A 90° rotation of the mitotic spindle changes the orientation of mitoses of zebrafish neuroepithelial cells

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SUMMARY

In the neural plate and neural tube in the trunk region of the zebrafish embryo, dividing cells are oriented parallel to the plane of the neuroepithelium, while in neural keel/rod, cells divide perpendicular to it. This change in the orientation of mitosis is brought about by a 90° rotation of the mitotic spindle. As the two halves of the neural primordium in keel/rod stage are in apposition, the perpendicular orientation of mitoses in this stage determines that daughter cells become allocated to both sides of the neural tube. To assess the role played by cell junctions in controlling the orientation of dividing cells, we studied the expression of components of adherens and tight junctions in the neuroepithelial cells. We find that these proteins are distributed irregularly at the neural plate stage and become polarised apically in the cell membrane only during the keel/rod stage. The stereotypic orientation of mitoses is perturbed only weakly upon loss of function of the cell junction components ASIP and aPKCα, suggesting that mitotic orientation depends in part on the integrity of cell junctions and the polarity of the epithelium as a whole. However, the 90° rotation of the spindle does not require perfectly polarised cell junctions between the neuroepithelial cells.

Movies available online

Key words: Zebrafish, Neurulation, Mitoses, Orientation, Spindle rotation, Neuroepithelial polarity, ASIP, Histone2A.F/Z:GFP, tau:GFP

INTRODUCTION

The zebrafish, like other teleosts, undergoes secondary neurulation (Kimmel et al., 1990; Kimmel et al., 1995; Schmitz et al., 1993). In the course of the convergence movements that follow gastrulation, the neural plate folds inward at the midline to form the so-called neural keel. The neural keel eventually rounds up and detaches from the adjacent epidermis to form the neural rod. The neurocoel, the lumen of the neural tube, forms secondarily by cavitation of the neural rod, when the neuroepithelial cells retract their apical processes. As the two halves of the neural primordium are in apposition in the midline at keel/rod stage, dividing cells very frequently contribute progeny to both sides of the developing neural tube (Kimmel and Warga, 1986; Kimmel et al., 1990; Kimmel et al., 1994; Schmitz et al., 1993; Papan and Campos-Ortega, 1994; Papan and Campos-Ortega, 1997; Papan and Campos-Ortega, 1999: Concha and Adams, 1998). A bilateral distribution of clonally related cells within the neural tube is a specific feature of the zebrafish, and perhaps of other animal species with secondary neurulation; it has not been observed in frogs, newts or chicken (Hartenstein, 1989; Hartenstein, 1993; Lumsden et al., 1994; Soula et al., 1993; Artinger et al., 1995; Leber and Sanes, 1995). Sometimes the cells of the neural plate give rise to clones of very similar composition on both sides of the neural tube. However, more frequently the siblings of a given clone differentiate into different cell types on either side (Papan and Campos-Ortega, 1997; Papan and Campos-Ortega, 1999).

During neurulation, mitotic divisions of neuroepithelial cells are polarised. On the basis of single cell labelling experiments, Kimmel et al. (Kimmel et al., 1994) reported that mitotic orientation of dividing neural progenitor cells changes from preferentially anteroposterior at embryonic division 15 to mediolateral at division 16, this latter mitosis leading to bilateral segregation of progeny cells. Papan and Campos-Ortega (Papan and Campos-Ortega, 1994) followed fluorescein-labelled neural plate cells that gave rise to progeny disposed on either side of the midline of the neural rod. Concha and Adams (Concha and Adams, 1998) used time-lapse microscopy and Nomarski optics to follow individual cells in the epiblast during gastrulation and early neurulation stages, and showed that mitoses in the neural plate are planar, oriented parallel to the apical surface of the epithelium, and occur initially in a preferred anteroposterior direction. However, at the onset of neural plate infolding, mitoses were oriented preferentially in the mediolateral direction. In addition, Concha and Adams (Concha and Adams, 1998) concluded that ‘The orientation of cell division for those cells that will contribute to the CNS will tend towards several mutually orthogonal alignments during the course of gastrulation and neurulation.’.

To follow the behaviour of zebrafish neuroepithelial cells in
vivo, we established strains carrying stable transgenic insertions of the zebrafish histone2A::F/Z gene fused to a DNA fragment encoding the green fluorescent protein (GFP) (Pauls et al., 2001). Expression of the Histone2A:F/Z::GFP fusion provides an ubiquitous chromatin marker from the 7th-8th cleavage onwards. To visualise mitotic spindles directly, we injected mRNA encoding tau::GFP. We find that mitotic spindles show a stereotyped orientation throughout neurulation, lying parallel to the plane of the neuroepithelium. However, we find that in divisions during the keel/rod stage the mitotic spindle itself rotates by 90° so that it lies perpendicular to the apical surface; the ensuing divisions are therefore oriented orthogonally.

A rotation of the mitotic spindle has also been observed at two different instances in neural development in Drosophila. Neuroectodermal cells undergo planar mitoses, but in delaminated neuroblasts the spindle rotates so that it is perpendicular to the neuroectoderm (Kalthschmidt et al., 2000). Similarly, in the lineage of sensory bristles, the progenitor cell pla divides within the plane of the epithelium, whereas the spindle in pla changes its orientation and the cell divides along the apico basal axis (Gho and Schweissguth, 1998; Roegiers et al., 2001). Recently, Lu et al. (Lu et al., 2001) and Le Borgne et al. (Le Borgne et al., 2002) presented evidence for a role of junctional proteins in controlling the shift in spindle orientation in the divisions of neuroblasts and pIb cells, respectively.

