Spectrins are components of the cortical cytoskeleton of many cell types, participating in diverse biological functions involving the cell membrane: membrane reinforcement, organelle maintenance, protein trafficking, regulation of exocytosis, maintenance of cell-cell junctions, and partitioning of cellular domains and polarity (Bennett, 1990; De Matteis and Morrow, 1998; Dubreuil and Grushko, 1998; Lippincott-Schwartz, 1998). As such, spectrins are essential in cell morphogenesis during differentiation and maintenance of cell membrane stability. The importance of spectrins in cell morphogenesis is underscored by studies of spectrin mutants in Drosophila and Caenorhabditis elegans (Deng et al., 1995; Dubreuil et al., 2000; McKeown et al., 1998; Thomas et al., 1998). Human mutations in erythroid spectrins SPTA1 and SPTB result in hemolytic anemia, classified by the abnormal cell shapes into hereditary spherocytosis (HS), elliptocytosis (HE) and pyropoikilocytosis (HPP) (Lux and Palek, 1997; Tse and Lux, 1999). Similarly, mouse mutants spherocytosis (sph/sph) and jaundiced (ja/ja) suffer from hemolytic anemia and harbor mutations in erythroid α-spectrin (Spnα1) and β-spectrin (Spnβ1), respectively (Bloom et al., 1994; Bodine et al., 1984; Brookoff et al., 1982; Wandersee, 1998).

Erythroid spectrins are the largest proteins of the red cell membrane cytoskeleton and account for much of the structural framework essential for stability of the membrane bilayer (Dubreuil and Grushko, 1998; Viel and Branton, 1996; Winkelmann and Forget, 1993). All spectrins share a repeat motif of 106 amino acids, termed the spectrin repeat, which forms a triple helical bundle (Y an et al., 1993). There are two α-spectrins (~280 kDa) in humans: SPTA1 is erythroid specific and SPTAN1 is present in all cells except erythrocytes (Leto et al., 1988). Several human β-spectrins (~250 kDa) have been

**INTRODUCTION**

Spectrins are components of the cortical cytoskeleton of many cell types, participating in diverse biological functions involving the cell membrane: membrane reinforcement, organelle maintenance, protein trafficking, regulation of exocytosis, maintenance of cell-cell junctions, and partitioning of cellular domains and polarity (Bennett, 1990; De Matteis and Morrow, 1998; Dubreuil and Grushko, 1998; Lippincott-Schwartz, 1998). As such, spectrins are essential in cell morphogenesis during differentiation and maintenance of cell membrane stability. The importance of spectrins in cell morphogenesis is underscored by studies of spectrin mutants in Drosophila and Caenorhabditis elegans (Deng et al., 1995; Dubreuil et al., 2000; McKeown et al., 1998; Thomas et al., 1998). Human mutations in erythroid spectrins SPTA1 and SPTB result in hemolytic anemia, classified by the abnormal cell shapes into hereditary spherocytosis (HS), elliptocytosis (HE) and pyropoikilocytosis (HPP) (Lux and Palek, 1997; Tse and Lux, 1999). Similarly, mouse mutants spherocytosis (sph/sph) and jaundiced (ja/ja) suffer from hemolytic anemia and harbor mutations in erythroid α-spectrin (Spnα1) and β-spectrin (Spnβ1), respectively (Bloom et al., 1994; Bodine et al., 1984; Brookoff et al., 1982; Wandersee, 1998).

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identified, including SPTB, which has an erythroid isoform (βE1) and a brain-muscle isoform (βE2), SPTBN1 (βH- spectrin), which is non-erythroid and found abundantly in the brain, and SPTBN2 (βH-spectrin), which is associated with the Golgi (Hu et al., 1992; Stankewich et al., 1998; Winkelmann et al., 1990a,b). Additionally, a novel βH-spectrin of over 400 kDa has been described in Drosophila and C. elegans, and more recently in humans (βV-spectrin) (Dubreuil et al., 1990; McKeown et al., 1998; Stabach and Morrow, 2000; Thomas et al., 1998). Most of these β-spectrin genes, and isoforms thereof, encode proteins containing a N-terminal actin-binding domain (ABD), 17 spectrin repeats and a C-terminal pleckstrin homology (PH) domain. βH-spectrin shares the same domain arrangement but has 30 spectrin repeats, where repeat 5 is homologous to SH3 domain. Importantly, the well-studied erythroid β-spectrin of mouse (Spnb1) and human (SPTB, βE1 isoform) are unique in that the PH domain is replaced with a short C-terminal tail by alternative splicing (Chu et al., 1994).

