Cell-autonomous action of zebrafish spt-1 mutation in specific mesodermal precursors

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In zebrafish, as in Xenopus, the well-orchestrated cell movements of gastrulation can be dissected into several components, including epiboly, involution, convergence and extension. Embryos homozygous for the recessive lethal mutation spt-1(b104) or ‘spadetail’ have a complex set of defects in the trunk of the embryo that may arise secondarily after loss of one of these movements, convergence, from those precursors that would normally have given rise to trunk somitic mesoderm (Fig. 1). We have now tested this hypothesis by transplanting cells between wild-type and mutant embryos, to identify the cells that spt-1 affects directly. Our results show that the mutation autonomously affects only those mesodermal precursors located along the lateral margin of the early gastrula blastoderm. Other mesodermal cells and all ectodermal precursors seem not to require function of the wild-type gene. Our findings reveal an unexpectedly delicate genetic control of vertebrate gastrulation.

For each experiment, separate groups of cells from two distinctively labelled donor embryos were cotransplanted at the late blastula stage into an unlabelled wild-type host embryo (Fig. 2) or, occasionally, into a spt-1 host. This design permitted the direct and simultaneous comparisons of the movements and fates of wild-type and mutant cells beginning gastrulation together at exactly the same position. Combinations in which both donors were of the same genotype (that is wild type: wild type and spt-1:spt-1) served as controls.

When labelled wild-type and spt-1 cells were cotransplanted into the lateral marginal zone of either wild-type or mutant host embryos, the labelled cells from wild-type donors converged dorsally in a normal fashion. In contrast, the cotransplanted spt-1 cells failed to converge, even though they were placed in the same environment as the wild-type cells transplanted with them (Fig. 3a). The spt-1 cells moved caudally during gastrulation and entered the tailbud that forms after epiboly. Thus, by this stage, the wild-type and mutant cells had segregated as

FIG. 2 Comparison of a, wild-type and b, spt-1 zebrafish embryos at 24 h. The trunk area of the mutant, delimited by the small arrowheads, is grossly deficient in muscle cells and lacks the segmental chevron-shaped pattern of organized myotomes. Also, note the characteristically bent notochord and excess of cells at the end of the tail.

METHODS. Embryos for experiments were produced by crossing heterozygotes for spt-1, yielding wild-type and spt-1 embryos in a 3:1 ratio. As the mutant phenotype is not visible before 12 h, the choice of donors and hosts for the transplantation procedures is done blindly. Donor embryos were labelled, some with rhodamine-dextran (RDA) and others with fluorescein-dextran (FDA), both dyes from Molecular Probes, 5 mg ml$^{-1}$ in 0.2M KCl, by intracellular injection of dye into the yolk cell before the 16-cell stage (1.5 h). Hosts, sibs of the donors, were not labelled. Genotypes of donor embryos were inferred from their later appearing phenotypes. Pipettes used for transplantations were made on a Flaming-Brown puller using non-capillary electrode glass. Tips of pipettes were either bevelled or broken off to an inner diameter of 10–30 µm and polished on a microforge. Often, a sharp point was tooled onto the end of the pipette to aid penetration of outer tissue layers. Pipettes were filled with mineral oil and attached via polyethylene tubing to an oil-filled Hamilton syringe. Transplantation procedures were monitored using a Zeiss UEM microscope equipped with ultraviolet epillumination and Silicon Intensified Target video camera (Dage-Mit, Inc., Model ST 66). Before gastrulation (5 h) a small group of 5–10 cells from one labelled embryo was gently drawn up into the transplantation pipette followed by another group of cells taken from a second, differently labelled donor embryo. Donor cells were collected from a mid anteroposterior position in the blastoderm but randomly with respect to dorsal–ventral position because dorsal–ventral polarity is not yet apparent. Both groups of cells were then simultaneously transplanted into an unlabelled host embryo. The locations of the labelled cells were recorded immediately after transplantation and again at 12, 24 and 46 h of development. At 12–14 h, the phenotypes of the donor embryos were recorded to establish the identities of the RDA- and FDA-labelled donor cells. Individual images were stored on a Panasonic optical disk recorder model TQ-303LF. Images were then superimposed and pseudocoloured using the imaging software 'NeuroVideo' running on a Mac II computer.

