 39°C for 1 h (24 to 25 hpf), and proliferating retinal cells were then examined by labeling with BrdU for 6 h (50–56 hpf). Immunostaining patterns with anti-BrdU and GFP are shown in panel A, and GFP-positive cells that had exited from the cell cycle are indicated by arrowheads. L, lens. The ratio of BrdU/GFP double-positive cells to total GFP-positive cells is shown in panel B. The difference between the control and either p57Kip2 or dnPKA-expressing samples is statistically significant (P < 0.001 in each case). (C) A plasmid GFP:HSE:p57Kip2 was coinjected with zRab11-FIP4-MO1 or control-MO into 1- to 4-cell embryos. Differentiation of photoreceptors was examined by immunostaining with zpr-1 at 60 hpf. Zpr-1/GFP double-positive cells are pointed out by arrowheads. Scale bars indicate 50 μm.
et al., 1996; Unger and Moon, 1996). A previous report demonstrated that, in zebrafish embryos, the phenotypes induced by ectopic expression of Hedgehog (Hh) were mimicked by the ectopic expression of a dominant-negative form of PKA (dnPKA) without induction of the ligand expression (Unger and Moon, 1996). Therefore, we examined whether the activation of the Shh signaling by dnPKA could rescue the defects in the retinal development of the morphant. dnPKA-EGFP fusion gene (Unger and Moon, 1996) was expressed, and the proliferation of the retinal cells was examined by BrdU incorporation (50–56 hpf) assay using the same method for p57Kip2 (Figs. 6A and B). We found that most of dnPKA-expressing retinal cells were localized as scattered single cells and failed to incorporate BrdU (Figs. 6A and B), indicating that activation of the Shh signaling by dnPKA activity could rescue the delayed cell-cycle exit in the zRab11-FIP4 morphant retina.

We next examined whether p57Kip2 could reverse the delay of photoreceptor differentiation by immunohistochemistry. However, we could not find zpr-1-reactive cells among p57Kip2-expressing retinal cells in the zRab11-FIP4 morphant (Fig. 6C). Similar results were obtained in the case of cells expressing zpr-3 antigen (data not shown). These results suggest a separate mechanism independent of p57Kip2 to regulate photoreceptor differentiation in the morphant. In a series of experiments, dnPKA also failed to reverse the delayed photoreceptor differentiation. However, in contrast to the case of p57Kip2 expression, dnPKA-expressing retinal cells did not induce the photoreceptor differentiation even in the control embryos regardless of localization at ONL (Supplementary Fig. 3).

Discussion

Function of zRab11-FIP4A in retinal development

In this study, we identified Rab11-FIP4 in zebrafish as a gene predominantly expressed in neural tissues including the retina. zRab11-FIP4 gene was shown to be transcribed as 2 isoforms, A and B, which differed only in their 5′-terminal sequence. We suppose the A-form to be the major type of transcript according to its expression pattern. We recently found that the protein structures as well as spatiotemporal expression patterns of the A- and B-forms were highly conserved even in mouse (manuscript in preparation). Furthermore, we found that only the A-form led to the lethal phenotype by ectopic expression and that the A-form-specific knockdown in embryos resulted in the small eye and head phenotypes, suggesting the A-form to be the major functional form.

The small eye phenotype found in the zRab11-FIP4 morphant was likely due to the slow rate of proliferation of the retinal cells rather than to apoptosis. Interestingly, the proliferation of the retinal progenitors in this morphant was maintained for a longer period than that in the normal embryo, suggesting that zRab11-FIP4A regulates the rate of proliferation as well as the withdrawal from the cell cycle of the retinal progenitors. Consistent with the prolonged proliferation period, the differentiation of various lineages of retinal cells was significantly retarded in the zRab11-FIP4 morphants. We surmise that repressed expression of ath5 in the morphants caused the delay in RGC differentiation. However, the delayed differentiation of amacrine and photoreceptor cells in the zRab11-FIP4 morphants was not likely due to the impaired expression of ath5; for the lakritz mutant, in which ath5 activity is eliminated (Kay et al., 2001), it had defective differentiation of RGCs only, but not that of other retinal cells. Such findings therefore suggest that genes other than ath5 also mediate the differentiation of the retinal cells under the regulation of zRab11-FIP4A.

