Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo

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Midline signaling by Hedgehog (Hh) family members has been implicated in patterning the vertebrate embryo. We have explored the potential regulatory role of cAMP-dependent protein kinase A (PKA) in these events. Zebrafish embryos injected with RNAs encoding Sonic hedgehog (Shh), Indian hedgehog (Ihh), or a dominant-negative regulatory subunit of PKA, PKI, have equivalent phenotypes. These include the expansion of proximal fates in the eye, ventral fates in the brain, and adaxial fates in somites and head mesenchyme. Moreover, ectopic expression of PKI partially rescues somite and optic stalk defects in *no tail* and *cyclops* mutants that lack midline structures that normally synthesize Shh. Conversely, all cell types promoted by ectopic expression of *hhs* and PKI are suppressed in embryos injected with RNA encoding a constitutively active catalytic subunit of PKA (PKA^{*}). These results, together with epistasis studies on the block of ectopic Hh signaling by PKA^{*}, indicate that PKA acts in target cells as a common negative regulator of Hedgehog signaling.

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Members of the *hedgehog* (*hh*) gene family encode signaling proteins involved in induction and patterning processes in vertebrate and invertebrate embryos. In vertebrates, *hhs* constitute a multigene family. *Sonic hedgehog* (*Shh*), which has received most of the experimental attention, has been isolated from mouse (Echelard et al. 1993; Chang et al. 1994), chick (Riddle et al. 1993), rat (Roelink et al. 1994), *Xenopus* (Ekker et al. 1995), and zebrafish (Krauss et al. 1993). The early expression is limited to the notochord, the floor plate and its anterior extension in the brain, and the zone of polarizing activity (ZPA) in the posterior mesenchyme of the limb bud. All of these sites have been shown to act as organizing centers patterning neighboring tissues by the secretion of morphogenetic signals (e.g., Saunders and Gasseling 1983; Brand-Saberi et al. 1993; Pourquie et al. 1993; Yamada et al. 1993). Recent evidence indicates that Shh is involved in these interactions.

For example, ectopically expressed *Shh* leads to a ventralization of large regions of the mid- and hindbrain in mouse and zebrafish, as revealed by the dorsally expanded expression pattern of *HNF-3 β* and its zebrafish homolog *axial* (Echelard et al. 1993; Krauss et al. 1993). In explants of intermediate neuroectoderm at spinal cord levels, Shh protein induces, in a dose-dependent fashion, floor plate, and motor neuron development (Martí et al.

1995a; Roelink et al. 1995; Tanabe et al. 1995). In explants taken at midbrain and forebrain levels, Shh also induces the appropriate ventrolateral neuronal cell types, dopaminergic (Heynes et al. 1995; Wang et al. 1995) and cholinergic (Ericson et al. 1995) precursors, respectively. In chick somites (Johnson et al. 1994) and mouse presomitic explants (Fan et al. 1995), Shh promotes the expression of sclerotome-specific markers like *Pax1* and *Twist* at the expense of the dermamyotomal marker, *Pax3*. Together with Wnt signals, Shh also promotes myotomal development (Münsterberg et al. 1995). Furthermore, when ectopically expressed in the anterior region of chick limb buds, *Shh* leads to a mirror-image duplication of digits similar to transplants of the ZPA (Riddle et al. 1993; Chang et al. 1994).

Less is known about the properties of other vertebrate Hedgehogs (Hhs). In the mouse, two additional *Hh* genes have been identified. *Indian hedgehog* (*Ihh*) is expressed in the growth zone of the developing cartilage and in intestinal epithelium, whereas *Desert hedgehog* (*Dhh*) is expressed by Sertoli cells in the testes (Bitgood and McMahon 1995). Mutational studies have demonstrated that *Dhh* is required for normal spermatogenesis (Bitgood et al. 1996). Other *hh* genes isolated from *Xenopus* and zebrafish are expressed earlier, beginning with the onset of gastrulation, and exhibit distinct and partially overlapping expression patterns (Ekker et al. 1995a,b). One of these genes, *tiggy-winkle hedgehog* (*twhh*) is co-expressed with *shh* in the ventral central nervous system

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(CNS), and like *shh*, has been shown to promote proximal cell fate in the zebrafish eye (Ekker et al. 1995b).

In *Drosophila*, only a single *hh* gene has been identified and shown to have a pivotal role in the regulation of many aspects of larval and adult pattern formation. For example, specific cell fates in the larval cuticle appear to be regulated by concentration-dependent Hh signaling (Heemskerk and DiNardo 1994). In the eye imaginal disc, Hh produced by newly differentiating photoreceptors regulates the movement of the morphogenetic furrow and, therefore, recruitment of uncommitted cells to differentiated fates (Heberlein et al. 1993; Ma et al. 1993). In the leg and wing imaginal discs, Hh produced by cells in the posterior compartment regulates gene expression at the compartment boundary, which is essential for normal patterning and outgrowth of these appendages (e.g., Basler and Struhl 1994; Tabata and Kornberg 1994; Fietz et al. 1995; Ingham and Fietz 1995).

Drosophila studies have identified several genes that appear to mediate Hh signaling or are targets of the Hh signaling pathway (for review, see Forbes et al. 1993; Perrimon 1995), although the Hh receptor itself has not been identified. The transmembrane protein Patched, the serine-threonine kinase Fused, and the zinc-finger protein Cubitus interruptus are all involved in the intracellular regulation of Hh signaling in the responding cells (Ingham et al. 1991, 1993). Moreover, several recent studies point to a role for cAMP-dependent protein kinase A (PKA) as a regulator of Hh signaling. Loss of PKA activity leads to the cell-autonomous, Hh-independent, transcriptional activation of Hh target genes (*patched*, *wingless*, and *decapentaplegic*) in eye, leg, and wing imaginal discs (Jiang and Struhl 1995; Lepage et al. 1995; Li et al. 1995; Pan and Rubin 1995; Strutt et al. 1995; for review, see Kalderon 1995; Perrimon 1995). Thus, PKA acts as a negative regulator of the Hh signaling pathway in *Drosophila*.