FIG. 1 Fate map (redrawn from ref. 6) of the zebrafish embryo at 50% epiboly, the onset of gastrulation (5.2 h). At this stage the blastoderm, in the form of a cup inverted atop the single large yolk cell, contains approximately 8,000 cells. The�staged region delineates the mesodermal precursors, which are in the lateral marginal zone, that are affected by the spt-1 mutation. In wild-type embryos, these precursors involute at the lateral margin and converge dorsally to form the paraxial mesoderm, which generates somites. Later, the somites form myotomes, blocks of body muscle. The dorsal marginal zone involutes to form the axial mesoderm, primarily notochordal. D, dorsal; V, ventral; AP, animal pole; LM, lateral margin; DM, dorsal margin.
groups into very different locations within the embryo (Fig. 3b).

Wild-type cells transplanted into the lateral margin of hosts of either genotype later expressed a fate appropriate for the lateral marginal zone of wild-type embryos, that is, myotomal muscle in the trunk. The *spt-1* cells transplanted into the lateral margin of hosts of either genotype later expressed a fate appropriate for the lateral marginal zone of mutant embryos, namely, mesenchyme in the tail (Fig. 3c). In the three cases where wild-type and *spt-1* cells were transplanted into mutant hosts, the wild-type cells converged as expected, forming trunk muscle cells, and the mutant cells formed numbers of derived cells in the tail. We interpret these results to mean that *spt-1* acts autonomously in the precursors of the trunk somitic mesoderm. In wild-type embryos, cells beginning gastrulation together never separate so completely with respect to both cell movement and eventual fate. Also, separation of donor cells did not occur when the cells transplanted to the lateral marginal zone were from donors of the same genotype. In these controls, all the donor cells migrated together and their progeny were frequently intermingled. As expected, wild-type: wild-type cotransplants tended to express trunk myotomal muscle fate (Fig. 3d), and *spt-1*: *spt-1* cotransplants tended to express tail mesenchyme (Fig. 3e).

Is function of the wild-type gene (*spt-1+*) required in other gastrula cells? Changes in the early movements of other mesodermal precursors were not previously detected in *spt-1* mutants, although changes in some nonmesodermal derivatives are present later. One of these is the notochord, which fails to lengthen normally, becoming kinked and twisted.

As in frogs, the notochord in zebralish forms from dorsal mesoderm by extension movements driven by cell intercalations, and it is possible that the notochordal defects in *spt-1* embryos result from a failure of normal extension movements. In our experiments, however, when wild-type and mutant cells were cotransplanted into the dorsal marginal zone, the transplanted cells of both genotypes intermingled with the unlabelled host cells in the notochord (Fig. 3f). As *spt-1+* function is required for normal cell intercalations of paraxial mesoderm, this result indicates that the intercalations that produce extension of dorsal axial mesoderm occur independently of *spt-1+* function, and that the kinking of the notochord in mutants must arise secondarily to its extension.

Ectodermal precursors lateral to the dorsal midline normally converge to form the spinal cord, just as mesodermal precursors converge to form the somites. The spinal cord is present and approximately of normal size in the trunk of *spt-1* mutants, suggesting that the convergence in the ectoderm is normal. We tested this directly by transplanting the mixtures of cells to the lateral nonmarginal zone of the blastoderm, which generates the spinal cord (Fig. 1). We consistently observed that cotransplanted wild-type and mutant ectodermal precursors converged dorsally together, and remained mixed together during their movements that form the spinal cord, irrespective of the host genotype (Fig. 3g). We conclude that the *spt-1+* product is not necessary for the proper convergence of ectodermal precursors. We suspect that the ectodermal irregularities seen in mutant embryos, that is mislocation of pigment cells and abnormal motoneuron axons, are secondary consequences of the lack of trunk somites in *spt-1* embryos.

