Published in final edited form as:

Dev Dyn. 2011 January ; 240(1): 278–287. doi:10.1002/dvdy.22517.

Identification and Expression Patterns of Members of the Protease-Activated Receptor (PAR) Gene Family During Zebrafish Development

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Abstract

Protease-activated receptors (PARs) play critical roles in hemostasis in vertebrates including zebrafish. However, the zebrafish gene classification appears to be complex and the expression patterns of *par* genes are not established. Based on analyses of genomic organization, phylogenetics, protein primary structure and protein internalization, we report the identification of four zebrafish PARs: *par1*, *par2a*, *par2b*, and *par3*. This classification differs from one reported previously. We also show that these genes have distinct spatiotemporal expression profiles in embryos and larvae – with *par1*, *par2a* and *par2b* expressed maternally and ubiquitously during gastrula stages and their expression patterns refined at later stages, and *par3* expressed only in 3-day old larvae. Notably, the expression patterns of zebrafish *par1* and *par2b* resemble those of their mammalian counterparts, suggesting that receptor function is conserved among vertebrates. This conservation is supported by our findings that Par1 and Par2b are internalized following exposure to thrombin and trypsin respectively.

Keywords

Protease-activated receptors (PARs); expression pattern; zebrafish

INTRODUCTION

Protease-activated receptors (PARs) are G protein-coupled receptors (GPCRs) that play critical roles in hemostasis, thrombosis, vascular development and the inflammatory response (Camerer et al., 2000; Macfarlane et al., 2001; Hollenberg and Compton, 2002; Ramachandran and Hollenberg, 2008; Peters and Henry, 2009). PARs are activated by multiple proteases, through a unique and irreversible proteolytic mechanism that involves receptor binding by a protease and subsequent protease-mediated cleavage of the receptor N-terminus. The new N-terminus generated by this event acts as a tethered ligand that subsequently interacts intramolecularly with the second extracellular loop of the cleaved receptor, leading to its activation (Vu et al., 1991; Coughlin, 2000; Macfarlane et al., 2001;

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Hollenberg and Compton, 2002). Notably, synthetic peptides that mimic the tethered ligand sequences can activate PARs in the absence of proteolysis (Macfarlane et al., 2001).

Four PARs (1-4) have been identified in mammals. PARs 1, 3, and 4 can be activated by thrombin and are regarded as thrombin receptors. PAR2 is not activated by thrombin but by multiple trypsin-like serine proteases, including trypsin itself and tryptase (Nystedt et al., 1994; Nystedt et al., 1995; Ishihara et al., 1997; Molino et al., 1997; Xu et al., 1998; Camerer et al., 2000). PAR1 and PAR3 have similar affinity for thrombin; PAR4 has a low affinity for thrombin and is activated only at much higher concentrations of thrombin. It has been proposed that PAR3 serves as a cofactor for PAR4, facilitating PAR4 activation without itself activating a downstream molecule (Nakanishi-Matsui et al., 2000). PAR1 is regarded as the prototype of the thrombin receptor. It mainly triggers $G_{\alpha_{q/11}}$ to activate phospholipase C, which leads to Ca^{2+} mobilization and PKC activation. In addition, PAR1 can activate other G proteins, including $G_{\alpha_{i/o}}$, $G_{\alpha_{12/13}}$, and $G\beta\gamma$ (Macfarlane et al., 2001; Hollenberg and Compton, 2002; Traynelis and Trejo, 2007). It has been reported that PAR2 and PAR4 signal through G_{α_q} and $G_{\alpha_{12/13}}$ (Traynelis and Trejo, 2007).

Thrombin is generated at sites of vascular injury, where it triggers the coagulation cascade and serves as the main regulator of platelet secretion and aggregation during clot formation. PAR1, PAR3, and PAR4 are expressed prominently in cells of the circulatory system, including platelets, endothelial cells, fibroblasts, leukocytes, and smooth muscle cells, and have been shown to play critical roles in blood hemostasis and thrombosis (Dery et al., 1998; Coughlin, 2000; Traynelis and Trejo, 2007). Notably, whereas human platelets express PAR1 and PAR4 (Kahn et al., 1999; Faruqi et al., 2000), mouse platelets express PAR3 and PAR4 (Kahn et al., 1998; Coughlin, 2005; Traynelis and Trejo, 2007). It also has been reported that whereas PAR1 and PAR3, but not PAR4, are highly expressed in human endothelial cells, PAR1 and PAR4 are the predominant receptors in mouse endothelial cells (Macfarlane et al., 2001; Kataoka et al., 2003; Traynelis and Trejo, 2007). In the case of PAR3, expression is not limited to the vascular system; the PAR3 mRNA is present in a wide range of human tissues including stomach, small intestine, trachea and bone marrow (Ishihara et al., 1997). In mouse, by contrast, PAR3 is restricted to certain tissues such as bone marrow (megakaryocytes) and spleen (Ishihara et al., 1997). Similarly, in humans the PAR4 mRNA is expressed in a variety of tissues; the highest levels outside the circulatory system are in the lung, pancreas, thyroid gland and intestine (Xu et al., 1998).