The mechanism by which Hh signals are transduced in vertebrates is less well understood. However, there is evidence that PKA may be involved, as a variety of drugs that stimulate PKA activity block Shh-mediated induction of *Pax-1* in somite explants and tyrosine hydroxylase-expressing neurons in mesencephalic explants (Fan et al. 1995; Heynes et al. 1995). We have explored the role of Hh and PKA signaling in patterning the zebrafish embryo. Our experiments demonstrate that suppression of PKA activity mimicks Hh-specific inductions in many different Hh-responsive cells, whereas constitutive PKA activity blocks the effects of endogenous and ectopic Hh signaling. Thus, PKA acts in target cells as a common negative regulator of Hh signaling pathways in vertebrate embryogenesis.

Results

Inhibition of PKA and ectopic expression of hhs leads to similar alterations in eye patterning

Expression of *shh* at the ventral midline of the forebrain has been implicated in patterning the vertebrate eye (Ek-

ker et al. 1995b; Macdonald et al. 1995). Proximal cell fates, which appear to depend on Shh signaling, are expanded in zebrafish embryos ectopically expressing *shh*. Concomitantly, distal retinal fates are reduced, resulting in a small eye phenotype (Ekker et al. 1995b; Macdonald et al. 1995). To determine whether Shh may act by regulating the activity of PKA, we compared the phenotype generated in zebrafish embryos ectopically expressing a dominant-negative form of PKA, PKI, with those induced by ectopically expressed *shh*, and several other vertebrate *hh* family members.

In the inactive state, PKA consists of a tetramer composed of two identical regulatory subunits and two identical catalytic subunits. Binding of cAMP by the regulatory subunits leads to dissociation of the complex, release of the catalytic subunits, and as a result, phosphorylation of target proteins. Clegg et al. (1987) have reported that cAMP-mediated activation of PKA is effectively blocked in tissue culture cells expressing a regulatory subunit containing a point mutation in the cAMP-binding domains. Thus, injection of RNA encoding this subunit into the zebrafish embryo provides a strategy by which endogenous PKA activity may be inhibited specifically.

Embryos injected with RNAs encoding zebrafish Shh, mouse Shh and *Ihh*, and mouse PKI display a similar reduced eye development. The frequency and severity of the phenotype is highest in embryos ectopically expressing zebrafish *shh* (158 of 169; 93%), mouse *Ihh* (104 of 120; 87%) and PKI (105 of 130; 81%), but weaker for those expressing mouse *Shh* (43 of 85; 51%) (Fig. 1A–E). Injection of a *lacZ* control RNA gave no phenotype (0 of 105).

Previous reports (Ekker et al. 1995b; Macdonald et al. 1995) have demonstrated that the small eye phenotype is the result of an expansion of the *pax2* and a reduction of the *pax6* expression domain. *pax2* is normally expressed proximally in the presumptive optic stalk, adjacent to a domain of *shh* expression at the ventral midline of the forebrain, whereas *pax6* expression is restricted to more distal regions of the eye primordia (Krauss et al. 1991; Fig. 1F,I). As with ectopic *shh* expression, ectopic expression of *Ihh* (50 of 52, 96%) and PKI (48 of 73, 66%) leads to expansion of the *pax2* expression domain (Fig. 1G,H), whereas *pax6* expression is substantially reduced (*Ihh*: 16 of 39, 41%; PKI: 38 of 49, 76%) (Fig. 1J,K). Therefore, ectopic expression of PKI mimicks the phenotypic consequences of ectopic *hh* expression in the developing eye. Moreover, it is clear that *Ihh*, whose expression is normally restricted to the gut and cartilage (Bitgood and McMahon 1995), phenocopies the effects of *Shh*. It is therefore, likely that different *hh* family members share similar signaling properties.

In *cyclops* (*cyc*; Hatta et al. 1991b) mutant zebrafish embryos, which lack *shh* expression in the ventral forebrain, the expression of *pax2* in the region of the optic stalk is reduced strongly at early somite stages (Krauss et al. 1993) and absent completely by early pharyngula stages (Fig. 1L,M). Thus, the optic stalk deficiencies in *cyc* mutants might result from the lack of *shh* expression

Figure 1. The effect of expression of *hh* family members and PKI on eye patterning in wild-type and *cyc* mutant embryos. (A,F,I,L) Control embryos. Embryos in all other panels were injected with the indicated RNAs. (A–E) Four-day-old larvae, lateral view of head. (F–K) Twenty-six-hour-old wild-type embryo, dorsal view of head. (F–H) Expression of *pax2* (p), distal expansion in eye primordia (arrow), and *shh* (s). (I–K) Expression of *pax6*. (L–O) Twenty-six-hour-old embryos, lateral view of head; expression of *pax2* and *shh*. (M–O) *cyc* mutants. Rescued *pax2* expression in optic stalk (p), ectopic *shh* expression in dorsal regions of the midbrain (s) (cf. with Fig. 2F). (os) Optic stalk; (mhb) midbrain–hindbrain boundary; (vb) ventral base.

(Macdonald et al. 1995). In agreement with this model, injection of RNA encoding *Ihh* and PKI partially rescues *pax2* expression in *cyc* mutants (*Ihh*: 7 of 14, 50%; PKI: 9 of 19, 47%) (Fig. 1N,O), in the absence of any rescue of *shh* expression in the ventral forebrain. Consequently, embryos still develop a fused eye phenotype. Ectopic *shh* expression is detected in PKI-injected *cyc* mutant embryos, but this is restricted to the dorsal midbrain region, similar to wild-type embryos injected with PKI-encoding RNA (cf. Fig. 1O with Fig. 2F). These experiments suggest that early expression of *pax2* in the presumptive optic stalk depends on Hh-mediated inhibition of PKA activity in target cells. Moreover, it is unlikely that the *cyc* gene product acts downstream of a Hh signal, as both *Ihh* and PKI rescue *pax2* expression.

