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Floor Plate and Motor Neuron Induction by Different Concentrations of the Amino-Terminal Cleavage Product of Sonic Hedgehog Autoproteolysis

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Summary

The differentiation of floor plate cells and motor neurons can be induced by Sonic hedgehog (SHH), a secreted signaling protein that undergoes autoproteolytic cleavage to generate amino- and carboxy-terminal products. We have found that both floor plate cells and motor neurons are induced by the amino-terminal cleavage product of SHH (SHH-N). The threshold concentration of SHH-N required for motor neuron induction is about 5-fold lower than that required for floor plate induction. Higher concentrations of SHH-N can induce floor plate cells at the expense of motor neuron differentiation. Our results suggest that the induction of floor plate cells and motor neurons by the notochord in vivo is mediated by exposure of neural plate cells to different concentrations of the amino-terminal product of SHH autoproteolytic cleavage.

Introduction

During vertebrate embryogenesis, the patterning of cell types along the dorsoventral axis of the neural tube is controlled by inductive factors that direct the fate of neural progenitor cells. The differentiation of cell types generated in the ventral half of the neural tube depends on factors provided by axial mesodermal cells of the notochord (Jessell and Dodd, 1992; Smith, 1993). The notochord appears to be the source of a contact-dependent factor that induces floor plate differentiation in midline neural plate cells (van Straaten et al., 1988; Placzek et al., 1990, 1991, 1993) and a diffusible factor that induces motor neurons and other neuronal cell types found in the ventral region of the neural tube (Yamada et al., 1993; Tanabe et al., 1995). Floor plate cells subsequently exhibit similar or identical contact-dependent and diffusible inductive activities (Yamada et al., 1991; Hatta et al., 1991; Placzek et al., 1993; Ericson et al., 1995).

Vertebrate homologs of a secreted protein encoded by the Drosophila gene *hedgehog* (*hh*) (Nüsslein-Volhard and Weischaus, 1980; Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993) have been implicated in the induction of ventral cell types in the neural tube. One of these genes, *Sonic hedgehog* (*Shh*), also known as *Vhh-1* or *Hhg-1*, is expressed by cells of the notochord and floor plate over the period that these two midline cell groups exhibit their inductive activities (Riddle et al., 1993; Krauss et al., 1993; Echelard et al., 1993; Roelink et al., 1994; Chang et al., 1994; Ruiz i Altaba et al., 1995a). Misexpression of *Shh* in vertebrate embryos leads to the differentiation of floor plate cells at ectopic locations within the neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ruiz i Altaba et al., 1995a; Ekker et al., submitted). The differentiation of floor plate cells in vitro can also be induced in neural plate explants grown in contact with cells transfected with *Shh* (Roelink et al., 1994; Tanabe et al., 1995; Ericson et al., 1995). In addition, motor neuron differentiation can be induced in neural plate explants by a diffusible factor derived from cells transfected with *Shh* or by transfection of *Shh* directly into neural plate cells (Tanabe et al., 1995). Under these conditions the induction of motor neurons appears not to depend on floor plate differentiation, providing evidence that SHH independently can induce floor plate cells and motor neurons.

The Drosophila HH protein is synthesized as a precursor that undergoes autoproteolytic cleavage to yield smaller amino- and carboxy-terminal domain products (Lee et al., 1994; Porter et al., 1995). The autoproteolytic processing of HH results from an activity inherent in the carboxy-terminal domain of the precursor protein, and mutations in the carboxy-terminal domain that impair cleavage severely attenuate the normal patterning activities of HH in Drosophila development (Lee et al., 1994; Porter et al., 1995). Autoproteolytic cleavage of HH thus appears to be required for its full biological activity. Expression of HH in cultured insect cells generates both the amino- and carboxy-terminal cleavage products, and under these conditions the amino-terminal product is retained on the cell surface, whereas the carboxy-terminal product is freely diffusible (Lee et al., 1994).