In the case of PAR2, expression at both the transcript and protein levels is high in the gastrointestinal tract (including the stomach, intestine, and colon), in digestive-tract organs such as the pancreas and liver (both human and mouse), and in the kidneys (Nystedt et al., 1994; Nystedt et al., 1995; Bohm et al., 1996). Consistent with the fact that trypsin is most abundant in the intestines, *in situ* hybridization revealed that mouse *Par2* is highly expressed on the luminal surface of epithelial cells of the small intestine (Bohm et al., 1996). This expression profile has implicated PAR2 in gut motility, inflammation, and secretory function. In addition, both mouse and human PAR2 are expressed in the smooth muscle, where they contribute to vascular relaxation (Hwa et al., 1996; Molino et al., 1997; D'Andrea et al., 1998; Ramachandran and Hollenberg, 2008).

PAR transcripts are also detected in other organ systems, including heart, central nervous system, airway and epidermis, as well as in many cell types of the immune system (e.g. macrophages, monocytes, neutrophils, and mast cells) (Macfarlane et al., 2001; Miotto et al., 2002; Ramachandran and Hollenberg, 2008; Rattenholl and Steinhoff, 2008; Sokolova and Reiser, 2008). The expression of PAR proteins suggests that their functions are diverse. However, except in the context of the circulatory system, the roles of these proteins remain to be characterized in depth.

PAR proteins also appear to play an important role in embryonic development. PAR1 has been implicated in blood vessel formation; *Par1*-knockout mice display bleeding and disorganized blood vessel formation during embryonic development, with 50% fatality occurring by midgestation (Connolly et al., 1996; Griffin et al., 2001). This phenotype is rescued by expression of a *Par1* transgene in endothelial cells, demonstrating that PAR1 is critical for proper endothelial-cell function in developing blood vessels (Connolly et al., 1996; Griffin et al., 2001). Interestingly, mice in which the $G\alpha_{13}$ -encoding gene was disrupted specifically in endothelial cells displayed the same phenotype as *Par1*-deficient mice, suggesting that $G\alpha_{13}$ is the main mediator of PAR1 signaling during vessel development (Ruppel et al., 2005). Mice in which other *Par* genes are knocked out developed normally to adulthood (Hirano, 2007).

Zebrafish has become a useful vertebrate model for studying embryonic developmental processes, due to the transparency and rapid external development of the embryos, and the availability of a broad array of genetic tools (Grunwald and Eisen, 2002). Notably, with respect to the circulatory system, it has been shown that trypsin is expressed in zebrafish (mouth, nose, gill and pancreas), and that active trypsin obtained from zebrafish water can efficiently induce thrombocyte activation *in vitro* (Kim et al., 2009). Furthermore, the zebrafish genome has been reported to harbor 27 genes encoding Par proteins, with some of these genes expressed in thrombocytes and responding to thrombin and trypsin (Kim et al., 2009). These findings suggest that the role of Par proteins in hemostasis has been conserved over the course of evolution. However, the expression patterns of the *par* genes have not been reported, and the reported gene classification is inconsistent with the most recent version of the zebrafish genome. Here we present our identification and cloning of the zebrafish *par* genes, our characterization of their genomic organization and phylogenetics, and our analyses of the protein primary structures, internalization, and spatiotemporal expression patterns during various stages of development.

RESULTS AND DISCUSSION

Identification and Cloning of the Zebrafish *par* Genes

In mammals, four PAR isoforms (PARs 1-4) have been identified. With respect to the 27 *par-like* genes previously reported to exist in the zebrafish genome (Kim et al., 2009), we found that many are in fact identical to one another, and that some of the genomic information provided in the study is not represented in the current version of the database. These discrepancies are probably due to mistakes and redundancies in the early versions of the zebrafish database used in that study (zebrafish genomic assembly version (Zv) 6 and 7). For our analysis, we initially conducted a new search of the EST and Ensembl Genome (Zv7) databases, using the human *PAR* gene sequences. We found four *par*-related genes (*pars 1, 2a, 2b, 3*)—corresponding to human *PAR1*, *PAR2*, and *PAR3*—in zebrafish, but failed to identify a homolog of the human *PAR4* gene (Fig. 2A). We also failed to find *PAR4* homologs in the Medaka and Fugu genomes (data not shown). However, in the course of revising this manuscript we discovered a gene encoding a Par4-like protein (XP_001333055) in the newest version of the zebrafish genome database (Zv8). Notably, despite 65% similarity with mammalian *PAR4* at amino acid level, syntenic analysis suggests that the genomic organization of this *par4-like* gene is not similar to that of mammals. In addition, this gene lacks a similar tethered peptide that found in human *PAR4* (data not shown). Thus it remains to be determined if this is a true zebrafish counterpart of the mammalian *PAR4* gene. The zebrafish *par1* gene (GenBank accession number GQ325252) contains an open reading frame (ORF) encoding a protein of 413 amino acids, which shares 46% identity and 70% similarity with human *PAR1* (Fig. 1A). Two closely related zebrafish *par2* genes, designated as *par2a* and *par2b* (GenBank accession number GQ325254 and GQ325253), were also found. The predicted encoded proteins share 55%

identity and 71% similarity with one another, and 50%/48% (respectively) identity and 70%/66% similarity with human PAR2, at the amino acid level (Fig. 1B). Also, using the new version of the Zv8 database, we identified a *par3* gene (GenBank accession number XM_002662087). It encodes a predicted protein that is 367 amino acids long and shares 37% identity and 59% similarity with human PAR1 (Fig. S1). However, we were able to clone only the first 774 base pairs of the cDNA, which encode the first 258 amino acids of the predicted Par3 protein (GenBank accession number GQ325255).