Despite the reduced propagation, the initial expression of ath5 was not influenced by zRab11-FIP4A depletion. Recently, RGC-derived Hh (Shh and/or Tiggy-winkle hedgehog [Twhh]) signaling was shown to be required for the propagation, but not the initiation, of ath5 expression and RGC differentiation (Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003), implying that expression or signaling of Hh could be impaired in the zRab11-FIP4 morphants. Although we were not able to detect the shh transcript in the retina, probably due to its low level of expression (data not shown), the phenotypes observed in the zRab11-FIP4 morphants were quite similar to those of the shh mutant sonic-you (syu, Shkumatava et al., 2004; Stenkamp et al., 2002). The syu mutant exhibited the small eye phenotype, being caused by a slow rate of the retinal cell proliferation rather than by an increased level of apoptosis; and it showed severe defects in the differentiation of both RGC and photoreceptors. In addition, it was earlier demonstrated that shh expression in RGC and amacrine cells, where zRab11-FIP4 was also expressed, was required for the differentiation of RGC and all other retinal cells (Shkumatava et al., 2004; Stenkamp and Frey, 2003). Although we have no direct evidence, all these observations support the idea that zRab11-FIP4A may functionally interact with the Shh signaling in the regulation of retinal neurogenesis.

Functional interaction between zRab11-FIP4 and Shh signaling

Recently, it was reported that the loss of p57Kip2 activity led to eye phenotypes similar to those of the shh mutant and that overexpression of p57Kip2 rescued the defects in cell-cycle exit in the shh mutant retina (2005 EMBO report paper). These findings suggest that cell-cycle exit of retinal progenitors is normally regulated by p57Kip2 induced by Shh. Interestingly, downregulation of cyclin D1 as well as the expression of p57kip2 was hardly observed in the zRab11-FIP4 morphant, raising the possibility that zRab11-FIP4 may functionally interact with the Shh signaling to regulate cell-cycle exit in the developing retina. Consistently, the morphant zebrafish were rescued from delayed cell-cycle exit in their retina partially but significantly by the overexpression of p57Kip2. Several lines of evidence indicate that reducing PKA activity by dnPKA is sufficient to mimic the activation of Hh signaling (Unger and Moon, 1996). We also found that dnPKA rescued the cell-cycle exit in the zRab11-FIP4 morphant retina, further supporting the...
idea of a functional interaction between Rab11-FIP4 and Shh signaling.

Although we demonstrated that both p57Kip2 and dnPKA could rescue the morphant retina from the defects in cell-cycle exit, dnPKA was more effective in the rescue than p57Kip2, suggesting that dnPKA regulates multiple molecules including p57Kip2 to stop the cell cycle. In the mammalian retina, multiple cdk inhibitors such as p57Kip2 and p27Kip1 are known to regulate cell-cycle exit in distinct populations of retinal cells (Dyer and Cepko, 2001). However, in the zebrafish retina, p27Kip1 is mainly expressed in the lens (Masai et al., 2005). On the other hand, cell-cycle progression is known to be regulated by the balance between cdk inhibitors and cyclin–cdk complexes (Li and DiCicco-Bloom, 2004). It was recently shown that PKA activity regulates Wnt-dependent activation of cyclin D1 expression in the zebrafish retina (Masai et al., 2005). As shown here, zRab11-FIP4A regulated the expression levels of both p57Kip2 and cyclin D1 in the developing retina, suggesting that downregulation of cyclin D1, in addition to the p57Kip2 expression, was simultaneously required to rescue the cell-cycle defects in the zRab11-FIP4 morphants. We also observed that p57Kip2-expressing cells formed the radially extended clusters, each of which consisted of clonal cells derived from a single progenitor, and proportion of cells exiting from cell-cycle seemed to be varied among particular clones. This observation may represent the differential sensitivities among the retinal progenitors to mitotic stimulation such as Wnt to promote cell cycle probably through the induction of the expression of cyclin D1.