**FIG. 3** The *spt-1* mutation specifically and cell autonomously affected the movements of lateral marginal zone cells. a Zebrafish embryo just before gastrulation (5 h) and immediately following cell transplantation. Two groups of cells from two different donor embryos were transplanted into the lateral marginal zone of a wild-type host embryo (n = 11). The FDA-labelled cells (coloured red) came from an embryo mutant for *spt-1* and the RDA labelled cells (red) came from a wild-type donor. b Same embryo after gastrulation (12 h). The two groups of cells segregated into different areas of the animal; the RDA labelled wild-type cells converged to the dorsal axis, whereas the mutant cells moved to the tail bud region. Diameter of the embryo shown in a and b is 500 μm. c Same embryo at 30 h. The wild-type cells, in the trunk, were anterior to the *spt-1* cells, in the tail. Wild-type and *spt-1* donor cells showed the same general pattern in both wild-type (n = 11) and *spt-1* hosts (n = 3). We never observed the reverse, that is, *spt-1* cells anterior to wild-type cells (Fisher sign test, P < 0.001). d Length of the embryo in c is ~1.5 mm. Orientation for a, b and c is animal pole (or anterior) towards the top of the page and dorsal to the right. c Upper inset, transplanted, RDA labelled cells of the same embryo at 48 h. The transplanted wild-type cells gave rise to striated myotomal muscle cells. c Lower inset: tail region of the same embryo at 48 h showing that the transplanted, FDA labelled *spt-1* cells gave rise to the fin rays and other associated mesenchymal derivatives. Orientation for the two insets is anterior to the left and dorsal towards the top of the page. Scale bar, 100 μm. d Cotransplanted wild-type cells did not segregate. When cells from two wild-type embryos were transplanted into the lateral marginal zone of a wild-type host (n = 17), the two groups of donor cells migrated together and the intermingled progeny cells differentiated into trunk myotomal muscle by 48 h. e Cotransplanted mutant cells did not segregate. When both groups of donor cells were of the mutant *spt-1* genotype and transplanted to the lateral marginal zone of a wild-type host (n = 3), both groups of cells took up positions within the tail where they formed predominately tail mesenchyme by 48 h. f The *spt-1* mutation did not affect the movements of precursors at the dorsal margin. FDA-labelled mutant cells and RDA labelled wild-type cells did not segregate apart when placed within the dorsal marginal zone (n = 3). By 30 h both groups of cells gave rise to intermingled notochord cells along the length of the axis, including the tail region as shown here in a wild-type host. g The *spt-1* mutation did not affect the movements of ectodermal precursors. FDA labelled mutant cells and RDA labelled wild-type cells migrated together and formed ectodermal derivatives when placed in the nonmarginal zone of a wild-type host (n = 25). This figure shows the intermingling of floor plate cells in the spinal cord at 24 h. Similar results occurred with neuronal and epidermal precursors. Orientation of a, e, f and g is anterior to the left and dorsal towards the top of the page. Scale bars, 100 μm.
Cloning and expression of a cDNA encoding an endothelin receptor

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ENDOTHELINS are a newly described peptide family consisting of three peptides (ET-1, ET-2 and ET-3) which are the most potent vasoconstrictive peptides known.1-3. They are crucial in the regulation of vascular smooth muscle tone.4-7. The diverse functions of endothelins are thought to be mediated by interaction with many different receptors coupled to the inositol phosphate/calcium ion messenger pathway. However, because of the structural resemblance of the three peptides, the presence and nature of multiple endothelin receptors remain to be elucidated. We report here the cloning of a complementary DNA encoding a bovine endothelin receptor, which has a transmembrane topology similar to that of other G protein-coupled receptors and shows specific binding, with the highest selectivity to ET-1 in animal cells transfected with the cloned cDNA. This receptor messenger RNA is widely distributed in the central nervous system and peripheral tissues, particularly in the heart and lung. Our results support the view that there are other receptor subtypes.