Given the discrepancies between our results and those reported in the earlier study (Kim et al., 2009), we re-examined the data on which that study was based. First, we found that many of the genes that had been classified into particular groups based on similarity in fact represent the same gene (see Supplementary Table). Briefly, five groups of Par genes had been identified, with group 1-3 representing *par1* homologs and group 4-5 representing *par2* homologs (Kim et al., 2009). The group1 genes are identical to the *par1* that we describe. The group 2 genes likewise represent a single gene, which is predicted to encode a Par2-like protein (XP_001334308, Zv8). Although it was suggested that PAR1-21A (group 2) encodes a thrombin receptor based on the fact that the protein could be activated modestly by human thrombin (Kim et al., 2009), whether it represents a second Par1 isoform remains to be determined since, firstly, the tethered peptide of this protein (PTQQL) differs significantly from that found in mammalian Par1 (SFLLRN and SFLLRN in human and mouse, respectively) and, secondly, there is no direct evidence that thrombin-based activation of this protein involves cleavage of the peptide. Similarly, the group 3 genes represent a single gene encoding a protein that is predicted to be Par2-like (XP_001096094, Zv8). This protein probably cannot be classified as a thrombin receptor because the transcripts are not expressed in thrombocytes, and a synthetic peptide based on its sequence failed to induce thrombocyte activation (Kim et al., 2009). In case of the group 4 and group 5 genes, which were thought to encode Par2 proteins according to the previous analysis, we again found that the members of each group likely represent a single protein. In the case of group 4, they are similar to the *par2b* described here, and in the case of group 5, they are similar to our *par2a*. With regard to the remaining two representatives of the Par family reported in the earlier study, we found that PAR2-14A represents the Par4-like protein described above, whereas PAR2-19A is now annotated as a rCG59702-like protein (XP_002665501, Zv8) and unrelated to Par proteins. Given these considerations, and the fact that the findings presented here are further supported by our analyses of genomic organization and phylogenetics (see below), we believe that our characterization of the Par genes is accurate.

Characterization of the Zebrafish Par Proteins

Analysis of multiple sequence alignments for each identified zebrafish *par* gene revealed that the predicted Par proteins contain seven putative transmembrane domains (indicated by solid lines above the sequences, Fig. 1 and Fig. S1), a characteristic feature of G protein-coupled receptors. The predicted amino acid sequences of these domains in the zebrafish PARS are highly conserved with those of their human and murine PAR counterparts, supporting the notion that zebrafish *par* genes are the orthologs of mammalian PAR-encoding genes (Fig. 1 and Fig. S1). Interestingly, each zebrafish Par protein has the conserved “CHD motif” in its second extracellular loop (indicated by asterisks, IT/VTCHDV in Par1 and Par 2, Fig. 1 and LVTCHDI in Par 3, Fig. S1); this region has been implicated in the ligand docking that leads to receptor activation (Gerszten et al., 1994; Xu et al., 1998).

Mammalian PARs are known to trigger cellular responses through a unique activation mechanism whereby a protease cleaves the N-terminus of the receptor, generating a new N-terminus that acts as a tethered ligand. This newly generated autoligand then binds to and activates the receptor (Camerer et al., 2000). Consistent with the conservation of such a

mechanism in zebrafish, Par1 harbors a putative protease cleavage site (Arg-28/Ser-29, indicated by the arrow, Fig. 1A), as well as a putative tethered ligand sequence (SFSGFF, dashed line box, Fig. 1A), within its extracellular N-terminal region. This peptide has been shown to induce thrombocyte activation (Kim et al., 2009). These features are similar to those of human and mouse PAR1 proteins, in which Arg-41/Ser-42 function as the cleavage site, and SFLLRN and SFLLRN function as the tethered ligand sequence in human and mouse, respectively. These findings support the notion that zebrafish Par1 is a protease-activated receptor. Also, a putative protease cleavage site (Lys-37/Thr-38) and a tethered ligand (TFTGTI) similar to those in human and mouse PAR3 were identified in zebrafish Par3 (Fig. S1). Although the N-terminus of mammalian PAR2 also contains a protease cleavage site (Arg-36/Ser-37, indicated by the arrowhead, Fig. 1B) and tethered ligand sequences (SLIGKV in human, SLIGRL in mouse, indicated by dashed line boxes, Fig. 1B), we failed to identify precisely the same features within the N-termini of zebrafish Par2a and Par2b. However, in Par2b, we found a similar potential protease cleavage site (Arg-27/Met-28, indicated by the arrow, Fig. 1B) and a tethered ligand sequence (MMIVIQ, dashed line box, Fig. 1B). This tethering peptide is different from that reported previously (SLVVIQ) (Kim et al., 2009), probably due to differences in the genetic backgrounds of zebrafish strains. Although a cleavage site (Ser-31/Gly-32, indicated by the arrow, Fig. 1B) and tethered ligand sequence (GFTEET, dashed line box, Fig. 1B) have also been proposed in Par2a, a synthesized version of this tethering peptide failed to induce thrombocyte activation (Kim et al., 2009). Thus, activation of these zebrafish Par2 receptors may involve cleavage sites different from those that have been defined for the mammalian homologs (Fig. 1B).