Studies of spectrins in mammalian red cells suggest that the α- and β-spectrins associate in an anti-parallel manner, followed by self-association of the αβ dimer to form a α2β2 tetramer (reviewed in De Matteis and Morrow, 2000; Dubreuil and Grushko, 1998; Lux and Palek, 1997; Viel and Branton, 1996). This rod-like tetramer interacts with other cytoskeletal proteins primarily through several modular domains of the β subunit. β-spectrin is responsible for binding of ankyrin (via repeat 14 and 15) and protein 4.1 (via N-terminal region), which anchor the spectrin tetramers to the cell membrane by attaching to transmembrane proteins band 3 and glycophorin C, respectively. The ABD of β-spectrin mediates crosslinking of actin microfilaments at the ends of extended α2β2 rods.

As a specialist in circulatory gas exchange, the vertebrate erythrocyte has evolved morphological features to withstand the mechanical deformation stresses of circulation. During vertebrate red cell development, the immature pro-erythroblasts are spherical with open chromatin, and develop in close association with stromal cells (Dieterlen-Lievre, 1975; Liao and Zon, 1999; Zon, 1995). In most vertebrates, terminal erythroid differentiation into circulating red cells is accompanied by morphogenetic changes, characterized by condensed chromatin, ovoid nucleus and a biconcave elliptical cell shape (Glomski et al., 1992, 1997). In mammals, the mature erythrocytes are biconcave, spherical and enucleated (Cohen et al., 1998; Koury et al., 1987). Vertebrate erythrocyte membrane is supported by an underlying cytoskeleton consisting of several major structural proteins: actin, adducin, α-spectrin, β-spectrin, ankyrin, band 3, protein 4.1 and other accessory proteins. In addition, the vertebrate red cell membrane is reinforced with a bundle of microtubules termed the marginal band (MB), which circumscribes the cortical membrane in the horizontal plane of the cell (Cohen et al., 1990; Ginsburg et al., 1989; Glomski et al., 1992; Goniatkowska-Witalinska and Witalinski, 1976). The MB is thought to confer structural support for the cell membrane and impart vertebrate red cells with their elliptical cell morphology. Organization of microtubule bundles into MB is not found in red cells of most mammals (Abdo et al., 1990; Cohen et al., 1998; Repasky and Eckert, 1981). It is not known whether the spectrin-associated actin cytoskeleton interacts with the MB, and how this interaction may affect cell shape and membrane stability.

The zebrafish Danio rerio has been used as a vertebrate genetic system in the study of hematopoiesis. Large-scale chemical mutagenesis screens have led to the identification of mutants affecting various aspects of blood formation, including stem cell, erythroid and lymphoid development (Orkin and Zon, 1997; Paw and Zon, 2000; Trede and Zon, 1998). The mutant riesling (ris) was identified by a marked reduction in the number of circulating cells observed 4 days post-fertilization (dpf). Initial phenotype studies classified ris in a group of eight mutants with decreasing blood counts, all characterized by normal initiation of embryonic hematopoiesis followed by a precipitous decrease in the number of circulating blood cells after 3 dpf (Ransom et al., 1996). Within this group of decreasing blood count mutants, the phenotype of ris is most similar to that of cabernet (cab), chablis (cha), merlot (mot) and retsina (ret), with regard to the onset and severity of the blood defect.

We report here the cloning of ris and identification of zebrafish β-spectrin, hereafter referred to as spth. We show that the defect in ris erythrocytes is analogous to that of human hereditary spherocytosis, with the added features of apoptosis and MB microtubule reduction. This is the first study of erythroid β-spectrin gene in a non-mammalian vertebrate, and illustrates the evolution of Spth structure and function in erythroid morphogenesis.

MATERIALS AND METHODS

Meiotic and radiation hybrid mapping

The risTB237 allele was generated on the Tübingen (Tü) strain in a large-scale chemical mutagenesis screen (Ransom et al., 1996). risTB237 allele was crossed to standard wild-type strain AB for maintenance, and polymorphic strains DAR and SJID for genetic mapping (SJD strain was a gift from Steve Johnson, St Louis, MO). Embryos were collected from hybrid (AB/DAR or AB/SJID) diploid ris/+ , scored for anemic phenotype at 4 dpf, frozen dry in 96-well PCR plates and stored at –20°C. Embryo DNA was extracted as described (Zhang et al., 1998). Primer sequences of simple strand length polymorphism (SSLP) markers used in mapping (shown in Fig. 1) were obtained from the Massachusetts General Hospital Zebrafish Server website (http://zebrafish.mgh.harvard.edu), and synthesized by Research Genetics or GibcoBRL. SSLP analysis was performed as described (Liao and Zon, 1999a,b). Half-tetrad analysis to assign chromosomal linkage was performed as described (Johnson et al., 1995). Radiation hybrid (RH) mapping was carried out as described (Geisler et al., 1999), using primers F (5'-TGACTTGGGAGAGACTTCACGC3'-G) and R (5'-GGATGCTTGTGGTGAGGAGG-3'), and typed on the Goodfellow RH panel (Kwok et al., 1997).