In Caenorhabditis elegans, a protein complex consisting of two PDZ proteins, PAR-3 and PAR-6, and an atypical protein kinase C (aPKC) controls the polarity of the first cleavages (Kemphues et al., 1988; Cheng et al., 1995; Guo and Kemphues, 1996). This complex is phylogenetically conserved, and is localised at the adherens junctions of neuroepithelial cells of vertebrates and in the subapical region of Drosophila epithelial cells (Horne-Badovinac et al., 2001; Manabe et al., 2002) (for reviews, see Ohno, 2001; Wodarz, 2002). ASIP (aPKC specifically interacting protein), a vertebrate homologue of PAR-3, contributes to the control of epithelial polarity (Izumi et al., 1998; Suzuki et al., 2001). A similar role has been postulated for the vertebrate homologue Bazooka (Kuchinke et al., 1998; Wodarz et al., 1999; Wodarz et al., 2000) (see also Müller and Wieschaus, 1996). We have analysed the expression of a zebrafish ASIP homologue, as well as that of aPKC/ and several other apical markers in the neuroepithelium. We find that these markers become apically localised for the first time late in the neural keel stage. The orientation of the cleavage plane is weakly but significantly impaired following injection of morpholinos directed against ASIP. Defects in mitotic orientation of similar degree are found in heart and soul mutants (hasm129) (Schier et al., 1996; Stanier et al., 1996; Malicki et al., 1996), which express an inactive aPKCa (Horne-Badovinac et al., 2001; Peterson et al., 2001). Therefore, the rotation that the mitotic spindles undergo in dividing neural keel/rod cells appears not to require firmly established cell junctions.

**MATERIALS AND METHODS**

**Animals**

Zebrafish embryos were obtained from spontaneous spawnings. The embryos were staged according to Kimmel et al. (Kimmel et al., 1995). For time-lapse movies, embryos were kept in a modified version of Embryo Rearing Medium (ERM) (Westefield, 1994). Embryos were anesthetised with MS222, manually dechorionated and placed in holes cut in a thin layer of 1.2% agarose gel (in ERM) supported by a coverslip [see Cooper et al. (Cooper et al., 1999)] (for a detailed description of the procedure). We used embryos of three different genotypes: wild type, animals with the insertions Tg(H2A.F/Z)ka37 and/or Tg(H2A.F/Z)ka66, and heart and soul embryos [allele m129, a premature stop codon leads to a C-terminal deletion of 73 amino acids (Horne-Badovinac et al., 2001)] carrying the insertion Tg(H2A.F/Z)ka37 (hasm129/has129; Tg(H2A.F/Z)ka37/ka37).

**Multilevel confocal time-lapse imaging and statistical analysis**

An LSM 410 confocal microscope (Zeiss) attached to an inverted Zeiss Diavert microscope with 40× and 63× immersion objectives was used to collect up to 12 different vertical stacks of images (z-series) at intervals of 45 or 180 seconds, each stack separated by between 0.75 and 7 μm from the next, depending on the experiment. z-series were transferred to a Macintosh Power PC for image processing using NIH-Image and Adobe Photoshop 6.0. Anaphase orientation was determined as described by Concha and Adams (Concha and Adams, 1998) for cytokinesis. The midline of the neural plate was first labelled on each frame and its mediolateral extent was subdivided into three equal parts. The orientation of each anaphase was determined by drawing a line parallel to the plane of cleavage and projecting it to the midline. The angles subtended at the midline of the neural plate were measured and mitoses within each region of the plate were grouped in intervals of 10°. Circular statistics for multimodal samples (Batschelet, 1981) were employed. Angles were measured on both sides of the neural plate and data were doubled to obtain a circular distribution of the angles. The average length of the vector was calculated and a Raleigh significance test (Batschelet, 1981) was applied.

Orthogonal sections (z-scans) of the neural plate of experimental embryos were obtained from sequences of 250 frames each (60 second intervals). A line was determined across the neural plate, and all sections from each sequence were referred to that line and transformed by the software of the confocal microscope.

**Cloning of a zebrafish ASIP homologue and design of morpholinos**

Based on the published 319 bp sequence of the zebrafish EST clone fe4811.1 (dbEST Id: 3215681), we designed the PCR primers 5′-TATCGTAGTCTTCTCGTGATG-3′ and 5′-CAGTAAGGGAC-AACTGGGAT-3′ to amplify a 276 bp fragment encoding amino acids 203 to 294 of the predicted ASIP homologue. A cDNA library was screened using this PCR fragment as a probe and a 3.8 kb cDNA clone was obtained. Alignment of this sequence with the corresponding sequences from human and rat revealed that the cDNA clone contained the complete coding region of a zebrafish ASIP gene, specifying a protein of 1127 amino acids (Accession Number AF465629).

A morpholino-modified antisense ASIP oligonucleotide (Nasevicius and Ekker, 2000), designed according to the manufacturer’s recommendations, was obtained from Gene Tools, LLC. The ‘standard control oligonucleotide’ provided by the manufacturer was used as a negative control. Their sequences were as follows: ASIP-Morpholino, 5′-ACACCGTCATCTTCTTACAGTCTCCACAC-3′; standard control oligo, 5′-CTTATATTGTACCAATTT-ATA-3′. Stock solutions of the ASIP morpholino (5 mM; 41.7 ng/μl) and the standard control oligo (1 mM) were prepared by resuspension in water. Stock solutions were diluted to working concentrations ranging from 2.6 ng/μl to 20.9 ng/μl in 1× Danieau medium containing 0.2% Phenol Red, and 5 nl aliquots of the different working solutions
were injected into the yolk of Tg(H2AF/Z)kca57 and Tg(H2AF/Z)kca66 zygotes. To test their efficacy in blocking translation, the morpholino was also injected (10.4 ng/ml) into wild-type zygotes together with mRNA encoding ASIP:GFP. Western blots were incubated with rabbit anti-GFP antibody (Torrey Pine Biolabs, Houston) and goat anti-rabbit HRP. Antibodies were detected by chemiluminescence ECL (Amersham Life Science, Arlington Heights, IL).

**GFP fusion proteins and mRNA injections**

For mRNA injections, four different constructs were synthesised, three of which encoded variants of ASIP fused to the enhanced variant of GFP (EGFP) (Cormack et al., 1996), while the fourth coded for tau:EGFP. An expression vector was made by cloning the EcoRI-XhoI fragment encoding the enhanced GFP variant from the pEGFP-C1 vector (Clontech Laboratories, Accession Number U55763) into the SalI-XhoI-digested pCS2+ vector (Turner and Weintraub, 1994). The coding region of zebrafish ASIP was amplified by PCR from the full-length cDNA clone using the primer pair ASIP-5EC 5'-TCGGAAATTCGTTGAACATATG-3', ASIP-3Sal 5'-AAGCCGTCA-ACGTACCCTGAAG-3'.