The SHH protein also undergoes autoproteolysis to generate amino- and carboxy-terminal cleavage products (Chang et al. 1994; Lee et al., 1994; Porter et al., 1995; Bumcrot et al., 1995). This raises the question of the contribution of proteolytic processing of SHH to its inductive activities on neural plate cells. It is possible that the amino- and carboxy-terminal cleavage products of SHH exhibit distinct biological activities, with one product inducing floor plate cells and the other motor neurons. Alternatively, the uncleaved precursor or a single cleavage product could mediate both the floor plate– and motor neuron–inducing activities of the notochord. In this case, the differentiation of distinct neural cell types could be the consequence of the exposure of neural plate cells to different concentrations of a single SHH cleavage product.

We show here that the notochord processes SHH into an amino-terminal cleavage product that exhibits both floor plate– and motor neuron–inducing activity. Low concen-

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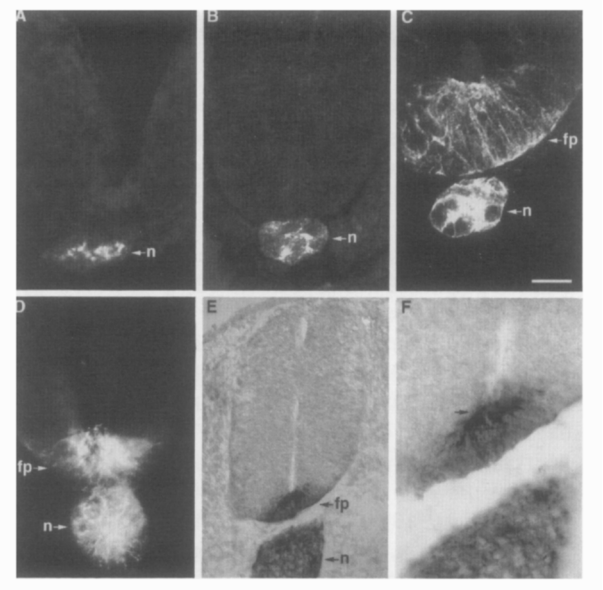

Figure 1. Localization of SHH in the Notochord and Floor Plate

Antibodies against the amino-terminal domains of mouse or rat SHH were used to localize SHH in rat (A and B), mouse (C), and chick (D–F) embryos by immunohistochemistry.

(A) Expression of SHH by the notochord (n) in an embryonic day 10 (E10) rat embryo. At this caudal level, the neural folds have not fused, and there is no expression of SHH by neural cells.

(B) High levels of SHH expression by the notochord (n) in an E11 rat embryo, at a level at which neural tube closure has occurred.

(C) Expression of SHH by notochord (n) and floor plate cells (fp) in a section of an E9 mouse embryo. The surface of notochord cells is labeled, and there is prominent labeling of the basal and apical surfaces of floor plate cells.

(D) Expression of SHH by notochord (n) and floor plate (fp) cells in a section through the anterior region of a stage 10 chick embryo. High levels of immunoreactivity are associated with the surface of notochord and floor plate cells.

(E) Expression of SHH in a section of a stage 22 chick embryo showing high levels of protein on notochord (n) and floor plate (fp) cells.

(F) High power view of ventral spinal cord showing the prominent apical localization (arrow) of SHH protein in the floor plate.

Scale bar indicates 20 μm in (A), 25 μm in (B), 20 μm in (C), 30 μm in (D), 50 μm in (E), and 15 μm in (F).

trations of the amino-terminal cleavage product induce motor neurons but not floor plate cells, whereas high concentrations induce floor plate cells at the expense of motor neurons. These results suggest that the induction of floor plate cells and motor neurons in vivo is triggered at different concentration thresholds of the amino-terminal cleavage product of SHH.

Results

Expression of SHH by the Notochord and Neural Tube

To provide information on the range over which SHH might act within the neural tube, we examined the distribution of SHH. In rat, mouse, and chick embryos, antibodies directed against the amino-terminal domain labeled the surface of notochord and floor plate cells prominently (Figure 1), with a temporal and spatial pattern similar to that of *Shh* mRNA expression (Echelard et al., 1993; Chang et al., 1994; Riddle et al., 1993; Roelink et al., 1994; Ericson et al., 1995). The pattern of expression of SHH within the ventral spinal cord of rat embryos coincided with that of FP4, a surface marker of floor plate cells (data not shown) (Placzek et al., 1993; Roelink et al, 1994), showing that high levels of SHH are restricted to the floor plate. No specific labeling was detected with antibodies directed against the carboxy-terminal domain of SHH (data not shown).