Isolation of cDNA clones

A partial 2.3 kb cDNA of zebrafish spth (BScd) was fortuitously identified in a screen for an unrelated gene from a late-somite oligo-dT cDNA library (gift from D. Gunwold, University of Utah, Salt Lake City, UT). Additional spth cDNAs were identified from an adult zebrafish kidney oligo-dT cDNA library by hybridization with clone BScd. The largest partial cDNA clones BS12 and BS31 (4.6 kb) were obtained from over 70 tertiary positives, out of over 3 million total phage plaques screened. Further 5’-full length sequence (3.5 kb) was obtained by RT-PCR from total embryo (23 hpf) RNA, using the SMART-RACE cDNA Amplification Kit (Clontech) with a 3’ gene-specific primer gsp238 (5'-CTGGGCCCTCCTCACGCGAGAAATT-
CAAC-3'). Protein sequence prediction and alignment were performed using DNASTAR Megalign software, applying Clustal method with PAM250 residue weight table.

**Mutation analysis using RT-PCR and allele-specific oligonucleotide (ASO) hybridization**

For mutation analysis, total RNA was isolated from homozygous embryos (from *ris/ris* intercross) at 23 hpf, reverse transcribed (Superscript II reverse transcriptase, GibcoBRL) and amplified using high-fidelity Taq polymerase mix from the Advantage2 PCR Enzyme System (Clontech). The full-length *sptb* sequence from 5'-UTR to 3'-UTR was covered by five overlapping fragments (primer sequences used to amplify these fragments will be provided upon request). ASO hybridization was used to confirm that the observed mutation from sequence analysis of *ris* RT-PCR clones was in fact present in the genomic DNA. A 250 bp fragment spanning the mutated nucleotide position was amplified from three wild-type and three *ris/ris* genomic DNA. A 250 bp fragment spanning the mutated nucleotide sequence analysis of *ris* hybridization was used to confirm that the observed mutation from used to amplify these fragments will be provided upon request). ASO sptb high-fidelity Taq polymerase mix from the Advantage2 PCR Enzyme (Superscript II reverse transcriptase, GibcoBRL) and amplified using intercross) at 23 hpf, reverse transcribed *ris/ris* embryos (from *ris/ris* animals using primers F (5'-GGTGAATCTTTGGGTTGAGGC-3') and R (5'-ATTCAAGGTGGCTACAGCTTCTG-3'). ASO hybridization and washes were performed essentially as described (Farr et al., 1988), with γ-P³² end-labeled wild-type ASO (5'-CTTGCCAGAGCGGGACAGAGATG-3') and ris ASO (5'-CTTGCCAGATAGGAAGATG-3').

**In situ hybridization, o-dianisidine staining, histological analysis and TdT-mediated dUTP nick-end labeling (TUNEL) assay**

In situ hybridization and riboprobe synthesis were performed as described (Schulte-Merker et al., 1992), with modifications (Liao et al., 1998). Antisense riboprobes were synthesized using either the BScd (+5412 to poly A tract), BFI1/238 (+130 to +3084) or BSPH (+6615 to +7054) clones. The BFI1/238 clone was obtained by 5'RACE. The BPH clone containing only the *sptb* PH domain was generated using the following primers: F: 5'-ATGGAGGGAACTC-TAGCCCGCAAG-3'; R: 5'-TTCTTCCTAAGCAAACGACTAAA-3'. Staining of hemoglobin by o-dianisidine was performed as previously described (Detrich III et al., 1995). Histological analysis of the embryonic blood, adult peripheral blood and kidneys was performed as described by Ransom et al. (1996). TUNEL assays on whole embryos was performed as described by Abellallah et al. (1996), employing Apoptosis Detection Kit-Peroxidase (Oncor). TUNEL assays on adult tissues were performed on freshly collected blood or kidney preparations (Ransom et al., 1996), fixed in 4% paraformaldehyde and assayed using the Apoptosis Detection System, Fluorescein (Promega).