The PCR fragment was digested with EcoRI/SalI and cloned into the EcoRI/SalI-digested pEGFP-N3 vector (Clontech Laboratories, Accession Number U57609). The ASIP fragment was excised with EcoRI/Xmal and cloned into the EcoRI/Xmal-digested pCS2+EGFP vector, resulting in a construct encoding amino acids 1-1127 of ASIP fused to the N terminus of EGFP. Starting from this full-length asip:egfp clone, we made the following pair of deletion constructs. The codons for amino acids 688-1127 were deleted, resulting in a construct (asip-A37:egfp) encoding a fusion of residues 1-687 to the N terminus of EGFP; and a fragment encoding the C-terminal part of ASIP (asip-A557:egfp) was cloned into the pCS2+EGFP vector, resulting in a construct encoding a fusion of amino acids 658-1127 to the N terminus of EGFP. For the tau:egfp construct, a EcoRI-XhoI fragment encoding a bovine tau fused to EGFP from pCaspeR (UAS:tau-mEGFP), kindly provided by Andrea Brand and Nick Brown (Cambridge, UK) (see Kaltschmidt et al., 2000), was cloned into the pCS2+ vector.

Capped RNA was synthesised in vitro by transcription with SP6 polymerase from the constructs described above. mRNAs were injected in 5 nl aliquots (2.5 ng) into the yolk of zygotes of either wild type or Tg(H2AF/Z)kca57 and/or Tg(H2AF/Z)kca66.

**Immunohistochemistry**

For immunohistochemistry, we used the following antisera: rat polyclonal anti-PKCζ (C20) at 1:500 (Santa Cruz Biotechnology); rabbit anti-β-catenin at 1:500 (a gift from P. Hauser) (see Schneider et al., 1996); mouse anti-ZO-1 at 1:100 (a gift of S. H. Tsukita) (see Itoh et al., 1993); and a mouse anti-phosphotyrosine antibody (PY20) at 1:100 (Transduction Laboratories). Secondary antibodies for detection were conjugated to Cy2 and Cy3 (Amersham Pharmacia). For whole-mount antibody staining, embryos were fixed for 2 hours at room temperature in 4% paraformaldehyde in PBS. All incubations for antibody staining were performed in solutions containing 5% DMSO and 1-1.5% normal goat serum. For DNA staining, Sytox Green (Molecular Probes) was used according to the manufacturer’s protocol. To visualise F-actin, embryos were incubated in rhodamine-conjugated phalloidin (Molecular Probes).

**RESULTS**

The following is a short description of neurulation in the zebrafish at the level of the first to fifth somite (prospective cervical spinal cord), where all observations were made (Fig. 1) (Kimmel et al., 1990; Kimmel et al., 1995; Schmitz et al., 1993; Papan and Campos-Ortega, 1994). In the course of convergence movements after gastrulation, the neural plate folds inward at the midline to form the neural keel (Fig. 1B). Neural keel formation takes place between the 6- and 10-somite stages (13.3 hours post-fertilisation, hpf). During the 10- to 14-somite stage, the neural keel progressively rounds up and forms a nerve rod, which is finally overlaid by the adjacent epidermis at about 16 hpf. The neural rod is a massive cellular conglomerate in which no lumen is discernible (Fig. 1C). Neurocoel formation starts in the 17- to 18-somite embryo, when the cells retract their apical processes from the midline, beginning ventrally in the spinal cord and progressing towards dorsal levels; the process is complete by the 30-somite stage (~24 hpf).

Throughout neurulation, the zebrafish neuroepithelium remains essentially pseudostratified, consisting of columnar cells that extend apically from the basement membrane. As in many other epithelia, neuroepithelial cells round up and divide apically (Hinds and Ruffet, 1971). Injections of mRNA encoding tau:GFP allows to follow neural plate cells during convergence movements and division (Fig. 2). To divide, neural plate cells detach from the neuroepithelium and come to be located above it (Fig. 3). Although we cannot exclude the possibility that dividing neural plate cells still remain connected to the basement membrane by thin processes, as in the cerebral cortex of the mouse embryo (Noctor et al., 2001; Miyata et al., 2001), any such connection must be below the level of resolution of our technique. Some dividing cells develop polarised processes above the epithelium, which are clearly oriented towards the midline (Fig. 2; see and Movie 1 at http://dev.biologists.org/supplemental/). During and after division, the cells maintain essentially the same neighbours as before leaving the epithelium, undergoing convergence in register with the underlying cells (Fig. 2; see Movie 1 at http://dev.biologists.org/supplemental/). After division, the daughter cells reorganise into essentially the same relative positions of the progenitors in the epithelium (Fig. 3).
The orientation of mitotic divisions in the neuroepithelium

Embryos carrying the insertions Tg(H2A.F/Z)kca37 and/or Tg(H2A.F/Z)kca6 were analysed by confocal LSM. Time-lapse movies were made over periods of 3-15 hours and used to determine the orientation of the anaphase in mitoses during neural plate, keel/rod and tube stages. In addition, the mitotic spindle was directly observed in embryos expressing tau:EGFP. To assess mitotic orientation, we distinguished two different systems of reference: with respect to the body axes and with respect to the neuroepithelium. Thus, mitoses can be oriented with respect to the mediolateral, anteroposterior and dorsoventral body axes; or mitoses can be planar, oblique or orthogonal, when the anaphase lies parallel, oblique or perpendicular to the epithelium, respectively.

Our analysis of the neural plate was restricted to the one- to three-somite stage, immediately preceding midline infolding. Owing to convergence, the mediolateral extent of the neural plate encompassed in our LSM photographs changed from 150 μm at the one-somite to 90 μm at the three-somite stage; the depth of the epithelium increased from 10-15 μm to 25-36 μm.