Regional ventralization of the CNS by PKI and *hh* signaling

Ectopic expression of *shh* in a variety of vertebrate embryos and application of purified Shh to CNS explants has demonstrated that Shh is a ventralizing factor in the vertebrate CNS (see above). Moreover, the related tran-

scriptional regulators, *HNF-3 β* (chick, rat, mouse) and *axial* (zebrafish), which are normally expressed at the ventral midline of the CNS, are apparent targets of the Shh signaling pathway.

Ectopic expression of zebrafish *shh* (Krauss et al. 1993) (36 of 45, 80%; Fig. 2B) and PKI (153 of 203, 76%; Fig. 2D) in the zebrafish embryo gave almost identical results, a dramatic dorsal expansion of *axial* expression in the midbrain, hindbrain, and anterior spinal cord. Ectopic expression of mouse *Ihh* (61 of 103, 59%; Fig. 2C) and mouse *Shh* (3 of 50, 6%; data not shown) gave a weaker ventralization restricted to the midbrain. Thus, suppression of endogenous PKA activity leads to a strongly ventralized phenotype, closely resembling that generated by the most potent ventralizing signal, zebrafish Shh. PKI expression results in only a slight broadening of the *shh* expression domain at the diencephalic–midbrain border (32 of 49, 65%) and a weak activation in the posterior midbrain (2 of 24, 8%; Fig. 2F). Thus, widespread ectopic *axial* expression presumably reflects a direct activation of the intracellular Shh signaling pathway and not the indirect consequence of induction of an endogenous Shh signal.

Figure 2. The effect of expression of *hh* family members and PKI on CNS patterning. (A–D) Expression of *axial*, 26-hour-old embryos, lateral view of head. The segmented ectopic expression in the hindbrain in B and D is indicated by arrowheads. (E,F) Expression of *shh*, 26-hour-old embryos, lateral view of head. The expanded expression at the diencephalic–mesencephalic boundary and the ectopic expression in the posterior midbrain is indicated by arrowheads. (G,H) Expression of *otx2* and *axial*, 25-somite stage, lateral view of head. The ectopic expression of *axial* in the hindbrain is indicated by arrowheads; the expression of *otx2* in the ventral forebrain by an arrow. (I–L) Expression of *isl1*, 26-hour-old embryos; (I,J) dorsal view of hindbrain, rostral to the left; (K,L) optical cross-section at level of arrowhead in M and N. The neural tube is outlined. Sites of expanded *isl1* expression in the hindbrain and the anterior spinal cord are indicated with arrows in I and L. (M,N) Expression of *wnt1*, 26-hour-old embryos, lateral view of head. (allg) Anterior lateral line ganglion; (dmb) diencephalic–mesencephalic boundary; (mhb) midbrain–hindbrain boundary; (mn) motoneurons; (tg) trigeminal ganglion.

Interestingly, no ectopic induction of *axial* expression is observed anterior to the position where expression normally terminates, at the diencephalic–midbrain boundary, suggesting that the anterior limit of *axial* expression is set by a change in the cellular competence to respond to Hh signaling in the forebrain. In support of this conclusion, expression of the homeo domain-containing transcriptional regulator *otx2* (Li et al. 1994) in the ventral forebrain remains unaltered in embryos exhibiting a strong PKI-induced ventralization of the hindbrain (0 of 64, Fig. 2H). In the hindbrain there is also evidence for differential responsiveness within adjacent rhombomeres, as ectopic expression of both zebrafish *shh* and PKI leads to a marked segmental organization to the domains of ectopic *axial* expression (Fig. 2B,D).

To determine whether expression of PKI leads to other aspects of ventralization we examined the expression of *isl1*, a LIM domain transcription factor expressed in motor neurons (Korzh 1993; Inoue et al. 1994; Appel et al. 1995). Motor neurons arise at ventrolateral positions along the CNS, most likely in response to induction by Shh (Martí et al. 1995a; Roelink et al. 1995). Embryos expressing PKI have a substantial increase (PKI: 23 of 36, 64%; *Ihh*: 3 of 20, 15%) (Fig. 2I,J) and a dorsal expansion

(Fig. 2K,L) in *isl1* expression in the hindbrain and the anterior spinal cord, but no alteration in forebrain expression (data not shown).

In summary, inhibition of PKA mimics several aspects of Shh-mediated ventralization in the CNS. Moreover, the effects are restricted to only those regions that are also responsive to the ectopic expression of *shh*, and related *hhs*. Although we observe extensive ventralization in the mid- and hindbrain regions, *wnt1* expression at the dorsal midline (Molven et al. 1991) (0 of 59, Fig. 3M,N) is largely unaltered. Thus, the most dorsal cell fates are not suppressed by ventralizing signals in these experiments.