Phylogenetic and Syntenic Analyses of Zebrafish *par* Genes

We examined the evolutionary relationships between zebrafish Pars and their mammalian counterparts by building a phylogenetic tree using the Neighbor-Joining (NJ) method (<http://www.ebi.ac.uk/Tools/clustalw2>). Our results indicate that the zebrafish Par proteins are closely related to their mammalian orthologs (Fig. 2A). Syntenic analyses indicate that the genomic organization of the genes encoding Par proteins is similar in zebrafish and mammals. The human and mouse *PAR1–3* genes are clustered in a small region (~200 kb) of a single chromosome, on 5q13 and 13D1, respectively (Fig. 2B and O'Brien et al., 2001), with *PAR2* and *PAR1* separated from *PAR3* by the *IQGAP2* gene (encoding the IQ motif containing GTPase activating protein 2). The *PAR3* gene is flanked by the *IQGAP2* and *SV2C* genes (encoding synaptic vesicle glycoprotein 2C), and the *PAR2* gene is positioned between the *CRHBP* (encoding corticotropin-releasing hormone binding protein) and *SI00Z* genes on one side, and the *PAR1* gene on the other. In both human and mouse, the *PAR4* gene is located on a second chromosome (human, 19p12; mouse, 8B3.3) (O'Brien et al., 2001). In a striking synteny, the zebrafish *par1* and *par3* genes on chromosome 5 are clustered and separated by *iqgap2*. In addition, *par3* is flanked by the *iqgap2* and *sv2c* genes (Fig. 2B), although *par1* is not flanked by *par2*. In fact, both *par2a* and *par2b* are located on chromosome 21, next to the *crhbp* gene (Fig. 2B). These observations suggest that zebrafish *par2* underwent a tandem duplication and was translocated, along with *crhbp*, from *par1* and *par3* to a different chromosome.

Internalization of Zebrafish Par Proteins

Many GPCRs undergo internalization in response to agonist stimulation—i.e. they are redistributed from the plasma membrane to the cytosol. Such internalization of the receptors plays a critical role in terminating receptor signaling (Hoxie et al., 1993; Trejo, 2003). Similarly, internalization of mammalian PAR1 is triggered by thrombin (Trejo, 2003), and the previous study of zebrafish Par proteins had shown that human thrombin can stimulate zebrafish Par1 to induce a Ca^{2+} response in *Xenopus* oocytes (Kim et al., 2009). To evaluate

Par1's response to thrombin, we generated a zebrafish Par1-GFP fusion protein in which GFP is attached to the C-terminus of the receptor and transiently expressed it in HEK293 cells. As shown in Fig. 3A, in cells in the basal state, Par1-GFP was mainly detected on the plasma membrane. However, in cells stimulated with thrombin for 30 minutes, the majority of the receptor was internalized into intracellular compartments that were visible as discrete punctate accumulations (Fig. 3B), suggesting that Par1 can respond to thrombin to undergo internalization. By contrast, treatment with trypsin did not change the membrane localization of Par1-GFP (Fig. 3C). These results are consistent with the notion that zebrafish Par1 is a thrombin receptor.

Mammalian PAR2 is not activated by thrombin, but rather by trypsin-like serine proteases (Dery et al., 1999). Similarly, zebrafish Par2b showed a significant Ca^{2+} response upon trypsin activation (Kim et al., 2009). To investigate directly how zebrafish Par2 responds to thrombin and/or trypsin, we constructed a Par2b-GFP fusion protein and expressed it in HEK293 cells. Like Par1-GFP, Par2b-GFP was detected largely on the plasma membrane (Fig. 3D). Not surprisingly, the distribution of Par2b-GFP was not altered upon exposure to thrombin; the fusion protein was still present on the cell membrane 60 minutes after stimulation (Fig. 3E). In contrast, 10 μM trypsin induced internalization similar to that of mammalian PAR2 (Fig. 3F), whereas 100 nM trypsin failed to promote this internalization (data not shown). These results are consistent with the finding that the concentration of bovine trypsin required to reduce gill bleeding is much higher than that of zebrafish trypsin, since the latter is ten-fold more active than bovine trypsin when applied to zebrafish (Kim et al., 2009). Another possibility is that the protease cleavage site of the zebrafish Par2b receptor is different (see above) from that of mammalian PAR2, and that it does not respond well to the pig trypsin used in our study. Taken together, these results support previous findings that Par1 is a thrombin receptor and Par2b is a trypsin receptor.