As mitoses take place apically, a single level of focus was chosen to study mitoses during the plate stage. A total of 327 mitoses were analysed in this stage in four different embryos, and all were found to be planar, i.e. essentially parallel to the plane of the neuroepithelium (Fig. 4, see Movies 1 and 2 at http://dev.biologists.org/supplemental/). Most of the metaphase plates observed underwent striking planar rotations of 10° to 90°, which ceased when the cells entered anaphase (Fig. 4A,B,F,G). These movements are similar to, although not as pronounced as, the rotations of the metaphase plate observed by Adams (Adams, 1996) in the ventricular zone of the developing cerebral cortex in the rat, which extended up to 360°. Nevertheless, as in the rat cortex, chromosomal

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Fig. 2. A sequence of six confocal micrographs of a planar view of the neural plate of an embryo that had received an injection of mRNA encoding tau:GFP. A cell about to divide (arrow in 0') has left the epithelium and come to be located apically (arrows track its progress). The cell has developed processes oriented to the midline (thin white line in each photograph). During convergence, the dividing cell maintains the same neighbours (the asterisk on the neighbouring cells is for reference).

Fig. 3. Two different embryos (A-D and E-H) that have received an injection of mRNA encoding tau:GFP. The photographs show orthogonal sections (z-scans) obtained from sequences of 250 frames (60 second intervals). The white line above the neural plate cells in each plate corresponds to the section of the coverslip (refer to Materials and Methods). The drawings beneath each photograph illustrate the profile of the epithelial cells. One cell (arrows in A-D) has lost the connections to the basement membrane at time point 0, and divides apically 9 minutes later (B). One of the daughter cells reintegrates into the epithelium (D) in the plane of focus. The embryo at E-H is slightly older. The photographs show one cell (arrows) leaving the epithelium (E-G) to divide apically 60 minutes later (H). not, notochord.
Mitotic spindle orientation in zebrafish neurulation

complements separate at anaphase without major additional changes in spindle orientation. For statistical analysis, the neural plate was subdivided into three equal parts and the orientation of anaphase was measured with respect to the midline. The anaphase can be oriented in any direction with respect to the mediolateral or anteroposterior axes of the neuroepithelium. However, mitoses within the medial region of the neural plate are preferentially oriented in a mediolateral direction at $86.6^\circ\pm35.9^\circ$ ($P=0.05$). Mitoses within the other two regions of the neural plate did not show any preferential orientation ($68.2^\circ\pm39^\circ$ $0.27<P<0.7$ for intermediary and $12.38^\circ\pm37.94^\circ$ $0.24<P<0.49$ for lateral regions).

In keel and rod stages (6- to 15-somite stage), mitoses were orthogonal to the apical face of the epithelium, which now forms the zone of apposition between the two halves of the neural anlage, and occurred throughout the entire dorsoventral depth of the keel or rod at comparable frequencies (Fig. 5, see Movies 3 and 4 at http://dev.biologists.org/supplemental/). Deviations from a strictly orthogonal orientation of the mitoses were determined by irregularities in the infolding epithelium. At rod stage, deviations did not exceed $\pm25^\circ$ in a total of 291 mitoses of Histone2A.F/Z:GFP-expressing cells. The oblong nucleus first moved apically to the region of apposition between the two walls of the anlage (Fig. 5A-B); once there,
the nucleus rounded up and the metaphase plate formed (Fig. 5C). In mitotic neural keel/rod cells expressing tau:EGFP (n=267, counted in four different embryos) the centrosome divided prior to dissolution of the nuclear membrane, the two centrosomes migrated to opposite poles and the spindle formed (Fig. 5J-L). In 89.5±3.6% of the tau:EGFP-labelled mitoses analysed so far at keel/rod stage, the spindle formed roughly parallel (±15°) to the epithelial plane, but within 1-2 minutes the spindle apparatus turned by 90° at 12 minutes 45 seconds (C). The nucleus then divides within 3 minutes and 45 seconds (D), and the two daughter nuclei are incorporated into different sides of the neuroepithelium (arrows). Note that the plane of cleavage in this division, as well as in other divisions shown in the remaining photographs, is parallel to the neuroepithelium. (J-R) A mitosis at the neural rod stage of an embryo that expresses tau:GFP. The arrows in J and L indicate the centrosome before and after division, those in M-P indicate the plane of rotation. Anterior is towards the top in all photographs.

In neural tube stage, the mitotic spindle formed always parallel to the plane of the neuroepithelium and divisions were planar (Fig. 4I-P, see Movie 5 at http://dev.biologists.org/supplemental/). We analysed 2733 mitoses in two different embryos within a region of the developing spinal cord that comprised three consecutive neuromeres, and over a period encompassing a significant fraction of embryonic neurogenesis. Time-lapse movies lasting for 15 hours were made at 12 different z-levels throughout the depth of the neural tube. To avoid ambiguities, we disregarded the regions around the floor plate and the roof plate, and restricted our observations to the intermediate region of the developing spinal cord where the neurocoel forms a vertical cavity. In 2716 mitoses, the orientation was parallel to the neurocoel, deviating only ±5-15° from a perfectly parallel orientation; 17 cells divided along a cleavage plane that was oriented obliquely (at an angle of between 15° and 25°) to the epithelium. Besides these 2733 mitoses, a relatively large number of mitoses were followed at various stages throughout the entire embryonic
observed that one of the daughter cells migrated into progeny could be followed for a relatively long time, we from mitoses during this stage. In 52 of the divisions whose wondered about the behaviour of the daughter cells resulting in neural tube stage in the developing zebrafish spinal cord, we subventricular regions. As no perpendicular mitoses were seen the ventricular surface whereas the other migrated into perpendicular to the ventricular surface and produced progeny Roughly 15% of all mitoses detected were oriented orientation of the mitosis (Chenn and McConnell, 1995).

In the developing cerebral cortex of the ferret, the behaviour of the neural tube. The embryo is oriented a little obliquely, such that the two halves of the neural tube are not in register. One cell in metaphase is indicated by an arrow at 0°. After division at 12° (arrows indicate both daughter nuclei), one of the nuclei (left arrow) can be seen to move towards the subventricular region to join differentiating cells in the external region of the spinal cord. Notice that the most external nuclei are separated from the others by a dark line, most probably corresponding to an incipient neuropil (at 12° and thereafter). Anterior is towards the left in all photographs.

period. Prior to entering anaphase, the metaphase plate underwent planar rotations of up to 90° at the neurocoel. Perpendicular divisions were never observed.