The effects of Ihh and PKI on the patterning of somites

Shh expression is also implicated in the regulation of somite pattern in the paraxial mesoderm (for review, see Bumcrot and McMahon 1995). For example, sclerotome formation by the somite occurs in response to midline signaling by the notochord (Brand-Saberi et al. 1993; Pourquie et al. 1993), and Shh is able to activate several sclerotome markers in presomitic mesoderm (Johnson et al. 1994; Fan et al. 1995), mimicking the inducing effect

Figure 3. The effect of expression of *hh* family members and PKI on the specification of the myotome in wild-type and *ntl* mutant embryos. (A–C) Fifteen-somite-stage embryos, lateral view. (D–I) Fifteen-somite-stage embryo, dorsal view of posterior part of axis; (D–G) expression of *myoD*; (H,I) expression of *shh*. (J,K) Expression of *en* in 25-somite-stage embryo, lateral view. (L,M) Expression of *en* in *ntl* embryos at 30-somite stage, lateral view of tail region.

of the notochord. In contrast to the chick and mouse, the sclerotome of young zebrafish somites is much smaller than the myotome and is located in ventral medial positions at some distance from the notochord (Kimmel et al. 1995). The positions of the paraxial mesoderm adjacent to the notochord are occupied by a specific subset of myotomal cells that express *myoD* (Weinberg et al. 1996) in the presomitic mesoderm. After somite formation, these adaxial cells give rise to the muscle pioneer cells, which can be identified by their enhanced expression of a zebrafish *engrailed* gene (*en*; Hatta et al. 1991a). It is likely that induction of the muscle pioneers is dependent on notochordal signals as these cells are lost in *no tail* (*ntl*) mutants in which the notochord precursors fail to differentiate (Halpern et al. 1993; Schulte-Merker et al. 1994).

To address the role of Hh signaling in zebrafish somite patterning, we injected RNAs encoding various *hhs* and PKI. Embryos injected with zebrafish *shh*, mouse *Shh* or *Ihh*, and PKI display a clear somitic phenotype. At the

15-somite stage, somites do not form the normal chevron shape (Fig. 3A–C), and in a smaller proportion of the embryos, the horizontal myoseptum, which lies at the midline of the dorsal–ventral axis of the somite at later stages of development, is irregular. In the presomitic mesoderm, *myoD* expression, which is normally restricted to the adaxial mesoderm immediately adjacent to the notochord, extends laterally over the entire width of the presomitic mesoderm (*mShh*: 30 of 48, 62%; *Ihh*: 28 of 51, 55%; PKI: 22 of 25, 88%) (Fig. 3D–G). Consequently, there is a corresponding increase in *en*-expressing muscle pioneers. Instead of five to six per somite as observed in uninjected control embryos, or controls injected with *lacZ* RNA (35 of 35), embryos ectopically expressing *Ihh* have ≤ 15 (*mShh*: 18 of 27, 67%; *Ihh*: 41 of 57, 72%), and those expressing PKI as many as 30 muscle pioneer cells per somite (58 of 82, 71%) (Fig. 3J,K; data not shown). Moreover, these *en*-expressing cells are dispersed over the entire mediolateral width of the somite, whereas in uninjected embryos they are exclusively medial (Fig. 4I). Thus, ectopic expression of *hh* family members and PKI leads to an expansion of medial muscle cell fate in the paraxial mesoderm.

myoD expression is also expanded in the somites themselves, most dramatically in PKI-injected embryos, consistent with an enlargement of the myotome (Fig. 3D–G). As we detect no ectopic expression of the endogenous *shh* gene in caudal regions of PKI-injected embryos (0 of 25; Fig. 3H,I), the activation of targets of the Hh signaling pathway occurs in the apparent absence of a Hh signal.

The finding that ectopic *Shh*, *Ihh*, and PKI induce the formation of extra muscle pioneers in wild-type embryos suggests that the reduction of a Hh signal might be the reason for the somite alterations in *ntl* mutant embryos. Although *ntl* mutants show some *myoD* staining in adaxial positions of the presomitic mesoderm, *En*-positive muscle pioneers never form (Halpern et al. 1993). Injection of mRNA encoding *Ihh* or PKI leads to a broadening of the adaxial expression of *myoD* in the presomitic mesoderm of *ntl* mutants (*Ihh*: 6 of 8, 75%; PKI: 5 of 6, 83%; data not shown). Moreover, in 80% of the PKI-injected *ntl* mutants, *En*-positive muscle pioneer cells are observed at later stages of development (10 of 12; Fig. 3L,M). Therefore, suppressing PKA activity in target cells can rescue the defects that result from the loss of Hh signaling by the notochord. Interestingly, we failed to rescue muscle pioneers by injection of *Ihh* mRNA. Thus, although *Ihh* can activate *myoD* expression in the absence of the notochord, it does not appear to be sufficient for the induction of muscle pioneers.

We also examined *twist* (Yan et al. 1995) expression in *hh* and PKI-injected embryos. *twist* is expressed in both the sclerotome and the hypochord, a prominent midline rod-like structure of unknown function that lies immediately beneath the notochord in the fish embryo. *twist* expression is lost or reduced strongly in presumptive sclerotomal cells in ventromedial positions within the somite (*Ihh*: 8 of 14, 57%; PKI: 27 of 36, 75%; Fig. 4A,B; data not shown). Remaining *twist* expression is re-

Figure 4. The effects of expression of *hh* family members and PKI on expression of *twist* in the sclerotome, head mesenchyme, and hypochord. (A–D) 20-somite stage; (A,B) dorsal view; (C,D) optical cross-section at level of tenth somite; the notochord is outlined. In B, note the abrupt change in the response of *twist* expression at the level of the first somite, indicated with an arrow. In B and D, the enhanced midline expression of *twist* in PKI-injected embryos represents hypochordal expression. (E–H) Thirty-hour-old embryos; (E,F) lateral view of head; (G,H) optical cross-section at level of hindbrain. (I) Double-labeling of *twist* mRNA (blue) and En protein (brown) in a 25-somite-stage PKI-injected embryo that is only affected on one (lower) side; dorsal view. The stained hypochord in the ventral midline is marked by asterisks. (hc) Hypochord; (nc) notochord; (sc) sclerotome.

stricted to ventrolateral positions (Fig. 4C,D). The coincidence of the loss of sclerotomal and the gain of adaxial myotomal specification is revealed clearly in double-labeled embryos in which only one side of the axis is affected, presumably because of the asymmetric localization of the injected RNA (Fig. 4I; see Materials and methods). These findings suggest that high concentrations of Hh and PKI promote myotomal and repress sclerotomal specification in zebrafish somites. In contrast to the sclerotome of the trunk, *twist* expression is up-regulated in the hypochord (Fig. 4A–D).