Expression of the Zebrafish *par1* Gene

We performed whole-mount *in situ* hybridization to examine the spatio-temporal expression of each of the *par* genes during zebrafish embryogenesis. The *par1* transcript was detected at both the 8-cell and blastula stages (Fig. 4A-B), indicating that it was maternally deposited. During the gastrula stage, *par1* expression was detected ubiquitously throughout the embryo (Fig. 4C-E), suggesting that this gene may play a role during early embryogenesis. At the 10-somite stage, *par1* expression was limited to the posterior and ventral mesoderm (Fig. 4G, H). By 1 day post fertilization (dpf), its expression was confined to the heart and veins. Specifically, *par1* expression was found in the common cardinal vein (CCV), posterior cardinal vein (PCV), caudal vein (CV) and intermediate cell mass of mesoderm (ICM) (Fig. 4I-N), suggesting that *par1* might play a specific role in vein development and/or function. At 2 dpf, in addition to being expressed in the heart, veins and caudal hematopoietic tissue (CHT), *par1* RNA was present in the branchial arches, notochord, otic vesicle, gut, pancreas and pronephric duct (Fig. 4O-S). At 3 dpf, *par1* expression persisted in the branchial arches, pancreas, heart and vein (Fig. 4T-V). Overall, these results indicate that *par1* expression is ubiquitous during early development, and then becomes confined to particular organs including the cardiovascular (heart, vein), digestive tract (gut, pancreas), and developing kidney. This expression pattern resembles that of the mammalian *PAR1* gene (Vu et al., 1991; Dery et al., 1998; Coughlin, 2000), suggesting that the function of Par1 and the regulation of its expression are conserved among vertebrates.

Expression of the Zebrafish *par2a* and *par2b* Genes

As in the case of the *par1* gene, *par2a* and *par2b* transcripts were detected at as early as the 8-cell stage, suggesting that they are also maternally deposited. Both were expressed ubiquitously during the blastula and gastrula stages (Fig. S2). However, by 1 dpf, *par2a*

expression was dramatically decreased, to a level that could not be detected by *in situ* hybridization (data not shown), whereas *par2b* transcript persisted in certain tissues, specifically in the pronephric ducts and otic vesicles (Fig. 5A-E). Transverse and sagittal sections revealed that the *par2b* transcript was enriched on the apical membrane of pronephric-duct epithelial cells (Fig. 5C-D). At 2 dpf, *par2b* expression persisted in those locations but was also observed in the gut and notochord (Fig. 5F-J). At 3 dpf, *par2b* expression in the gut became more prominent, and was strongest on the apical membrane of the gut epithelial cells. In addition, *par2b* was detected in the pharynx, branchial arches and liver (Fig. 5K-P). Thus, as in the case of *par1*, the expression of *par2b* is similar to that of its mammalian counterpart (Nystedt et al., 1995; Bohm et al., 1996; Grandaliano et al., 2003; MacNaughton, 2005). Notably, both human and zebrafish Par2 are highly expressed in epithelial cells of the intestinal surface (Bohm et al., 1996). Collectively, these data suggest that zebrafish Par2b is likely a functional counterpart of human PAR2, and that Par2a may not contribute to organogenesis.

Expression of the Zebrafish *par3* Gene

Although an RT-PCR approach enabled us to detect *par3* transcripts in 3 dpf embryos (Fig. S3), whole-mount *in situ* hybridization in embryos ranging from blastula stage through 3 dpf failed to detect any *par3* expression (data not shown). These findings suggest that the expression levels during this period are below the level of detection for this method. Furthermore, given that PAR3 has been shown to act as a cofactor for PAR4 activation rather than to directly generate a signal (Nakanishi-Matsui et al., 2000), and that we did not identify a convincing Par4 homolog in zebrafish, *par3* may not be required during early stages of zebrafish development.

SUMMARY

We have identified and cloned four zebrafish *par* genes. Analyses of the PAR family phylogenetic tree and the genomic organization of the PAR genes indicate that the newly discovered zebrafish genes are homologs of mammalian PARs—*PARs1–3*. Our characterization of internalization supports the previous findings that zebrafish Par1 is a thrombin receptor and Par2b is a trypsin receptor. *In situ* hybridization studies demonstrated that the zebrafish *par1–2* genes display distinct spatial and temporal expression patterns during development, with *par1* being expressed in the vascular system, gut and developing kidney, much like its mammalian counterpart. Although both *par2a* and *par2b* are expressed ubiquitously during the blastula and gastrula stages, *par2a* expression diminishes after segmentation is complete, whereas *par2b* is detected in the gut, pronephric duct and liver in embryos from 1 to 3 dpf; this expression profile is also similar to that of mammalian *PAR2*. Overall, our analysis of the expression patterns of the zebrafish *par* genes suggests that different Par proteins play distinct roles during zebrafish development. Our findings lay the foundation for future studies that will elucidate the functions of Par proteins during embryonic development.