Irregularities in the neural plate during infolding to form the neural keel precluded observations of the transition from plate to keel stage. However, the change from the orthogonal orientation of divisions in the neural keel/rod to the planar orientation in the neural tube could be readily observed (see Movie 6 at http://dev.biologists.org/supplemental/). This change occurred progressively, rather than suddenly. Mitoses with a pronounced oblique orientation first appeared in the ventral-most levels of the spinal cord, gradually progressing to dorsal levels; from the 15-somite stage onwards, planar mitoses appeared gradually, again beginning at ventral levels. Daughter cells resulting from mitoses with oblique orientation remained on the same side of the neurocoel and were not distributed bilaterally. As neurocoel formation follows the same ventral to dorsal course (Schmitz et al., 1993), these observations suggest that the transition from orthogonal to planar divisions occurs when the two halves of the neural anlage separate from each other.

**Postmitotic cells migrate into subventricular regions of the neural tube**

In the developing cerebral cortex of the ferret, the behaviour of neuroepithelial cells after mitosis correlates with the orientation of the mitosis (Chenn and McConnell, 1995). Roughly 15% of all mitoses detected were oriented perpendicular to the ventricular surface and produced progeny that behaved differently, in that one daughter cell remained at the ventricular surface whereas the other migrated into subventricular regions. As no perpendicular mitoses were seen in neural tube stage in the developing zebrafish spinal cord, we wondered about the behaviour of the daughter cells resulting from mitoses during this stage. In 52 of the divisions whose progeny could be followed for a relatively long time, we observed that one of the daughter cells migrated into subventricular regions, whereas the other remained at the neurocoel (Fig. 6, see Movie 7 at http://dev.biologists.org/supplemental/). It is very likely that the migrating cells differentiated as neurons or glia cells, as the subventricular region into which they moved contained differentiating cells and was clearly separated from the region with proliferating cells by a layer of neuropil (Fig. 6). Unfortunately, we could not ascertain whether any of the cells that remained at the neurocoel in these 52 mitoses divided again. The observations therefore indicate that mitotic spindle orientation does not correlate with the behaviour of daughter cells in the developing spinal cord of the zebrafish.

**There is a gradual increase in the apical concentration of cell junction markers during neurulation**

The orientation of mitoses has frequently been related to epithelial polarity (e.g. Reinsch and Karsenti, 1994; Adams, 1996). Recently, two different reports (Lu et al., 2001; Le Borgne et al., 2002) have emphasised the role played in *Drosophila* by proteins of the so-called subapical protein complex (Crumbs, Stardust and Discs lost) (Bachmann et al., 2001) and E-cadherin in controlling the rotation of spindle orientation that precedes the asymmetrical divisions of the neuroblasts and pIIb cells, respectively. The peculiar stereotypic orientation of the mitotic spindle during neurulation in the zebrafish poses the question as to whether apical junctions perform a similar function in the neuroepithelium of the zebrafish.

ASIP, a mammalian homologue of PAR-3, associates with the atypical protein kinase C (aPKC) and plays an important role in controlling epithelial polarity (Izumi et al., 1998; Suzuki et al., 2001; Ohno, 2001). A zebrafish *asip* homologue, whose product shows 65% identity to ASIP from the rat, was cloned and used to make three different reporter constructs: *asip:egfp* includes the entire coding sequence, *asip-Δ5′:egfp* is an amino-terminal deletion, and *asip-Δ3′:egfp* is a C-terminal deletion.
These constructs were used to synthesise mRNA, which was injected into the yolk of zygotes of either wild-type or Tg(H2AF/Z)kca37; Tg(H2AF/Z)kca66 heterozygotes, and the injected embryos were observed by confocal microscopy to follow ASIP:EGFP expression in vivo. As an additional control, mRNA encoding EGFP only was injected into other embryos. After injections of egfp mRNA, strong cytoplasmic labelling was observed in embryonic cells from mid-gastrula stages on. A similar cytoplasmic distribution can be observed following the injection of asip-D3:egfp mRNA. By contrast, injection of asip-Δ5:egfp mRNA resulted in membrane labelling. Whereas the C-terminal deletion occasionally also labelled other structures, such as the mitotic spindles, the full-length protein resulted in specific labelling of the apical cell membrane (data not shown; Fig. 7J-L).

The pattern of ASIP:EGFP labelling was stage-dependent. In the neural plate, a punctate labelling pattern was apparent (Fig. 7A-H), consisting of several bright dots at the membrane of the neural plate cells, particularly at points of contact with other cells. However, there was little or no evidence for a preferential localisation of labelling at apical levels. After neural plate infolding had started and the two halves had entered into apposition, the ASIP:EGFP protein became concentrated apically (Fig. 7I). The density of apical labelling increased such that in the neural tube stage a rim was present decorating the apical pole of the neuroepithelial cells now at the neurocoel (Fig. 7J-L).

The localisation of aPKC can be followed with an antibody that recognises two different isoforms, aPKCα and aPKCζ (Horne-Badovinac et al., 2001). In the neural plate (not shown), as well as dorsally in the neural keel, which corresponds to the region of infolding of the neural plate (Fig. 7M), we failed to detect any specific staining. In the neural keel, weak staining was observed apically in the neuroepithelial cells restricted to ventral levels (Fig. 7N) (i.e. the region where one wall of the neural keel has already made contact with the opposite wall). At rod and tube stages, the epitope detected by this antibody was found at the apical pole of the neuroepithelial cells, and the intensity of the staining increased with the age of the embryos.

Other markers of adherens and tight junctions (revealed using phalloidin to visualise F-actin and antibodies against β-catenin, phosphorylated tyrosine, and ZO-1) were found to behave in the same manner as ASIP:EGFP and, in part, aPKCα/ζ. The markers were diffusely distributed at the cell membrane in neural plate stage, and became concentrated at the apical pole of the neuroepithelial cells when the two halves of the neural primordium were in apposition at the neural keel stage (Fig. 8, and not shown). The markers continued to concentrate at the apical pole in the neural tube stage. All these observations indicate that important components of adherens and tight junctions are either expressed in an unpolarised fashion or even absent altogether at the neural plate stage, and that cell junctions mature over the period from the keel to the tube stage.