We next determined whether SHH synthesized by the notochord is subject to proteolytic processing. Antibodies directed against the amino-terminal domain of SHH detected an ~20 kDa band in chick notochord extracts (Figure 2A), similar in size to that of the amino-terminal cleavage product of SHH (Chang et al., 1994; Lee et al., 1994; Bumcrot et al., 1995), suggesting that SHH synthesized by chick notochord cells undergoes autoproteolytic cleavage.

Expression and Secretion of SHH and Its Cleavage Products by Transfected Cells

To generate SHH and its proteolytic products for use in induction assays, we transfected COS cells with cDNA-derived expression plasmids (Figure 2B) that encode the wild-type mouse SHH, a SHH variant (SHH-U_{CA}) in which the cleavage site cysteine residue has been changed to an alanine residue. This mutation blocks autoproteolytic cleavage of HH (Porter et al., 1995), a SHH variant (SHH-C) comprised of the SHH signal sequence fused to the carboxy-terminal domain, and a SHH variant (SHH-N) corresponding to the amino-terminal product generated by autoproteolytic cleavage (Porter et al., 1995).

Extracts of COS cells transfected with pShh expressed an ~20 kDa amino-terminal product (Figure 2C, lane 2), an ~29 kDa carboxy-terminal product (Figure 2C, lane 11), and the ~45 kDa SHH precursor (Figure 2C, lanes 2 and 11; see below). Medium conditioned by COS cells transfected with pShh contained low levels of the ~20 kDa amino-terminal product (Figure 2C, lane 6; data not shown), high levels of the ~29 kDa carboxy-terminal product (Figure 2C, lane 15), but not the ~45 kDa SHH precursor (Figure 2C, lane 6). Extracts of COS cells transfected with pShh-U_{CA} expressed only the ~45 kDa protein (Figure 2C, lanes 3 and 12), confirming that the ~45 kDa band corresponds to the SHH precursor. Medium conditioned by pShh-U_{CA}-transfected cells did not contain detectable immunoreactive proteins (Figure 2C, lanes 7 and 16). COS cells transfected with pShh-C expressed the ~29 kDa SHH-C product (Figure 2C, lane 13), but this protein was not detected in the medium (Figure 2C, lane 17), in contrast with the high levels of carboxy-terminal product detected in the medium of COS cells transfected with pShh (Figure 2C, lane 15). In COS cells, the amino-terminal domain of SHH may therefore be required for secretion of the carboxy-terminal domain.

COS cells transfected with pShh-N expressed only the ~20 kDa SHH-N product (Figure 2C, lane 5). Strikingly, medium conditioned by these cells contained large amounts of SHH-N (Figure 2C, lane 9), in contrast with the low levels of the amino-terminal product detected in

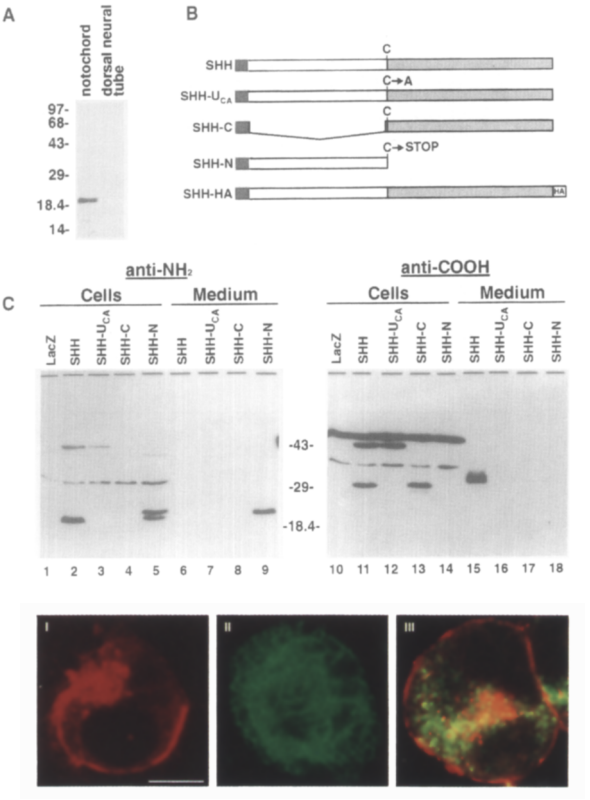

Figure 2. Expression and Subcellular Localization of SHH and Its Cleavage Products in Mammalian Cells