**Antibodies, immunohistochemistry and western blot analysis**

Polyclonal antisera (anti-BSc) was generated by challenging rabbits with a GST-fusion protein (GST-ri5) consisting of zFS repeats 15 and 16 (Charles River Pharmaservices). The GST-ri5 construct was generated by PCR using primers from +5434 (F: 5'-GTTGAATCTTTGGGTTGAGGC-3') to +5749 (R: CGGAATCTGGCTGAGCTGCTTTTCCTCTTCAGGC-3'), digested with EcoRI and XhoI, and cloned in frame into pGEX-6P-1 vector (Amersham Pharmacia). GST-fusion protein was produced in *E. coli* and purified over glutathione sepharose column prior to immunization (Charles River Pharmaservices). Whole-mount immunohistochemistry was performed essentially as described but without affinity purification (Schulte-Merker et al., 1992), with anti-BSc (1:300) followed by anti-rabbit HRP (1:300). Western blot analysis was performed on whole-blood lysates collected from single adult wild-type, *ris/+* and *ris/ris* fish, and luminol-developed using the ECL western blotting system (Amersham Pharmacia).

**Electron-microscopy (EM) analysis**

Whole blood collected from adult wild-type, *ris/+* and *ris/ris* fish was washed in chilled 1× PBS, fixed in 2% glutaraldehyde/0.1 M cacodylate buffer for 1 hour on ice, washed in 1× PBS and processed for scanning EM as previously described by Peters et al. (1992). Transmission EM was performed on sections of wild-type and *ris/ris* embryos at 4 dpf, as described (Zapata, 1980).

**GenBank Accession number**

AF262336 for full-length zebrafish *sptb* cDNA and protein sequence.

**RESULTS**

**Embryonic and adult blood defects in *ris***

One autosomal recessive allele *ris* was recovered in a large-scale ENU mutagenesis morphological screen (Haftter et al., 1996). The observed phenotype was a marked decrease of circulating blood cells during embryogenesis, which can be demonstrated by staining for hemoglobin with o-dianisidine (Fig. 1A). Histological analysis of *ris* embryonic blood revealed abnormal cell shape and increased number of nucleated cells (Fig. 1B). Hematopoietic differentiation appeared normal in *ris*, where the expression of genes corresponding to stem cell (scf) and early (gata-1) and terminal (alas-e) stages of blood formation were unaffected (Fig. 1C). Surprisingly, although *ris* embryos were profoundly anemic at 4 dpf, they could be raised to adults if care was exercised with water quality and feeding.

Peripheral erythrocytes of *ris* adults were severely malformed, with tear-drop or spherocytic cells containing nuclei that fail to condense fully (Fig. 2). The distorted shape of the mutant red cells with open nuclei reflected both aberrant cell morphogenesis and membrane instability. The number of cells in circulation of *ris* adults was also significantly less than wild type, apparent from blood collection where the wild-type blood had a deep red color but the *ris* blood was pale (data not shown). Furthermore, the kidney (organ of hematopoiesis) in *ris* was at least fivefold larger than that of wild type and extremely hyperplastic, containing large numbers of erythroid precursors (Fig. 2). The cardiac chambers of the adult *ris* homoyzogotes become dilated over time, indicative of cardiac compensation for the anemia (data not shown). The same histological analysis when applied to *ris* heterozygotes did not reveal any defects.

Together, the observed phenotypic features of *ris* homoyzogotes suggest that erythrocytes terminaly differentiate but assume an abnormal cell morphology. The mutant red cells presumably undergo hemolysis, resulting in severe anemia. As a compensatory response, the kidney marrow increases erythropoiesis, as reflected by the marked hyperplasia.