The behaviour of apical markers during mitoses in the course of neurulation

During mitoses in tube stage, the apical localisation of
ASIP:EGFP fluorescence and of the aPKC\(\lambda/\zeta\) epitope is preserved. Mitotic cells in the neuroepithelium round up as they proceed into prophase; however, strong fluorescence can be observed on the side of the cell facing the neurocoel. The apical localisation of ASIP:EGFP and aPKC\(\lambda/\zeta\) persists throughout anaphase and telophase (Fig. 9A-F). However, in mitoses during keel/rod stage, the behaviour of both markers is different. In mitoses that lead to bilateral progeny in rod stage, the apical localisation of ASIP:EGFP is lost and the marker is redistributed regularly over the circumference of the cell membrane as the cell rounds up at prophase. Apical localisation of ASIP:EGFP becomes evident again at telophase in both daughter cells (Fig. 9G-J). To summarise, imperfectly polarised junctional components of neuroepithelial cells in keel/rod stage lose their apical localisation at prophase; apical localisation is re-established at telophase. By contrast, the firmly established polarity of cell junctions in neural tube cells persists during mitosis.

The stereotypic orientation of mitoses is impaired by injection of morpholino-modified ASIP-antisense oligonucleotides and in heart and soul mutants

To test for a possible role of the Par3/Par6/aPKC protein complex in regulating the stereotypic orientation of the spindle during neurulation, we carried out two different sets of experiments. First, morpholinos against asip RNA were injected, at four different concentrations, into the yolk of Tg(H2AF/Z)\(\kappa\alpha\zeta7\), Tg(H2AF/Z)\(\kappa\alpha\zeta6\) zygotes, and the injected animals were analysed at the neural tube stage. In control injection of high concentrations (20.5 ng/ml), in which the epitope is clearly concentrated apically (arrows).

In experiments, ASIP:EGFP mRNA was injected into wild-type zygotes simultaneously with the morpholinos. No fluorescence was observed in these animals. Protein extracts were analysed with anti:GFP antibody after western blotting, and no GFP was detected (data not shown). Spindle orientation was essentially perpendicular to the plane of the neuroepithelium (60-90°) in 0.6 to 7.6% of all observed mitoses, depending on the concentration of the injected morpholinos (Table 1; Fig. 10A-D). In addition, between 2.8 and 23% of all mitoses were obliquely oriented (at angles of 15-45°). The integrity of the neuroepithelium was apparently normal in embryos injected with low doses (2.6-7 ng/ml), but it was clearly disturbed after injection of high concentrations (20.5 ng/ml). In these animals, neuroepithelial cells were loosely aggregated and divisions occurred not only at the neurocoel, as in untreated animals, but also in deeper regions of the neural tube (not shown).

Second, time-lapse movies were made on heart and soul\(m129\) embryos carrying the Tg(H2AF/Z)\(\kappa\alpha\zeta7\) transgene. In some regions of the neural tube of the homozygotes, the neurocoel had not formed properly and the compact organisation characteristic of the neural rod persisted into the neural tube stage (not shown). However, the wild-type orientation of mitoses and their location in the middle of the neural anlage was largely preserved within these regions. A total of 750 mitoses were observed at neural tube stage. In 603 cases (80.4%), the mitoses were planar, 68 (9.1%) had an oblique plane of orientation (up to 45°), and in 79 cases (10.5%) the

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Parallel (%)</th>
<th>Oblique (15°-45°) (%)</th>
<th>Perpendicular (60°-90°) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6 ng/ml</td>
<td>790 (96.6%)</td>
<td>23 (2.8%)</td>
<td>5 (0.6%)</td>
</tr>
<tr>
<td>4.2 ng/ml</td>
<td>97 (72.9%)</td>
<td>31 (23.3%)</td>
<td>5 (3.8%)</td>
</tr>
<tr>
<td>7 ng/ml</td>
<td>139 (79.9%)</td>
<td>25 (14.4%)</td>
<td>10 (5.7%)</td>
</tr>
<tr>
<td>20.9 ng/ml</td>
<td>458 (85.3%)</td>
<td>38 (7.1%)</td>
<td>41 (7.6%)</td>
</tr>
<tr>
<td>7 ng/ml*</td>
<td>589 (94.3%)</td>
<td>28 (5.5%)†</td>
<td>1 (0.2%)†</td>
</tr>
<tr>
<td>Uninjected</td>
<td>2716 (99.4%)</td>
<td>17 (0.6%)†</td>
<td>–</td>
</tr>
</tbody>
</table>

*Control morpholino.
†15°-25°.
mitosis was perpendicular (60° to 90°) to the neuroepithelium (Fig. 10E-H).

**DISCUSSION**

Two main conclusions can be drawn from earlier observations on mitotic orientation during zebrafish neurulation (Kimmel et al., 1994; Concha and Adams, 1998) and from our present results. First, neuroepithelial mitoses exhibit a preferential orientation with respect to the body axes and with respect to the neuroepithelium. Second, throughout neurulation, with some exceptions in keel/rod stage, the mitotic spindle forms parallel to the plane of the neuroepithelium, but in keel/rod stage it rotates by 90°. In the following, the orientation of mitoses and the rotation of the spindle will be discussed separately.

**On the orientation of mitoses during neurulation**

Technical differences make it difficult to compare our data to those described by Kimmel et al. (Kimmel et al., 1994). The latter authors injected rhodamine-dextran into individual
blastomeres and followed the development of the resulting clones within the prospective hindbrain and spinal cord. They found that neural progenitors undergoing the 15th embryonic division are oriented preferentially along the anteroposterior axis, whereas cells at the 16th division adopt a mediolateral orientation. The authors explicitly state that division 14 takes place in early gastrula, whereas division 16 splits the clones bilaterally. However, there is no direct indication as to when division 15 occurs during neurulation. Division 16 should take place either in keel or in rod stage, as only divisions in keel/rod stage lead to bilateral progeny (Papan and Campos-Ortega, 1994; Papan and Campos-Ortega, 1997; Concha and Adams, 1998) (this study, see below). Consequently, in the 11 developing cell clones studied by Kimmel et al. (Kimmel et al., 1994) the 15th embryonic division might have taken place in neural plate stage. As this division was oriented anteroposteriorly, it might have occurred early in plate stage, because, according to Concha and Adams (Concha and Adams, 1998), mitoses in the early neural plate are oriented anteroposteriorly. Therefore, according to this interpretation, the material analysed by Kimmel et al. (Kimmel et al., 1994) did not reveal any mediolaterally oriented mitoses in neural plate stage.