We previously reported an expression-cloning strategy for receptors that can be electrophysiologically assayed in Xenopus oocytes after injection of appropriate exogenous mRNAs.6-7. Because oocytes injected with the bovine lung mRNA showed a potent and specific electrophysiological response to application of endothelin, we adopted this strategy for the isolation of a functional cDNA clone encoding a bovine receptor. A bovine lung cDNA library was constructed in an RNA expression vector from an mRNA fraction giving potent receptor expression in Xenopus oocytes. A functional receptor cDNA clone was identified and purified from the cDNA library by testing for expression in oocytes after injection of the mRNAs synthesized in vitro from the subdivided cDNA mixture. The cDNA clone was verified to encode a functional receptor in the oocyte expression system. A potent electrophysiological response was induced by application of ET-1 or ET-2, whereas a moderate response was produced by ET-3, but not by any of the peptides unrelated to endothelin which we analysed (data not shown). Furthermore, the endothelin-evoking currents were fluctuating, long-lasting and were thus characteristic of the response mediated through activation of the inositol phosphate/Ca2+ second messenger pathway.

Figure 1 shows the 3,216-nucleotide sequence of the cloned cDNA and the amino-acid sequence deduced for the endothelin receptor, which was assigned from the longest open reading frame of the cDNA. The nucleotide sequence surrounding the initiation codon agrees well with the consensus sequence. The deduced amino-acid sequence of the receptor consists of 427 amino-acid residues with a relative molecular mass of 48,516. Hydrophathy and amino-acid homology analyses of the deduced sequence show the existence of seven hydrophobic segments and a significant sequence similarity with G protein-coupled receptors.10-12. The receptor probably has seven membrane-spanning domains with an extracellular N terminus and a cytoplasmic C terminus.

The endothelin receptor is not particularly closely related to other peptide receptors4-7 in terms of sequence similarity (23-26% identity in the core sequence covering transmembrane segments I-VII). It has a relatively long N terminus preceding transmembrane segment I, and this portion, as in lutropin-choroionadotropin and other large hormone receptors13-14, may be involved in binding a relatively large endothelin peptide. There are several potential sites for post-translational modification in the receptor: a possible site for cleavage of an N-terminal hydrophobic sequence as a signal peptide; two consensus sites for N-glycosylation15 (Asn 29 and Asn 62); six cysteine residues in the C terminus near transmembrane VII, one of which may be palmitoylated as in the β2-adrenergic receptor;16, and several proline residues for regulatory phosphorylation in the cytoplasmic domain.15-17. Another interesting feature is the similarity of the sequence (residues 251-261) near transmembrane V with the consensus sequence of domain 9 of the serine/threonine protein kinase family,18 but the relevance of this sequence similarity is unknown.

Previous pharmacological and ligand-binding studies suggested that there are several types of endothelin receptor in mammalian tissues.19,20. However, the precise characterization of the individual receptors has been hampered by the cross-reactivity of each receptor with other endotheins. We determined the affinities of the cloned receptor for endothelin peptides in monkey kidney COS cells after transcription of the cloned cDNA. Binding of 125I-labelled ET-1 to membranes prepared from cells transfected with the receptor cDNA was saturable with a dissociation constant (Kd) of 0.18 nM (Fig. 2a). No such binding was detected on membranes prepared from untransfected cells or from cells transfected with the vector DNA alone. Displacement experiments of 125I-labelled ET-1 binding indicated that ET-1 is the most potent inhibitor of radioligand binding, being about 10 times more effective than ET-2 (Fig. 2b) although these two peptides differ in only 2 out of 21 amino-acid residues.1 An endothelin-like venom toxin, sarafotoxin S6b, and ET-3 also displaced the radioligand binding, but these two peptides were much less effective than either ET-1 or ET-2. The values of the inhibition constant (K0) for ET-1, ET-2, sarafotoxin S6b and ET-3, were calculated to be

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