**Zebrafish *sptb* is the gene defective in *ris***

Genetic mapping was initiated to uncover the molecular defect underlying the *ris* mutation, and to provide insight into the defects of the other blood mutants (*cab, cha, mot* and *ret*) that exhibit a similar hematopoietic phenotype as *ris*. Using half-tetrad analysis, *ris* was found to be linked to the centromeric markers srs12 and z381 of linkage group (LG) 17. Subsequent linkage analysis with LG 17 SSLP markers refined the *ris* locus to a genetic interval flanked by z20006 (0.17 cM) proximally and z1408 (0.8 cM) distally (Fig. 3A). No recombinants were found between *ris* and z22901 and z11202 with analysis of 1148 meiotic events, suggesting a strong genetic linkage of less than 0.087 cM.
Examination of genes mapped to LG 17 revealed conserved synteny to human chromosome 14q, where the zebrafish and corresponding human genes of PAX9, OTX2, BMP4 and GSC are preserved in the same gene order. Closer examination of genes located on the human chromosome segment 14q suggested erythroid β-spectrin (sptb) as a candidate gene, a defect in which could cause the hematological phenotypes observed in ris. To genetically evaluate zebrafish sptb as a candidate gene, a defect in which could cause the hematological phenotypes observed in ris. To genetically evaluate zebrafish sptb as a candidate gene, a defect in which could cause the hematological phenotypes observed in ris, radiation hybrid mapping was carried out on closely linked SSLP markers z22901, z11202 and zebrafish sptb (Geisler et al., 1999). Typing of z22901 and sptb on the zebrafish radiation hybrid panel confirmed that they co-localized on LG17 within 15 cR of each other (LOD score=12.59). Together, the genetic data strongly suggested that sptb is the gene represented by the ris mutation. Consistent with this, RNA in situ analysis revealed that sptb expression in ris embryos was dramatically reduced (Fig. 3B), whereas other hematopoietic genes were expressed at wild-type levels (Fig. 1C).

A combination of kidney cDNA library screening and 5’-RACE PCR provided cDNA clones to assemble the full-length sptb cDNA sequence. RT-PCR and sequence analysis of sptb transcript from ris revealed a C to T transversion mutation at nucleotide +3130, changing Q(1044) to a premature stop codon (Fig. 3C). The mutation was confirmed in the genomic sequence of ris by ASO hybridization (data not shown). Polyclonal antiserum (anti-BSc) generated against recombinant Sptb repeats 15 and 16 specifically delineated erythrocytes by whole-mount immunohistochemistry, and detected a protein of approximately 250 kDa by western blot analysis of wild-type whole-blood lysates (Fig. 4). Consistent with the Q(1044)X mutation in risTB237, anti-BSc failed to detect Sptb product by whole-mount immunohistochemistry and western blot analysis of ris embryos and blood lysate, respectively.

**Phylogeny and conservation of Sptb**

Phylogenetic analysis of zebrafish Sptb and selected homologs from the fly, mouse and human predicted an ancient evolutionary divergence between mammalian erythroid and non-erythroid β-spectrin genes, consistent with intragenic duplication and divergence during evolution (Thomas et al., 1997). In fact, zebrafish and mammalian Sptb are more closely related than are β-spectrin genes within the mammalian species (Fig. 5A). Although erythroid β-spectrin protein of other non-mammalian vertebrates (such as avians) has been studied biochemically, its sequence has not been published to be included in comparison analysis. Primary protein sequence comparison of zebrafish Sptb to murine and human β-spectrin homologs revealed a high level of conservation, with highest overall identity of 62.3% to human.

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**Fig. 1.** Embryonic blood defects in ris. In this and all subsequent figures embryos are shown as lateral views, with anterior left and ventral down, at 10x magnification, unless specified otherwise. (A) Whole-mount o-dianisidine staining of wild-type and ris embryos at 4 dpf, showing absence of blood cells in ris (arrowhead). (B) Wright-Giemsa staining of embryonic blood cells collected from wild-type and ris embryos at 2.5 dpf. Wild-type primitive erythrocytes are spherical and nucleated, whereas ris primitive erythrocytes are often abnormally shaped (arrowhead) and binucleated (asterisk). 20x magnification. (C) In situ analysis of ris embryos at 23 hours post-fertilization (hpf), with antisense riboprobes of scl, gata-1 and alas-e. At 23 hpf, blood cells are restricted to the intermediate cells mass (ICM), dorsal to the yolk tube extension (arrow).

**Fig. 2.** Adult ris blood and marrow. Peripheral blood and kidney collected from wild-type and ris adults. Blood smear and kidney tissue preparation were Wright-Giemsa stained, and whole kidneys are shown in situ, situated in the retroperitoneal cavity of the adult animal (organ margin demarcated with broken lines). Mutant erythrocytes are spherical or tear-drop shaped, with round nuclei. More bi-nucleated cells are seen in the mutant (asterisk). In contrast, the wild-type red cells and nuclei are elliptical. Mutant kidney tissue is dramatically enlarged compared with wild type, with increased cellularity. Blood, gross kidney and tissue kidney are shown at 20x, 4x and 4x, respectively.
sptb is the defective gene in ris. (A) Genetic map of LG 17, showing a sample number of SSLP markers tested in the meiotic mapping (in red), and their relative distances from ris locus (in black). The number of recombinants/the total number of animals tested are indicated in parenthesis. (B) sptb whole-mount RNA in situ on wild-type and ris embryos at 23 hpf. Identical staining of ICM, myotube and somites is observed with riboprobes generated binding), and 69.8% in repeat 17 (ankyrin conservation between zebrafish and human, with 81.2% participate in protein interactions have higher levels of (domain II) between zebrafish and human SPTB is 60.5%, where a sample repeat (repeat 8) shares an identity of only 56.1%.