Concha and Adams (Concha and Adams, 1998) analysed the direction of cytokinesis, whereas we have observed anaphases; however, we can readily compare our data with theirs, as we use the same points of reference. The authors found changes in orientation during the neural plate stage, from anteroposterior (early) to mediolateral (late). The material we used for the analysis of mitoses in plate stage, which was restricted to the late neural plate (one to three somite), was processed in the same manner as that studied by Concha and Adams, and we can confirm their results: at the one- to three-somite stage, anaphase figures tend to be oriented mediolaterally in cells located medially in the neural plate, whereas in the remaining neural plate anaphase figures exhibit random orientation. We would like to emphasise that divisions mediially in the neural plate, although oriented mediolaterally, are unrelated to those that lead to bilateral progeny [the 16th division according to the terminology of Kimmel et al. (Kimmel et al., 1994)]; the latter divisions are also oriented mediolaterally, but take place in the keel/rod stage and are orthogonal following a 90° shift of the spindle (see below), whereas the former are planar.

With respect to the neuroepithelium, mitoses are planar (parallel) in plate and tube stage (Concha and Adams, 1998) (this study), and orthogonal (perpendicular) in keel/rod stage (Kimmel et al., 1994; Papan and Campos-Ortega, 1994; Concha and Adams, 1998). Our present observations are compatible with the assumption that all mitoses in plate stage are planar, for we observed no exceptions to this. However, the late neural plate is engaged in energetic convergence movements and it is frequently difficult to establish the orientation of a cell with respect to the epithelium. Therefore, exceptions are conceivable. Our material strongly suggests that all divisions in keel/rod stage are orthogonal, all of them leading to bilateral progeny, and that all mitoses in tube stage are again planar [as is implicit in figure 10 by Concha and Adams (Concha and Adams, 1998)]. As we observed the neural primordium from above, it was difficult to establish any preferential orientation of mitoses in tube stage along the dorsoventral and anteroposterior axes.

What determines mitotic orientation during neurulation in the zebrafish? Concha and Adams (Concha and Adams, 1998) have discussed how mechanical tension and other forces may contribute to steer the direction in which cells move in the epiblast and to align mitotic divisions during gastrulation and neurulation. Indeed, the planar orientation of mitoses in neural plate stage might well be mechanically determined by factors acting on the neuroepithelium, for example from the overlying enveloping layer. Concha and Adams (Concha and Adams, 1998) emphasise stretching of the epiblast as a major element in orienting mitoses, and propose that cells cleave in the direction of greatest elongation. Although stretching may well be operative in this respect earlier in the epiblast, the strong convergence movements that precede neural keel formation seem to preclude stretching in plate stage. However, convergence itself might contribute to the preferential mediolateral orientation of planar mitoses within the medial one-third of the neural plate. Similarly, in keel/rod stage, mechanical forces may be exerted by one half of the neural primordium on the other half, with which it is in direct contact, and thus contribute to spindle formation parallel to the zone of apposition of the two halves. Other factors may also operate in the neural tube to determine the planar orientation of the spindle in this stage.

Epithelial polarity and mitotic orientation

An additional factor might be represented by the polarity of the epithelium. The well-established function of the Par3/Par6/aPKC protein complex in controlling cell polarity in several animal species prompted us to ask whether this complex might be involved in controlling mitotic orientation in zebrafish neurulation. Indeed, Horne-Badovinac et al. (Horne-Badovinac et al., 2001) have described abnormalities in the orientation of dividing, has (aPKC defective) retina cells. For convenience, we concentrated on the neural tube stage, in which mitoses are always planar. Injection of ASIP morpholinos caused the appearance, in tube stage, of a few perpendicular mitoses as well as a relatively high proportion of obliquely oriented mitoses. Perpendicular mitoses were never seen in the neural tube of uninjected embryos, we found only one after injection of a control morpholino, and oblique mitoses were less abundant and less pronounced in uninjected and control embryos. The observations on the developing spinal cord of has mutants uncovered a similar behaviour. The two extant alleles of the has gene, m129 and m567, which encode C-terminal truncations of the aPKC family members that lack 73 and 69 amino acids, respectively, and have lost kinase activity, are assumed to be null alleles (Horne-Badovinac et al., 2001; Peterson et al., 2001). Homozygosity for the hasm129 allele leads to mild disruption of spindle orientation in neural tube stage in the time span encompassed by our observations: up to 10% perpendicular and another 9% oblique mitoses were found. One possible explanation for the weak effects of the ASIP morpholinos and those found in the spinal cord of has mutants is functional redundancy, as two isoforms of the aPKC family members are known, which are likely to be encoded by different genes. It is possible that additional ASIP genes may also exist. Therefore, we conclude that ASIP and aPKC are involved in controlling stereotypic spindle orientation in the
neuroepithelium, but that they represent only part of the controlling machinery.

**Orientation of mitotic divisions and behaviour of the daughter cells**

During neurogenesis in other animal species [for example in the cerebral cortex of the mouse (Smart, 1973) and ferret (Chenn and McConnell, 1995) and in the retina of the rat (Cayouette et al., 2001) or the zebrafish (Horne-Badovinac et al., 2001)], mitoses show a variety of orientations with respect to the neuroepithelium. Thus, in the cerebral cortex of the ferret, planar mitoses accounted for 85% of the cases, producing progeny that remained at the ventricle and continued to divide. These mitoses were termed symmetrical. An orientation orthogonal to the neuroepithelium was observed in 15% of all mitoses (Chenn and McConnell, 1995). Moreover, perpendicular mitoses were referred to as asymmetrical because the progeny behaved differently: one of the daughter cells continued to divide whereas the other migrated into subventricular regions to differentiate. In his study of the cerebral cortex of the rat, Adams (Adams, 1996) failed to find unambiguous evidence for perpendicular mitoses. Here, the mitotic spindle was found to be oriented invariably parallel to the plane of the neuroepithelium. Our present results indicate that perpendicular mitoses, if they occur at all, are extremely rare in the developing spinal cord of the zebrafish. Nevertheless, although all mitoses in tube stage were planar, one of the daughter cells was seen to migrate away from the ventricular region in 52 cases. This figure appears very low in light of the fact that we analysed several thousand mitoses in the neural tube. However, it is a minimal estimate, as it corresponds only to those cases in which both progeny cells remained within the same plane of focus and could be followed. In many other cases, the two daughter cells were seen to separate from each other for a given distance, but one cell then left the plane of focus and could not be followed any further. We thus conclude that during zebrafish neurogenesis spindle orientation cannot be correlated with equivalent or dissimilar behaviour of the progeny cells.