Many studies have addressed the inductive properties of notochordal signals on presomitic mesoderm. How-

ever, the notochord extends into the head, where it is surrounded by head mesenchyme. In wild-type zebrafish embryos, adaxial cells of the head mesenchyme adjacent to the notochord express *twist*. This expression is increased strongly and expanded laterally in embryos injected with *Ihh* or PKI mRNA (*Ihh*: 32 of 47, 68%; PKI: 43 of 59, 73%; Fig. 4A,B, E–H). Thus, Hh signaling may have a role in patterning the head mesenchyme, and as in all other sites studied, this induction is mimicked by suppression of PKA.

A constitutively active form of PKA represses structures that are promoted by Ihh and PKI

If PKA is a negative regulator of Hh signaling in responding cells, we would predict that stimulation of PKA activity should suppress endogenous Hh signaling. To test this model, we injected mRNA encoding a mutated form of the catalytic subunit of PKA (PKA*), which is rendered constitutively active because of a reduced affinity for the regulatory subunit (Orellana and McKnight 1992).

In contrast to the dominant-negative regulatory subunit, zebrafish embryos display an extreme sensitivity to the expression of the constitutively active catalytic subunit. Almost all embryos injected with 100 pg of RNA encoding PKI and *Ihh* survive, whereas all those injected with the same amount of RNA encoding PKA* die during blastula and gastrula stages. Lowering the mass of RNA injected enhances embryo survival (90% survive postgastrulation after injection of 5 pg of mRNA), and having passed the critical early stages, surviving embryos exhibit no increase in lethality. Approximately half (19 of 36) of these display a common phenotype: fused eyes (Fig. 5A,B), a short and laterally curved body axis and somites that lack their horizontal myoseptum (Fig. 5C,D). Both the notochord and the floor plate appear normal.

Similar to *cyc* mutant embryos, which lack midline *shh* expression, *pax2* expression in the optic stalk is greatly reduced or completely missing at the 15-somite stage of PKA*-injected embryos (61 of 82, 74%; Fig. 5E,F). At early pharyngula stages, some *pax2* expression can be detected in the forebrain of injected embryos; however, this expression remains at the midline rather than being split into two domains defining the proximal region of the developing eyes (47 of 62, 76%; Fig. 5G,H). In addition, the expression domains of *pax6*, which normally define distal regions of the eye vesicles, are shifted toward the midline (28 of 35, 80%; Fig. 5I,J).

As in *cyc* mutant embryos, the loss of *pax2* expression and fusion of the eyes in PKA*-injected embryos might be a secondary consequence of the failure of ventral midline specification in the forebrain. In agreement with this interpretation, the normal forebrain extension of *shh* (14 of 25, 56%; Fig. 5K,L) and *otx2* (14 of 20, 70%; Fig. 5M,N) expression is not observed in PKA*-injected embryos. Thus, Shh signaling may be required for normal ventralization of the forebrain, acting, at least in part, as an autoinductive signal.

Figure 5. Expression of PKA* suppresses ectopic and endogenous Hh signaling. (A,B) Forty-eight-hour-old embryo, ventral view of head. (C,D) Seventy-two-hour-old larvae, lateral view of trunk somites. (E–H) Expression of *pax2*. (E,F) Fifteen-somite stage, lateral view of head. (G,H) Twenty-four-hour-old embryos, dorsal view of head. (I,J) Expression of *pax6* in a 24-hr-old embryo, dorsal view of head. (K,L) Expression of *shh* in 24-hour-old embryos, lateral view of head. *shh* expression in the ventral forebrain is indicated by an arrow. (M,N) Expression of *otx2* in a 30-hour-old embryo, dorsal view of head. (M) *otx2* expression in the ventral forebrain is indicated by an asterisk; (N) *otx2* expression in proximal regions of the eyes is indicated by an arrow. (O,P) Expression of *axial* in 24-hr-old embryos, lateral view of head. (Q–S) Expression of *myoD* (*m*) and *pax2* (*p*) in a 15-somite-stage embryo, dorsal view of posterior trunk. (S) PKA* and *lhh* coinjected. (T,U) Expression of *en* in 24-hr-old embryos, lateral view of trunk. (ac) Adaxial cells; (dmb) diencephalic–mesencephalic boundary; (mhb) midbrain–hindbrain boundary; (op) otic placode; (os) optic stalk; (pn) pronephros.

At the mid/hindbrain level, ventral expression of *shh* appears unaltered. In contrast, expression of *axial* is reduced but never absent completely (38 of 56, 68%; Fig. 5O,P). In the floor plate of the spinal cord, expression of *shh* and *axial* are normal (data not shown) and dorsal midline expression of *wnt1* throughout the CNS is unaffected (16 of 16; data not shown). Thus, stimulation of PKA activity leads to ventral-specific defects in anterior CNS patterning that are consistent with the suppression of endogenous Hh signaling.

In the mesoderm, adaxial *myoD* expression in pre-somitic mesoderm is also missing at early somite stages (57 of 73, 78%; Fig. 5Q,R), with an accompanying loss of *en*-expressing muscle pioneers at later stages (26 of 28, 93%; Fig. 5T,U). Furthermore, *myoD* expression in the somites is reduced greatly. In summary, structures that

are affected by ectopic expression of *hh* genes and PKI are also modulated by PKA*, but with opposite results. Thus, specification of proximal fates in the eyes, ventral fates in the brain, and medial muscle fates in the somites are promoted by ectopic expression of *hhs* and inactivation of PKA, but suppressed by activation of PKA.