(A) Detection of SHH amino-terminal cleavage product in stage 10 chick notochord. An ~20 kDa immunoractive band is detected in extracts of notochord but not dorsal neural tube tissue. Sizes (in kilodaltons) of molecular markers are indicated.

(B) Schematic diagram showing the proteins encoded by constructs used to transfect COS or HEK-293 cells. SHH indicates wild-type SHH. Autoproteolytic cleavage occurs between Gly-198 and Cys-199 (C) (Porter et al., 1995). SHH-U$_{CA}$ indicates a modified form of SHH in which the Cys-199 has been changed to Ala (A). SHH-C indicates a truncated form of SHH in which residues 25–198 have been deleted. SHH-N indicates a form of SHH truncated after Gly-198, corresponding to the amino-terminal product generated by autoproteolysis (Porter et al., 1995). SHH-HA indicates a modified form of SHH in which an HA epitope has been fused to the carboxyl terminus.

(C) Expression of SHH, SHH-U$_{CA}$, SHH-C, and SHH-N after transient transfection of plasmids into COS cells. Cells were harvested and media collected after 48 hr, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose, and probed with antibodies direct against the amino-terminal (anti-NH$_2$) (lanes 1–9) or carboxy-terminal (anti-COOH) (lanes 10–18) domains of mouse SHH (Chang et al., 1994). Anti-NH$_2$ antibodies detect an ~33 kDa cross-reactive protein. In lane 5, the upper band probably represents the SHH-N domain from which the signal sequence has not been cleaved. In other experiments, the prominent ~20 KDa band in lane 9 corresponds in mobility to the ~20 kDa band detected in lane 5 (data not shown). Anti-COOH antibodies also detect ~40 KDa and ~37 KDa cross-reactive proteins. In lane 15, the altered mobility of SHH-C may reflect differences in glycosylation compared with the carboxy-terminal cleavage product in lanes 11–13. The size (in kilodaltons) of molecular markers is indicated.

(D) Confocal immunofluorescence images showing the localization of the amino-terminal and carboxy-terminal products of SHH in transfected COS and HEK-293 cells.

(i) Labeling of pShh-HA transfected COS cells with anti-NH$_2$ antibodies shows immunoreactivity intracellularly and prominently on the cell surface. Scale bar, 10 μm.

(ii) Labeling of pShh-HA transfected COS cells with anti-HA antibodies shows immunoreactivity intracellularly but not on the cell surface. Affinity purification of the HA-associated protein expressed in COS cells revealed a single ~30 KDa species (data not shown), showing that the HA epitope remains associated with the carboxy-terminal cleavage product.

(iii) Dual immunofluorescence localization of SHH in a HEK-293 cell line that stably expresses SHH-HA. Anti-NH$_2$ antibodies (red) detect immunoreactivity intracellularly and on the cell surface. Anti-HA-directed antibodies (green) detect the carboxy-terminal domain intracellularly but not on the cell surface.

the medium of COS cells transfected with pShh. Medium derived from COS cells transfected with pShh contained between 5 × 10^{-11} M and 10^{-10} M SHH-N, as determined by enzyme-linked immunosorbent assay (ELISA), whereas the concentration of SHH-N in the medium derived from COS cells transfected with pShh-N was about 10^{-7} M. This result suggests that autoprocessing of SHH is required for retention of the amino-terminal product by cells, as shown for HH (Porter et al., 1995).