**Mitotic spindles rotate by 90° in the neural keel/rod stage**

The orthogonal orientation of mitoses in keel/rod stage is brought about by a 90° rotation of the mitotic spindle. In most divisions (89%) during this stage, the spindle formed parallel to the plane of the neuroepithelium. However, the spindle apparatus rotated by 90° and the ensuing division was orthogonal. The striking correlation between apposition and separation of the two halves of the neural anlage and orthogonal or planar orientation of mitoses suggests a causal link between these phenomena. One can imagine that in keel/rod stage a signal passes from one wall of the neural anlage to the other and elicits the rotation of the spindle. This hypothetical signal would be interrupted as the neurocoel forms and the mitotic spindle would not rotate but remain planar in tube stage.

The 90° rotation of the mitotic spindle in neural keel/rod stage raises two questions. One is related to the mechanism and the molecular control of the rotation; the other is whether the change from a planar to an orthogonal orientation of the mitosis actually has any functional significance.

Two cases of mitotic spindle rotation occur during neural development in *Drosophila*, which are strikingly similar to the rotation described here. Although *Drosophila* neuroectodermal cells divide in the plane of the epithelium, after delamination, spindle orientation in the neuroblasts turns by 90° immediately prior to mitosis, and division takes place orthogonal to the neuroectoderm (Kaltschmidt et al., 2000) (for a review, see Knoblich, 2001). Two different mechanisms are involved in the control of mitoses in the neuroectoderm and the neuroblasts. The first mechanism is provided by adherens junctions and by the subapical protein complex, which exert control over the planar divisions in the neuroectoderm (Lu et al., 2001). When the neuroblasts delaminate in wild-type embryos, the junctions disappear and the rotation of the mitotic spindle is brought about by the second mechanism, i.e. the apical localisation of Insuteable (Kraut and Campos-Ortega, 1996; Kraut et al., 1996), in association with Bazooka (Schober et al., 1999; Wodarz et al., 1999), Partner of Insuteable (Pins) (Yu et al., 2000; Schaefer et al., 2000), *Drosophila* Par-6 (Petronzcki and Knoblich, 2001) and *Drosophila* aPKC (Wodarz et al., 2000). Lu et al. (Lu et al., 2001) have reported that in crumbs mutants or after misexpression of crumbs, the junctions disappear and, consequently, the plane of division of the neuroectodermal cells is randomised. A similarly decisive role has been proposed for junctional proteins in directing spindle orientation in dividing pIIa and pIIb cells in the lineage of *Drosophila* sensory bristles (Le Borgne et al., 2002). The pI progenitor divides asymmetrically in the plane of the epithelium, giving rise to pIIb, which delaminates partially, and pIIa, which remains in the epithelium. A strong accumulation of *Drosophila* E-Cadherin, α-Catenin and β-Catenin is observed in pIIb at its point of contact with pIIa, while the localisation of these markers in pIIa is the same as in the progenitor pl. The division of pIIa is also planar, suggesting that the junctional protein complex directs the orientation of the division. By contrast, in the partially delaminated pIIB cell, there is a strong accumulation of junctional proteins in an apical stalk that remains in contact with pIIa. In the stalk, Bazooka recruits Insuteable and Partner of Insuteable apically in the neighbourhood of the junctional complex, and thus ensures that the mitosis takes place orthogonally to the epithelium (Le Borgne et al., 2002).

We are reluctant to draw any firm conclusions as to whether junctional proteins play any role in controlling the rotation of the mitotic spindle in the zebrafish neural keel/rod. The present observations indicate that adherens and tight junctions mature in the period between neural keel and neural tube, i.e. during the phase in which mitotic spindles undergo the 90° rotation. Therefore, spindle rotation in the neural keel/rod does not seem to require perfectly polarised cell junctions, and the mechanism controlling spindle rotation in the zebrafish neuroepithelium might well be different from that in the two examples from *Drosophila*. However, the change in expression of ASIP:EGFP and aPKCζ during mitoses in keel/rod and tube stages is very striking, although its significance is not yet clear. Whereas apical localisation of these proteins in cells in keel/rod stage is lost upon entry into prophase, the apical localisation is retained when the cells of the neural tube divide. This may be simply a reflection of the fact that cell junctions are not firmly established and well polarised until the late rod or early tube stage. But one cannot exclude the possibility that the mitotic
spindle is reoriented as soon as the junctional proteins lose their apical localisation in dividing cells in keel/rod stage, thus determining the 90° rotation. Therefore, the question of causal relationships between junctional proteins and spindle rotation in the neuroepithelium of the zebrafish must remain open.

**Functional significance of the shift of mitotic spindle orientation**

The orthogonal divisions in neural keel/rod stage are obviously related to the bilateral distribution of progeny. As mentioned, cell clones with a bilateral distribution have been described at several levels on the zebrafish neuraxis after single cell labelling (Kimmel et al., 1990; Kimmel et al., 1994; Schmitz et al., 1993; Papan and Campos-Ortega, 1994; Papan and Campos-Ortega, 1997; Papan and Campos-Ortega, 1999). Our current results indicate that most, if not all, mitoses that take place in neural keel/rod stage lead to bilateral progeny. As sister cells within cell clones differentiate into distinct cell types on either side of the spinal cord (Papan and Campos-Ortega, 1997; Papan and Campos-Ortega, 1999), perpendicular mitoses leading to bilateral progeny might well be accompanied by a differential distribution of cytoplasmic determinants. Experimental support for this hypothesis is pending.

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