To determine whether PKA is epistatic to Hh signals, as would be expected from our analysis, we examined the effects of coinjection of *shh*, *Ihh*, and PKA* mRNA on patterning of the eyes, brain, and presomitic mesoderm. Even when *hh* RNAs are injected in a 10- to 100-fold molar excess, no ectopic *pax2*, *myoD*, or *axial* expression is observed. Moreover, the endogenous expression domains of these target genes are suppressed as also observed in embryos injected with PKA* mRNA alone (Fig. 5Q–S; Table 1).

Table 1. Coinjection of constitutively active PKA* and Hhs

Phenotype	zfShh (40 pg) + PKA* (4 pg)	zfShh (40 pg)	PKA* (4 pg)	Ihh (200 pg) + PKA* (2 pg)	Ihh (200 pg)	PKA* (2 pg)
Ectopic <i>myoD</i>	0/27 (0%)	15/24 (62%)	0/26 (0%)	0/28 (0%)	22/38 (59%)	0/26 (0%)
Loss of <i>myoD</i>	7/27 (26%)	0/24 (0%)	13/26 (50%)	15/28 (54%)	0/38 (0%)	22/26 (85%)
Ectopic <i>pax2</i>	N.D.	N.D.	N.D.	0/46 (0%)	36/38 (95%)	0/26 (0%)
Loss of <i>pax2</i>				34/46 (74%)	0/38 (0%)	23/26 (88%)
Ectopic <i>axial</i>	0/20 (0%)	14/23 (61%)	0/16 (0%)	N.D.	N.D.	N.D.
Loss of <i>axial</i>	10/20 (50%)	0/23 (0%)	16/16 (100%)			

Sibling embryos were either injected with *hh* RNA, or with PKA* RNA or with both, and were stained for *myoD* (in the presomitic mesoderm) or *pax2* RNA (in the promimal regions of the eyes) at the 20-somite stage, or for *axial* RNA (in the ventral base of the brain) at 26 hr of development.

(N.D.) Not done.

Discussion

Hh signaling is mimicked by repression of PKA

Our studies point to an evolutionarily conserved role for PKA as a negative regulator of the Hh signaling pathway. Ectopic expression of PKI, a potent dominant-negative regulatory subunit of PKA, leads to alterations in the patterning of the brain, eyes, and somites that mimic the effects of ectopic expression of several *hh* family members. In all cases, the responsive cells lie in close proximity to normal sites of *shh* expression in midline cell populations. In contrast, ectopic expression of a constitutively active form of PKA suppresses completely the effects of ectopic Hh signaling and partially suppresses the targets of endogenous Hh signaling. Thus, PKA is epistatic to Hh signals.

In general, the results are consistent with a simple model: Hh signaling by midline structures (the notochord and ventral CNS) acts on neighboring cells to overcome a PKA-mediated inhibition of Hh target genes. The signal may be Shh, or other midline-expressed family members. An alternative explanation, that PKI-mediated pattern alterations are a secondary consequence of the activation of endogenous *shh* expression, seems unlikely for a number of reasons. For example, we fail to observe significant ectopic expression of *shh* in PKI-injected embryos despite the dramatic expansion of *pax2* expression in the eye, *axial* expression in the brain, and *myoD* and *en* expression in the muscle pioneers and myotome of the somites. Furthermore, *pax2* expression in the eye primordium and *en* expression in muscle pioneers are rescued by PKI injection in *cyc* and *ntl* mutants, respectively, although these mutants lack the normal sites of Shh synthesis that are associated with the induction of these markers. Thus, increased Shh production by the normal midline signaling centers does not appear to be responsible for the PKI-mediated activation of Hh target genes. Rather, as has been observed in PKA-deficient clones in *Drosophila* imaginal discs (Li et al. 1995), PKI-mediated activation of Hh targets is most likely cell autonomous and Hh independent.

Interestingly, zebrafish embryos expressing PKI have no additional phenotypes compared with those ectopically expressing *hhs*. Thus, a major early role of PKA in

the fish appears to be the modulation of Hh signaling. This function is conserved in other vertebrates. For example, in the mouse, ectopic expression of *Shh* and PKI in the CNS both lead to the ectopic transcriptional activation of *HNF-3 β* , an *axial*-related gene, whose expression is normally restricted to the ventral midline. Furthermore, *Patched*, which codes for a transmembrane protein whose *Drosophila* ortholog is regulated by Hh and PKA, is ectopically transcribed (Echelard et al. 1993; Goodrich et al. 1996; D.J. Epstein, E. Martí, M.P. Scott, and A.P. McMahon, in prep.). Moreover, our observation that Ihh has similar inductive properties to Shh, and that signaling can be blocked by PKA*, suggests that Ihh, and possibly other Hh family members, may also act through a similar pathway. At present it is not clear whether PKA is directly in the Hh signaling pathway, or has an indirect role, modulating the activity of a common target (Kalderon 1995; Perrimon 1995). However, the observation that suppression of ectopic Hh signaling by PKA* is not overridden by an excess of Hh signal supports the view that PKA acts downstream of Hh signals in a linear pathway. A satisfactory resolution of this issue will require the identification of Hh receptors and a more thorough characterization of Hh signaling pathways.

Differential responses of the neuroectoderm to Hh signals

An interesting characteristic of Hh- and PKI-injected embryos is that ectopic expression of *axial* is limited to defined regions along the anteroposterior and dorsoventral axes. No ectopic *axial* expression is observed in the forebrain, which lies anterior to the position where normal *axial* expression ends in the CNS. Thus, this boundary is not set by an anterior limit to Hh signaling but by the inability of forebrain cells to respond to the Hh pathway in the same way as their midbrain neighbors. These results indicate that prior anterior–posterior patterning within the CNS dictates the response to Hh signaling. Furthermore, the profile of ectopic *axial* expression in the midbrain and hindbrain in response to zebrafish Shh and PKI is almost identical. Thus, the competence of these target cells to respond to Hh signaling may not be

limited by the distribution of a Hh receptor but by a component of the signal transduction pathway downstream of PKA, such as a transcription factor whose activity is regulated by PKA-mediated phosphorylation.