METHODS

Embryo Generation and Maintenance

WT zebrafish of AB*, India, TL and hybrid backgrounds used in this study were maintained either in the Animal Care Facility at the University of Iowa or the zebrafish facility at Vanderbilt University, as described previously (Lin et al., 2005). Embryos were obtained from natural matings, and staged according to morphology or by days post fertilization (dpf) at 28.5°C, as described (Kimmel et al., 1995). Embryos older than 1 dpf were treated with 0.003% phenylthiourea (PTU) to prevent pigmentation.

BLAST Search, Sequence Alignments and Phylogenetic Analysis

To clone zebrafish *par* genes, we searched the zebrafish Ensembl Genome Server (www.ensembl.org/Danio_rerio/) and EST (www.ncbi.nlm.nih.gov/BLAST/) databases using the human *PAR1*, *PAR2*, *PAR3* and *PAR4* genes. Multiple-gene alignments and phylogenetic analysis of human, mouse and zebrafish *Par*s were carried out using ESript 2.2 (<http://esript.ibcp.fr/ESript/ESript/>) and the Clustal-W program (<http://www.ebi.ac.uk/Tools/clustalw2/>), respectively.

Cloning of Zebrafish *par* Genes

PCR primers flanking the ATG initiation and stop codons of the zebrafish *par* genes were designed to amplify the full coding sequences. For zebrafish *par3*, primers flanking the EST sequence (GenBank accession number EE306722.1) were used to amplify the partial cDNA. RT-PCR (Titanium™ One-Step RT-PCR kit, Clontech) was performed using RNAs prepared from zebrafish embryos. PCR products were cloned into the PCR II-TOPO vector (Invitrogen), and sequences were verified. Subsequently, all *par* genes were subcloned into the pCS2 expression vector using the Gateway® (Invitrogen) strategy. GFP fusion constructs (*par1-gfp* and *par2b-gfp*) in which the *gfp* sequence was inserted at the 3' end of the *par* genes were generated using the MultiSite Gateway® (Invitrogen) kit.

Whole-Mount *In Situ* Hybridization

DIG-UTP-labeled sense and antisense RNA probes for each *par* gene were generated as described previously. Whole-mount *in situ* hybridization was performed as described (Lin et al., 2005). Frozen sections were generated from embryos labeled by whole-mount *in situ* hybridization using a cryostat.

Internalization of *Par* receptors in HEK293 cells

HEK (human embryonic kidney) 293 cells were transiently transfected with plasmid constructs encoding *Par1*-GFP or *Par2b*-GFP. At 24 hours post-transfection, cells were starved overnight in serum-free medium, and then stimulated with thrombin (5nM, Sigma) or trypsin (100 nM and 10 μM, Amresco) (or left untreated) for 30-60min. Cells were then fixed with 4% paraformaldehyde (PFA) and mounted in Vectashield® mounting medium (Vector Laboratories). Confocal images were acquired on a *Leica* Inverted DMI6000B Microscope.

Microscopy

Embryos subjected to whole-mount *in situ* hybridization were mounted in 75% glycerol/PBT, and photographed using an Axiophot2 microscope (Zeiss) and an Axiocam digital camera (Zeiss), or a *Leica* M165FC Stereofluorescent Microscope with a *Leica* DFC290 Color Digital Camera. For confocal imaging, a *Leica* Inverted DMI6000B Microscope with a *BD CARV II* Spinning Disk Confocal Imager was used. Images were acquired using a 63X oil objective and an EMCCD camera equipped with the IPLab software. All images acquired were compiled and edited using Adobe Photoshop® and Illustrator software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was initiated in the laboratories of Heidi Hamm and Lila Solnica-Krezel at Vanderbilt University, and completed at the University of Iowa. We acknowledge Joshua Clanton, Heidi Beck and Amanda Bradshaw of the Vanderbilt fish facility, and the zebrafish care team of the Animal Care Facility at the University of Iowa, for

excellent fish care. This study is supported by a NIH/NCRR K99R00RR024119 grant to FL and NIH/NIGMS R01GM77770 grant to LSK and HH.

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A

.....NLRLVLTGLALNGSASALPNNTFIRSES.....GFLLEVTDFFDYL.....DVOEGGSG
hsPar1 MGPRRLLLVAACFSLGCLPLLSARTRARRPESKATNATLDFEFLFLPNNDKYEPFW.....EDEEKNISG
mmPar1 MGRRLLLVALGLSLGCLPLLSRRVPMSCPESERTDATTNPSPEFLKNSPENTFELVPLGDEEEKNISG

drPar1 FSSGQETPTKRKEHLHPPAKKTYHFESEASSFLTGRVACANVITIVIVFVIFSLPNNLALVMFARKVRPK
hsPar1 LLEYRLVSNKSKS.SPLQKQLPARFSEDSAGKYLTSWLLDLFLVFSVFGVFFVSLPLNMAIVFLLKMKVK
mmPar1 LLEGRAVYLNIS.LPPHTPPPPFSESDASGYLTSPWLLFLFMPSSVYFIVFIVSLPLNLVLAIAVFVLRMKVK