Of particular interest is the observation that zebrafish Sptb contains a C-terminal pleckstrin homology (PH) domain in blood cells, which is normally eliminated by alternative splicing of sptb in mammalian erythrocytes (Fig. 5C). Presence of the PH domain in zebrafish erythroid sptb transcript was confirmed by RNA in situ analysis using only the PH domain of sptb as probe, which exhibited an identical expression pattern as seen with riboprobes that included only the spectrin repeat domains (Figs 3B, 5B). Western blot analysis of whole blood lysate demonstrated a single product >220 kD, suggesting that a single isoform of Sptb containing the PH domain is found in zebrafish red cells (Fig. 4B). Additionally, the same zebrafish sptb transcript was expressed in the embryonic myotube within a discrete temporal window, between 23-28 hpf (Fig. 5B, arrow). Low level expression of sptb is also observed in the somites (Fig. 5B, *). Histological analysis of cardiomyocytes and skeletal muscle of ris embryos did not reveal any defects (data not shown). Homozygote ris adults are fertile and grossly normal, except for the aforementioned hematological abnormalities.

Hemolysis in ris is accompanied by MB reduction and apoptosis

To further investigate changes in ris spherocytes leading to hemolysis, ultrastructural analysis was carried out on red cells collected from wild-type, ris homozygote and ris heterozygote adult fish. Scanning EM examination of zebrafish erythrocytes demonstrated that the wild-type cell membrane was smooth, biconcave and elliptical, with a central bulge produced by the nucleus (Fig. 6). In contrast, red cells from ris homozygotes were markedly smaller and spherocytic. Additionally, the homozygote ris cell membrane has spikulated projections and surface pitting, features indicative of cytoskeletal fragility and membrane loss. Scanning EM analysis of blood collected from ris/+ revealed that the erythrocytes had irregular membrane surface and lacked biconcavity, but still maintained the elliptical shape (Fig. 6).

Transmission EM analysis of wild-type or ris/+ erythrocytes demonstrated the presence of MB in the cortical membrane, typically consisting of 20-24 microtubules per MB in differentiated erythrocytes (Fig. 6). In ris homozygotes, the MB forms and localizes to the cortical membrane. However, the number of microtubules per MB in ris spherocytes is reduced to half that of wild type, suggesting that the Sptb cytoskeleton is necessary for proper aggregation of microtubules into MB.

Since adult fish red cells are nucleated and fail to condense in ris, we explored whether fragmentation of the chromosomes by apoptosis is associated with membrane damage. TUNEL assays performed on ris embryos at 23 hpf and 48 hpf, failed to detect increased apoptosis in circulating cells (data not shown). In contrast, the few remaining blood cells pooled in the cardiac chamber of 4 dpf ris embryo had undergone apoptosis (Fig. 7A). Similarly, apoptosis was observed in ris adult peripheral blood, but to a much lesser extent in the kidney (Fig. 7B). These results suggest that apoptosis is associated with circulating erythrocytes that have accumulated membrane damage, and is not initiated during erythroid differentiation in the early embryo or kidney.
DISCUSSION

Hereditary spherocytosis in zebrafish

The ris phenotype is characterized by spherocytosis and severe anemia. Remarkably, marrow hyperplasia and cardiac dilatation are adequate compensatory responses that allow the profoundly anemic embryos to reach adulthood. Genetic mapping of ris and examination of conserved synteny between zebrafish and humans suggested sptb as a candidate gene, as human SPTB mutations result in HS, a red cell membrane disorder with similar phenotypic features as observed in ris. A SSLP marker less than 0.087 cM from the ris locus co-localizes with sptb by RH mapping. Sequence analysis of sptb from ris reveals a C to T transversion mutation, resulting in premature termination in the middle of the transcript. Consistent with this nonsense mutation, the steady-state sptb transcript level is reduced in ris, which is probably a consequence of nonsense-mediated mRNA decay (Culbertson, 1999; Frischmeyer and Dietz, 1999; Lew et al., 1998; Ruiz-Echevarria et al., 1998). Likewise, anti-BSct polyclona failed to detect Sptb protein in ris embryos and adult blood. These results suggest that risTB237 represents a null phenotype of sptb.