It is also possible that the lack of differentiation of retinal cells in the zRab11-FIP4 morphants was caused by delayed cell-cycle exit. However, differentiation of the photoreceptors was not reversed by overexpression of p57Kip2 in the morphant retina, suggesting that the photoreceptor differentiation required an additional signal. This idea is supported by the finding that Shh itself, but not the p57Kip2, is able to rescue the photoreceptor differentiation in the shh mutant retina (Shkumatava et al., 2004; Shkumatava and Neumann, 2005). We therefore examined whether dnPKA can rescue the delay in photoreceptor differentiation. However, as in the case of p57Kip2, we failed to detect the rescue of photoreceptor differentiation by dnPKA in the morphant retina, and we could not detect normal photoreceptors expressing dnPKA even in the control embryos. Thus, Shh may transduce a distinct signal to promote photoreceptor differentiation in a PKA-independent manner, but we cannot exclude the possibility that dnPKA actively inhibits the differentiation. Alternatively, photoreceptor differentiation might also be regulated by some factors other than Shh and influenced by depleting zRab11-FIP4A activity.

There are 2 other genes whose mutants lead to eye defects similar to those of zRab11-FIP4 morphants. The receptor protein tyrosine phosphatase α (RPTPα) gene was shown to be essential for normal retinal development in zebrafish in experiments reducing its expression by use of a specific antisense MO (van der Sar et al., 2002). The RPTPα morphants exhibited small eyes with defects in both lamina formation and differentiation of retinal cells, but no increased cell death was observed. The perplexed mutant was also reported to have similar ocular defects (Link et al., 2001). This mutant showed the small eye phenotype, although the mutant retinas maintained proliferating progenitor cells for an extended period, even at 82 hpf. Cell death occurs only at a modest level in this mutant, and so the slow rate of proliferation is likely to underlie its small eye phenotype. The differentiation of perplexed retinal cells is also prevented, suggesting that this putative gene regulates the transition of retinal cells from proliferating progenitors to post-mitotic precursors. Recently, carbamoyl-phosphate synthetase 2-aspartate transcarbamylase-dihydropyrimidine synthetase (cad) gene, which is involved in de novo pyrimidine synthesis pathway, was identified as a gene responsible for the perplexed phenotype (Willer et al., 2005). Further investigations are required to clarify the actual interaction of zRab11-FIP4 with these genes.

Functions of the conserved domains of Rab11-FIP4

We showed Rab11-FIP4 to possess 2 distinct domains, the single EF-hand motif found only in the A-form and RBD shared between A and B-forms at N- and C-terminal regions, respectively. These domains are well conserved among various species. A preliminary experiment indicated that the phenotype of the zRab11-FIP4 morphants caused by suppressing the expression of the A-form was not rescued by the expression of the B-form (data not shown), suggesting the importance of the N-terminal region in the physiological function of zRab11-FIP4A. Although the N-terminal sequences of Rab11-FIP4 orthologues are diverse, the single EF-hand motif is highly conserved among various species. This motif is the most common Ca\textsuperscript{2+}-binding motif with the helix–loop–helix structure (Lewit-Bentley and Retey, 2000). In general, 2 EF-hand motifs are connected by a linker loop to form a single globular domain. The conformational change in this globular domain induced by Ca\textsuperscript{2+}-binding results in the interaction of the domain with the target molecules; and thus an even number of EF-hands has been found in many proteins (Lewit-Bentley and Retey, 2000). However, proteins not obeying the rule of EF-hand pairing, like Rab11-FIP4A, were also previously identified (Goll et al., 2003; Sugita et al., 2002). In those cases, the proteins contain an odd number of the EF-hands, and the unpaired EF-hand was shown to interact with other molecules, thus suggesting that some proteins might interact with Rab11-FIP4A through the conserved single EF-hand motif to express their function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.050.

References