drPar1 KPAAIYMMNLACADLLFLVLLPFRVAYNYNCNNVYGAGMCRVVTAAFYNMVGVLLIMCISVDRFLAV
hsPar1 KPAAVYMLLLADLVFLVSVLPFKISYFYFSGSDWFGSELCSRVTAAFYNMVSIILMTVISIDRFLAV
mmPar1 KPAVYVMLLLADLVFLVSVLPFKISYFYFSGSDWFGSGMCRSATAAFYNMVASIMLMTVISIDRFLAV

drPar1 VYPMGLSTWRSQPTAATCAAMNLLSVGCVPLLLISNOTIHLPLDLITTCCHDVLDIQHLREYVYVFFEF
hsPar1 VYPMGLSTWRSQPTAATCAAMNLLSVGCVPLLLISNOTIHLPLDLITTCCHDVLDIQHLREYVYVFFEF
mmPar1 VYPLGLSTWRSQPTAATCAAMNLLSVGCVPLLLISNOTIHLPLDLITTCCHDVLDIQHLREYVYVFFEF

drPar1 CSLFFEPILPILSTVCYLRILIQALCAANVENRAKKTRAMIMAVTVFAVVFVFCIFPTNILLSHYVRFAYKH
hsPar1 SAVIFFEPILPILSTVCYLRILIRCLSSAVENRSKKGRALFLSAAVFCIFIFICFPPTNVLLIAHYSFLSHS
mmPar1 SAVIFFEPILPILSTVCYLRILIRCLSSAVENRSKKGRALFLSAAVFCIFIFICFPPTNVLLIAHYSFLSDSP

drPar1 N.DASVAAVLVSCMCSISCSLDPLIYFFGSSCCOKGVLAFLRCQGVPGIERSAQFTSTRSSNLETLSK
hsPar1 TDEARYFAYLLVCVSVSSCSIDPLIYFASSECORYYVSLICCKESSD.PSSYNSGGQLMASKMDTCS
mmPar1 GTEARYFAYLLVCVSVSSCSIDPLIYFASSECORHLYSLICCKESSD.PNSCNSGQLMPSKMDTCS

drPar1 SVNSQ.VYIKLMA
hsPar1 NLNNSYIKLLT
mmPar1 HLNNSYIKLLA

B

drPar2aMAHGLTWISALLFLPIAVNLSGOTSINPNWD.....RGTFEEKREGVSVTSTALGV
hsPar2a MRSPAAWLLGAILLAAASLCSGT.....TGCTNRSKGRSLTCKVLTGTSHTVTKGKVTVETVTSVEFSASV
mmPar2a MRLSLAWLLGAILLAAASLCSGT.....TGCTNRSKGRSLTCKVLTGTSHTVTKGKVTVETVTSVEFSASV
drPar2bMAVSESY.RILFLACVIFASAQPGKNGRM.....MTVIGDAGHTFVSGITLHFL

drPar2a LNSKLTQIFPEVLYIVIVSVCLPTNMAIIVWVLFRRKKHPSISIFMANLALADLLVWVPLKIAHFNG
hsPar2a LTKLTQIFPEVLYIVIVSVCLPTNMAIIVWVLFRRKKHPSISIFMANLALADLLVWVPLKIAHFNG
mmPar2a LTKLTQIFPEVLYIVIVSVCLPTNMAIIVWVLFRRKKHPSISIFMANLALADLLVWVPLKIAHFNG
drPar2b LQSLTKTQIFPEVLYIVIVSVCLPTNMAIIVWVLFRRKKHPSISIFMANLALADLLVWVPLKIAHFNG

drPar2a NNLIFGEALCNVLLGFFGYGNMYCSILFITCLSVQRYWVAVPPLSQQRNNRNLAIAGVSVVNLVVALVLP
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drPar2b NNLIFGEALCNVLLGFFGYGNMYCSILFITCLSVQRYWVAVPPLSQQRNNRNLAIAGVSVVNLVVALVLP

drPar2a LYVYQDQVQVNVNMLITTCCHDV.....TRPSQSLYFCFIYFLTMGVVGVFVPLVCLVMSVQMLRA
hsPar2a LYVYQDQVQVNVNMLITTCCHDV.....TRPSQSLYFCFIYFLTMGVVGVFVPLVCLVMSVQMLRA
mmPar2a LYVYQDQVQVNVNMLITTCCHDV.....TRPSQSLYFCFIYFLTMGVVGVFVPLVCLVMSVQMLRA
drPar2b LYVYQDQVQVNVNMLITTCCHDV.....TRPSQSLYFCFIYFLTMGVVGVFVPLVCLVMSVQMLRA

drPar2a LKSSMNDPISVQKKRKAFLIVTVLVFLVLT.....PSNMIMVHVYALLFNGVKNNSGFGYFVITLCLASLNSC
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mmPar2a LKSSMNDPISVQKKRKAFLIVTVLVFLVLT.....PSNMIMVHVYALLFNGVKNNSGFGYFVITLCLASLNSC
drPar2b LKSSMNDPISVQKKRKAFLIVTVLVFLVLT.....PSNMIMVHVYALLFNGVKNNSGFGYFVITLCLASLNSC

drPar2a VDPFFVYVYFISDFREHVRNITFLCRSERTACRMVVSFSAKYKSKSTYTSISQNTQSSSY
hsPar2a VDPFFVYVYFISDFREHVRNITFLCRSERTACRMVVSFSAKYKSKSTYTSISQNTQSSSY
mmPar2a VDPFFVYVYFISDFREHVRNITFLCRSERTACRMVVSFSAKYKSKSTYTSISQNTQSSSY
drPar2b VDPFFVYVYFISDFREHVRNITFLCRSERTACRMVVSFSAKYKSKSTYTSISQNTQSSSY