Surprisingly, ventral development of the spinal cord is unaffected in embryos injected with RNAs encoding various Hhs, PKI, or PKA*, although the presomitic mesoderm at a similar axial level shows a strong response. This is in contrast to findings in vitro where explants of intermediate trunk neuroectoderm show a concentration dependent response to Shh signaling, inducing either floor plate (high concentration) or motor neuron (lower concentration) markers (Roelink et al. 1995). The different outcome of our experiments may have a trivial explanation. For instance, the induction of floor plate may occur later than adaxial specification in the presomitic mesoderm, so that the floor plate in the posterior spinal cord is less affected because of the progressive degradation of the injected synthetic RNA. However, we cannot rule out differences in the competence of the spinal cord to respond to a Hh signaling pathway in vivo versus in vitro. Furthermore, it is not clear whether differential suppression of endogenous PKA activity can lead to the induction of floor plate and motor neurons as separate events, as might be expected if PKA is a direct component of Hh signaling pathways.

Hh signaling in the zebrafish and other vertebrates

Our results raise some interesting questions about the evolution of Hh signaling in the development of different vertebrate structures. In all vertebrates examined, *Shh* is expressed at the midline along the whole length of the body axis. In addition, the fish has at least two additional members, *twhh* (Ekker et al. 1995b) and *moonrat hedgehog* (P. Ingham, pers. comm.) that are expressed in either the notochord or floor plate. We observed a response of neighboring tissues to the overexpression of *hhs* and the suppression or hyperactivation of PKA at all axial levels, consistent with a role for Shh, and possibly other midline expressed Hh family members, in the regulation of several different components of the early body plan.

In the brain, Shh promotes proximal specification in the eye premordia in the forebrain and ventralization of the CNS in the midbrain and hindbrain (Echelard et al. 1993; Krauss et al. 1993; Ekker et al. 1995b; Macdonald et al. 1995). Also in the head, notochordal Hh signaling may regulate adaxial specification in the head mesenchyme. In the trunk and tail, Hhs from the notochord appear to promote the specification of the hypochord, a midline structure lying ventral to the notochord in fish and amphibians, whose origin and function are poorly understood. Mammals and birds do not appear to have a hypochord. In addition, in the paraxial trunk mesoderm, Hh signaling promotes adaxial specification of muscle pioneers, a second cell type not found in chicks and mice, and myotomal specification of the somites.

Although the effects we detect for Hhs in the brain are consistent with results obtained in the mouse (Echelard et al. 1993), our observations on paraxial mesoderm pat-

ternings appear to conflict with results obtained in chicken and mouse experiments (Johnson et al. 1994; Fan et al. 1995). However, when considered in the context of vertebrate evolution from simple chordate ancestors, together with the architecture of different vertebrate body plans, the various results are consistent.

In chick embryos in ovo and in explants of mouse presomitic mesoderm, Shh has been shown to activate sclerotomal- and to repress dermatomal-specific gene expression (Johnson et al. 1994; Fan et al. 1995). Thus, the most ventral component of the somite, the sclerotome, which lies immediately adjacent to the Shh expressing notochord, appears to require Shh signaling for its development. In addition, Shh also induces the expression of the myotome-specific gene *myoD* in the chick and mouse (Johnson et al. 1994; Münsterberg et al. 1995). As the myotome develops at a more dorsal position within the somite than the sclerotome, this would suggest an even more extensive long-range interaction between the somite and Shh produced by midline cells. Surprisingly, the induction of *myoD* expression in vitro requires higher concentrations of Shh than the induction of sclerotome-specific gene expression (Münsterberg et al. 1995). Moreover, chick embryos after notochord removal (Pourquie et al. 1993) and notochordless mouse mutants (*HNF-3 β* ; Ang and Rossant 1994; Weinstein et al. 1994) still undergo myotomal development. Thus, although the myotome responds to Shh in chick and mice, Shh is not absolutely essential for its formation.

In contrast to amniotes, lower vertebrates such as the zebrafish have a quite different arrangement of somite derivatives. The sclerotome is proportionally much smaller, restricted to a medial position several cell diameters away from the notochord. In contrast, the large myotome abuts the notochord, and myotomal cells in contact with the notochord give rise to muscle pioneers. It is thought that the chordate ancestor of the vertebrates, like existing chordates such as *Amphioxus*, lacked sclerotome and, as a consequence, vertebrae, but still developed a large paraxial myotome adjacent to the notochord. Thus, sclerotomal development in the trunk is a recent vertebrate acquisition.

We suggest the following interpretation of the various observations. The initial function of Hh signaling in vertebrates was the induction of muscle development, which may have required different concentrations of Hh signals for different cell types, as has been observed in the ventral CNS (Roelink et al. 1995). Thus, muscle pioneers that develop in contact with the notochord may require higher concentrations of Hh, whereas more general myotome induction may require lower concentrations. The evolution of sclerotome presumably occurred when somitic cells evolved a new competence to respond to midline Hh signals but at some distance from the source. Therefore, sclerotome cells presumably required lower amounts of Hh for their induction than the adaxial muscle cells. Consistent with the view that different concentrations of Hh are required for positioning of somite derivatives, we observed that embryos injected with the constitutively active form of PKA showed some

decrease in the expression of *twist* in the sclerotome, whereas adaxial expression of *myoD* and *en* were lost almost completely (M. Hammerschmidt and A.P. McMahon, unpubl.). Clearly, if expression of PKA* only partially blocks Hh signaling, those cells that require the highest concentration of signal will be most susceptible to PKA*-mediated inhibition. Thus, PKA seems to act as a negative regulator of the Hh signaling pathway in both short- and long-range signaling processes. A moderate inhibition of PKA presumably leads to induction of sclerotomal gene expression and a strong inhibition to the induction of myotomal gene expression.