The ris mutation is similar to that described in the jalfja mouse in several ways. Both ris and jalfja exhibit hallmarks of HS, where the erythrocytes become spherical and undergo hemolysis. At the molecular level, both ris and jalfja mutations are caused by premature termination changes in the middle of the erythroid β-spectrin gene, R(1099)X in Snpb1 of jalfja, and Q(1044)X in Sptb of ris (Bloom et al., 1994). Both ris and jalfja represent null mutations of erythroid β-spectrin, where the transcript level is reduced and the protein product is absent in the homozygote mutant animals (Bloom et al., 1994; Bodine et al., 1984). Compensatory responses of marrow hyperplasia and cardiac dilatation are seen in both ris and jalfja. Like the jalfja mouse, hematopoietic tissue of ris is filled with erythroid precursors, indicative of efficient erythropoiesis. Therefore, the
continued anemia in *ris* is a consequence of rapid hemolysis of differentiated erythrocytes in circulation. Non-hematopoietic tissues that express erythroid β-spectrin do not appear to be affected in either *ris* or *jalja* (Kaysser et al., 1997). Histological analysis of cardiomyocytes and muscle cells from *ris* homozygotes did not reveal any defects (data not shown). Furthermore, both *ris* and *jalja* are recessive mutations, whereas most human reports of SPTB defects are inherited in a dominant manner (Lux and Palek, 1997; Mailet et al., 1996; Tse and Lux, 1999).

Unlike *jalja* mice, which rarely survive a few days after birth, *ris* homozygotes can survive to adulthood. This most probably reflects physiological differences between the mouse and zebrafish. In fact, it has been suggested that the neonatal lethality of the *jalja* mouse is a result of kidney failure, secondary to the overwhelming iron accumulation that results from severe hemolysis (Kaysser et al., 1997). Zebrafish iron metabolism may be sufficiently different from the mouse, such that renal failure caused by secondary hemochromatosis does not result. Alternatively, the *ris* homozygotes that survive to adulthood may be a consequence of breeding selection. Each given clutch of *ris* homozygote embryos is less viable than wild-type embryos, where only 5 to 10% of the *ris* clutch grow to reproductive adults. Once mature, the lifespan of the *ris* homozygotes is indistinguishable from wild type. These adult *ris* survivors suffer from severe anemia, but have been selected for additional genetic traits that are compatible with their maturation.

Morphological differences between zebrafish and mammalian erythrocytes preclude direct comparisons of the spheroctytic morphology observed in *ris* to that of *jalja* mouse or human HS. Nonetheless, the *ris* defect supports the regulatory role of β-spectrin in cytoskeletal assembly, where β-spectrin protein is the limiting component (Bodine et al., 1984; Geiduschek and Singer, 1979; Lehner and Lodish, 1988; Moon and Lazarides, 1983). The dosage effect of Sptb deficiency was reflected by the morphological changes of *ris/+* and *ris/ris* red cells. With reduced Sptb levels in *ris/+* animals, the biconcavity of red cells was lost and the membrane surface appeared irregular, but the MB and elliptical shape of the cell were maintained. With Sptb completely absent in *ris/ris*, the red cell became smaller and spheroctytic, microtubule aggregation into the MB was compromised and cell membrane was lost in spiculated projections. These phenotypes underscore the importance of Sptb concentration in morphogenesis and membrane stability, where reduction of Sptb results in altered cell shape and absence of Sptb leads to membrane loss.

**Role of PH domain in erythroid morphology and membrane integrity**

The zebrafish Sptb protein in red cells is distinct from the mammalian SPTB β1 protein, as its PH domain is not replaced with a short C-terminal tail (Winkelmann et al., 1990a,b). The alternative splicing activity that removes the PH domain from the transcript of mammalian erythroid β-spectrin is unique to erythroid differentiation (Chu et al., 1994). PH domains from human SPTB β1 isoform and SPTBN1 proteins have been shown to associate with the cell membrane in an ankyrin-independent manner (Lombardo et al., 1994). PH domains have also been implicated in localizing several general signaling components to the cortical membrane, such as inositol-(1,4,5)-trisphosphate, phosphotidylinositol-(4,5)-bisphosphate and βγ G proteins (Lemmon et al., 1996; Touhara et al., 1994). Nevertheless, the functional significance of removing PH domain from Sptb in the erythrocyte is not known.