Figure 1. Comparison of zebrafish and mammalian Par1 and Par2

Amino acid alignments of zebrafish (*Danio rerio*, dr), human (*Homo sapiens*, hs) and mouse (*Mus musculus*, mm) Par1 (A) and Par2 (B), generated using the ESPrnt 2.2 program (<http://esprnt.ibcp.fr/ESPrnt/ESPrnt>). Amino acids are numbered to the right of each line. The transmembrane (TM) domains are indicated by solid lines above the sequences. The putative protease cleavage sites and the tethered ligands are indicated by the arrow or arrowhead and dashed line boxes, respectively. The CHD motifs are indicated by asterisks above the sequences. Identical amino acids are highlighted in black boxes; similar amino acids are shown in black bolded characters in black frames.

Figure 2. Analysis of phylogenesis and genomic organization of *par* genes

(A) Phylogenetic tree depicting the evolutionary relationships among PAR proteins, as established by the Neighbor-Joining (NJ) method (<http://www.ebi.ac.uk/Tools/clustalw2>), in which proteins cluster together based on sequence similarity. Bootstrap values indicated at each branch point were calculated from 1,000 replicate runs. (B) Schematic diagrams of the synteny in the regions of zebrafish chromosomes 5 and 21 (site of *par* genes) and human chromosome 5 (site of PAR genes).

Figure 3. Internalization of zebrafish Par1 and Par2b upon thrombin or trypsin stimulation
HEK (human embryonic kidney) 293 cells transfected with cDNA encoding GFP-tagged zebrafish Par1 or Par2b were stimulated with either thrombin (50 nM, **B, E**) or trypsin (10 μ M, **C, F**) for 30-60 min. Untreated cells served as control (**A, D**). Scale bar: 10 μ m.

Figure 4. Expression of *par1* during zebrafish embryogenesis

Whole-mount *in situ* hybridization was performed on embryos at various stages: 8-cell (A); sphere (B); gastrula (C-E); 10-somite (F-H, arrows indicate posterior and ventral mesoderm); 1 dpf (I-N; I: lateral view of a embryo showing *par1* expression in the heart, posterior cardinal vein (PCV), cardinal vein (CV), intermediate cell mass of mesoderm (ICM); J: high-magnification image of anterior region of I; K: dorsal view of the anterior region of the embryo showing the *par1* expression in common cardinal vein (CCV); L: high-magnification image of the trunk region of I, location of the notochord (nc) is indicated; M-N: sagittal and transverse sections of the trunk area indicated by the black line shown in I); day 2 (O-S; O: Lateral view showing *par1* expression in the heart, branchial arches, caudal hematopoietic tissue (CHT); P: ventral view of the anterior region of O at high magnification; Q-R: lateral view of the trunk region of O at high magnification; S: transverse section at the trunk region showing *par1* expression in notochord (nc), vein, pronephric duct (pd) and gut); 3 dpf (T-V; T: lateral view showing *par1* expression in the heart, pharynx, CHT; V: ventral view of the anterior region at high magnification; *par1* expression in pancreas is indicated). Most embryos are shown in lateral view, with the

animal pole up. Exceptions include **G** (dorsal view), **H** (dorsal-posterior view), **P** (ventral view), and **K, P, V** (dorsal view). Scale bars: 100 μm .

Figure 5. Expression of *par2b* in embryos at 1-3 dpf, as detected by whole-mount *in situ* hybridization

(A-E) 1 dpf. **A**: Lateral view; **B**: high-magnification view of the trunk region of **A**; **C-D**: transverse and sagittal sections, respectively, of the trunk area indicated by the black line shown in **A**, with black arrows denoting *par2b* expression in the pronephric duct (pd). Note that *par2b* is highly expressed on the apical side of the epithelial cells of the pronephric duct; **E**: dorsal view of anterior region in **A**, showing *par2b* expression in the otic vesicle. (F-J) 2 dpf. **F**: Lateral view; **G-H**: lateral and dorsal views, respectively, of the anterior region of **F** at high magnification, showing *par2b* expression in the gut and notochord (nc); **I**: lateral view of the trunk region of **F** at high magnification, showing *par2b* expression in the gut and notochord; **J**: sagittal section of the trunk area. (K-P): 3 dpf. **K-L**: Lateral view, arrows denote pharynx and liver; **M**: ventral view, showing *par2b* expression in branchial arches and the liver; **N**: lateral view of the trunk region at high magnification, showing *par2b* expression in the gut and pronephric duct (pd); **O-P**: transverse (**O**) and sagittal (**P**) sections of the trunk area. Note that *par2b* transcript was enriched on the apical side of the epithelial cells of both the pronephric duct and gut. Scale bars: 100 μ m unless specifically labeled.