A second feature of vertebrate evolution is the development of a head skeleton from the cranial neural crest (Noden 1988). Our results suggest that cranial crest cells, which migrate to and surround the anterior notochord, also respond to Hh signals, activating *twist* expression. Thus, during vertebrate evolution, Hh signaling may have had a pivotal role as a common mechanism for the initiation of cartilaginous development in the head mesenchyme and sclerotome. In summary, the induction of muscle precursors might be the phylogenetically older function of Hh signaling, which later in evolution was complemented by an induction of vertebrae and head skeleton structures. The shift in the structure of the somites from myotome to sclerotome as the predominant adaxial component might have been accomplished by modifications in the dependence of the two structures on Hh signals. In the chick, for instance, Shh can only induce *myoD* expression in collaboration with Wnt signals, which most likely are generated by the dorsal ectoderm (Münsterberg et al. 1995).

Materials and methods

Expression constructs

cDNAs for in vitro transcription were cloned into the *Bgl*III site of the plasmid pSP64T (Krieg and Melton 1984). The following cDNA fragments were used. (1) Zebrafish Shh, see Krauss et al. (1993) (pSP64T-zfShh). (2) Mouse Shh, full-length cDNA containing ~250 bp of 5' and 3' untranslated region (UTR), respectively (pSP64T-mShh) (Bumcrot et al. 1995). (3) Mouse Ihh, a 1.2-kb fragment of full-length Ihh cDNA obtained by a complete *Sma*I and a partial *Bam*HI digest, containing the coding region plus 15 bp of 5' UTR and 40 bp of 3' UTR (pSP64T-Ihh). (4) Constitutively active PKA catalytic subunit PKA*, a 1.15-kb *Nco*I-*Apa*I fragment of plasmid pCQ87/R196 (a gift of G.S. McKnight, cf. Orellana and McKnight 1992) (pSP64T-PKA*). (5) Dominant-negative PKA regulatory subunit PKI, plasmid pMT-Rev^{AB} neo (a gift of G.S. McKnight, cf. Clegg et al. 1987), was digested with *Xba*I and resealed to remove the metallothioneine promoter. A 1.4-kb fragment of the religated vector obtained by a partial *Pst*I digest that contains the coding region of the mutated regulatory subunit plus 30 bp of 5' UTR and 20 bp of 3' UTR was cloned into Bluescript, and a 1.4-kb fragment obtained by a complete *Eco*RV and partial *Sma* digest of this construct was cloned into pSP64T (pPSP64T-PKI).

Formation of synthetic mRNA and injection of embryos

The plasmids pSP64T-Shh and pSP64T-Ihh were linearized with *Sal*I, pSP64T-PKI, and pSP64T-zfShh with *Bam*HI, and pSP64T-PKA* with *Xba*I. Capped mRNA was synthesized us-

ing the SP6 message machine kit (Ambion, Texas) according to suppliers' instructions. The quality of the synthetic RNA was determined by ethidium-agarose gel electrophoresis, and the concentration determined spectrometrically. All RNAs generated the expected protein products when translated in a rabbit reticulocyte lysate (GIBCO/BRL).

For injection into embryos, the diluted RNA solutions were supplemented with Phenol red (Sigma; final concentration of 0.05%) to allow a visual control of the injection process. Embryos were injected with ~2 nl of the RNA solution (100 pg, unless stated otherwise) using an Eppendorf microinjector 5242. Normally, the RNA was injected into the center of the yolk of one- to four-cell-stage embryos. Following the cytoplasmic streaming, the RNA is transported very rapidly to the animal pole of the embryo and rather uniformly distributed in all cells, as demonstrated by in situ hybridization at various blastula and gastrula stages (data not shown). In some cases, the RNA was injected into single blastomeres of four- to eight-cell embryos or into the yolk right underneath the blastomeres. RNA supplied this way stays restricted to the injected cells and their descendants, as revealed by in situ hybridization and immunostainings at late blastula stages (data not shown). This often leads to embryos that show the ectopic expression restricted to the left or right half of the embryo, with a sharp border in the dorsal midline (data not shown), thereby providing a negative control in the same embryo.

Previous studies with other mRNAs had shown that ~95% of the synthetic mRNA injected into one- to four-cell-stage embryos appeared full-length at the one-somite stage in Northern blot analyses (M. Hammerschmidt, unpubl.), suggesting that ectopic protein would be present at stages when Hh signaling is normally occurring. Mouse *Shh* and *Ihh* were strongly expressed in injected zebrafish embryos at blastula and gastrula stages, as revealed by whole-mount immunostaining stages with an affinity-purified anti-mouse Shh antiserum (Martí et al. 1995b), which detects both mShh and Ihh but fails to detect endogenous zebrafish Hhs (data not shown).

Fish maintenance, in situ hybridization, and immunostaining

Fish were maintained and bred as described in Mullins et al. (1994). Whole-mount in situ hybridization and antibody stainings were carried out as described previously (Hammerschmidt and Nüsslein-Volhard 1993). Anti-En monoclonal antibody supernatant from 4D9 hybridoma cells (a gift of C. Dreyer, Max Planck Institut für Entwicklungsbiologie, Tübingen, Germany) was diluted 1:1 with incubation buffer. For double-stainings, embryos underwent a standard in situ hybridization, followed by a fixation for 2–6 hr in 4% paraformaldehyde and a standard immunostaining.

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Note added in proof

Following submission of this paper, Weinberg et al. (1996) described an expansion of the presomitic *myoD* expression upon ectopic expression of zebrafish *shh* similar to results presented here.

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