Zebrafish Sptb is the first example of PH-domain-containing erythroid β-spectrin in a vertebrate red cell. The presence of PH domain in zebrafish Sptb may have a number of structural consequences on the overall geometry of the cortical cytoskeleton, since the PH domain could make direct contact with membrane lipids, or cause steric disruption of αβ dimer self-association. These conformational changes could manifest morphologically, conferring fish red cells with an elliptical shape. Notably, human red cells do become elliptical in hereditary elliptocytosis, when self-association of spectrin αβ dimer is disrupted (Mailet et al., 1996; Nicolas et al., 1998; Tse and Lux, 1999).

**Sptb and microtubule aggregation**

Scanning and transmission EM analysis of *ris* red cells demonstrated membrane instability, where membrane loss and defective MB formation were observed. These structural changes confirm that Sptb is essential for the proper assembly of a cortical cytoskeleton that consists of actin filaments and microtubules. Absence of Sptb in *ris* led to an abnormal cell
morphology and a fragile membrane, where the MB microtubule filaments failed to from properly and the actin cytoskeleton was not crosslinked by spectrin tetramers. These abnormally shaped cells were vulnerable to structural stress during circulation, where the accumulation of membrane damage led to hemolysis.

Recent work has shown that spectrin interacts with microtubule-based motors dynein/dynactin to mediate transport of membrane proteins from the Golgi apparatus (Burkhardt, 1998; De Matteis and Morrow, 1998; Devarajan et al., 1997). The N-terminal domain I of β-spectrin has been implicated to contain a Golgi targeting signal, acting in a proposed spectrin-ankyrin-adaptor protein trafficking system (SAATS) (Devarajan et al., 1997). Furthermore, PH domain has also been shown to be important in association of β-spectrin to the Golgi, where ADP-ribosylation can modulate spectrin recruitment by altering phosphatidylinositol 4,5-bisphosphate levels in the Golgi (Godi et al., 1998). Although these studies were not carried out in erythrocytes, they do demonstrate an interaction between spectrin and the microtubule.

Taken together with our observation that aggregation of microtubule filaments to form the MB is compromised in zebrafish red cells lacking Sptb, we propose that Sptb may be directly involved in organizing the microtubule for MB formation. Accordingly, Sptb is a central protein acting at the physical interface between actin and microtubule-based cytoskeletal elements. Therefore, zebrafish red cells may offer an unique system where interaction between actin and microtubule associated cytoskeletal proteins can be studied, in the context of Sptb. Here, specific mutations in the cytoskeleton represented by ris and other zebrafish mutants allow useful assessment of the specific roles of the proteins involved.

**Sptb and apoptosis**

TUNEL assays of ris embryos and adult tissues showed that apoptosis occurred in circulating red cells, but not in developing erythroid progenitors. This suggests circulatory deformation suffered by ris red cells may be the trigger for apoptosis. Alternatively, apoptosis of circulating spherocytes may be due to a lack of response to growth factor stimulation. The spherocytes may fail to receive survival signals that use phosphoinositol 4,5-bisphosphate levels in the Golgi (Godi et al., 1998). Although these studies were not carried out in erythrocytes, they do demonstrate an interaction between spectrin and the microtubule.

Moreover, ris is one of five mutants that have a decreased blood-count phenotype: others include cab, cha, mot and ret. Human HS can also be caused by defects in erythroid α-spectrin, ankyrin, protein 4.1 and band 3. In fact, cloning of the ret mutant identified the zebrafish band3 gene (B. H. P., unpublished). These results suggest that the zebrafish decreasing blood count mutants represent genes encoding various structural components of the erythroid membrane cytoskeleton. Cloning of the remaining members of this class of mutants will probably identify known structural partners such as erythroid α-spectrin, ankyrin and protein 4.1. Alternatively, the remaining mutants may lead to the identification of novel proteins that interact with the membrane cytoskeleton and are important for membrane stability.

Cloning of ris adds to the growing list of zebrafish mutations that are relevant to human disease, several of which are involved in hematopoiesis: sauternes (alas-e defect in sideroblastic anemia), yquem (urod defect in porphyria) and retsina (band3 defect in HS) (Brownlie et al., 1998; Wang et al., 1998; B. H. P., unpublished). Cloning of ris also demonstrates the utility of comparative syntenic, which was also applied in the cloning of you-too (gli2) (Karlstrom et al., 1999). Comparative genomics approaches to identify zebrafish genes will be greatly facilitated by the completion of the human genome sequencing. Recent identification of novel genes involved in zebrafish organogenesis may have congenital human disease correlates that remain to be defined (Donovan et al., 2000; Zhang et al., 1998; Zhong et al., 2000). These studies highlight the maturation of zebrafish as a vertebrate genetic system for gene discoveries that are important for both normal development and human disease.

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