The subcellular localization of SHH and its amino- and carboxy-terminal cleavage products was determined by transfecting into COS and HEK-293 cells, a pShh-HA construct that encodes an SHH protein modified at its carboxyl terminus by addition of an influenza hemagglutinin (HA) epitope (Figure 2B). The SHH-HA protein undergoes cleavage and is biologically active as determined by induction of ventral cell types in neural plate explants (data not shown). Antibodies directed against the amino-terminal domain of SHH reacted prominently with the surface membrane of transfected COS and HEK-293 cells (Figure 2D, parts i and iii). The HA-modified carboxy-terminal domain was detected in the cytoplasm and in the medium but not on the cell surface (Figure 2D, parts ii and iii; data not shown). These results indicate that the amino-terminal cleavage product is present on the cell surface and that the carboxy-terminal domain and the SHH precursor are not.

Floor Plate- and Motor Neuron-Inducing Activities of SHH and Its Cleavage Products Expressed in COS Cells

To examine the activities of SHH and its cleavage products, we assayed the ability of transfected COS cells to induce the differentiation of floor plate cells and motor neurons when grown in contact with chick neural plate explants. Floor plate cells were identified by expression of hepatocyte nuclear factor 3β (HNF-3β), a winged-helix transcription factor synthesized at early stages of floor plate differentiation (Lai et al., 1993; Ruiz i Altaba et al., 1993b, 1995b) and of SC1, an immunoglobulin-like protein (Tanaka and Obata, 1984). Motor neurons were identified by expression of the LIM homeodomain proteins Islet-1 (Isl-1) and Islet-2 (Isl-2) (Isl$^+$ cells) (Ericson et al., 1992; Tsuchida et al., 1994).

Neural plate explants grown in contact with COS cells expressing SHH contained HNF-3β$^+$ cells and Isl$^+$ cells (Figures 3A and 3F). Conditioned medium from these cells contained high levels of the carboxy-terminal cleavage product (see Figure 2) but did not induce HNF-3β$^+$ or Isl$^+$ cells (Roelink et al., 1994; data not shown). Neural plate

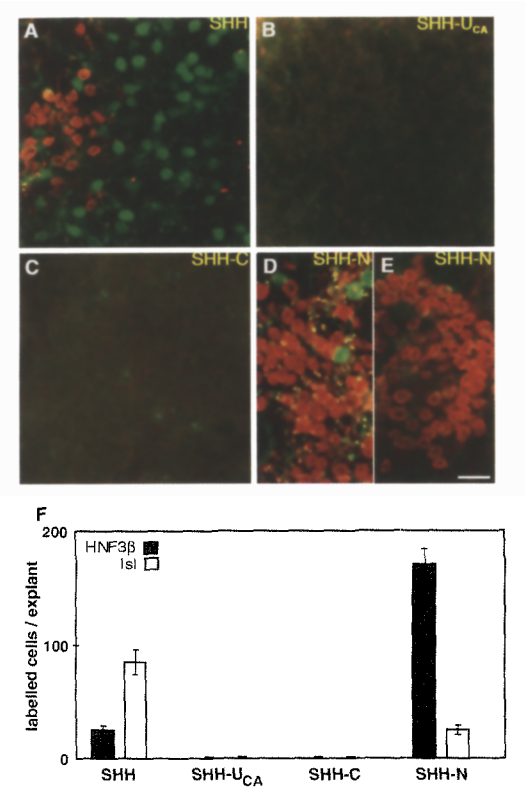

Figure 3. Floor Plate– and Motor Neuron–Inducing Activity of SHH and Its Proteolytic Products Expressed in COS Cells

Neural plate explants were grown for 44 hr on COS cells transfected with *Shh* expression constructs. Under these conditions the explants flattened to form a layer of one or two cells thick. After 44 hr, these explants contained ~700 cells.

(A) Neural plate explant grown in contact with COS cells expressing SHH. Nuclei that express HNF-3β (red) or Isl proteins (green) are present.

(B) Neural plate explant grown in contact with COS cells expressing SHH-U_{CA}. No HNF-3β$^+$ or Isl$^+$ nuclei are detectable.

(C) Neural plate explant grown in contact with COS cells expressing SHH-C. No HNF-3β$^+$ or Isl$^+$ nuclei are detectable.

(D and E) Peripheral region of two different neural plate explants grown in contact with COS cells expressing SHH-N. Large numbers of HNF-3β$^+$ nuclei (red) are present, interspersed among which are a few Isl$^+$ nuclei (green). Scale bar, 20 μm.

(F) Quantitation of HNF-3β$^+$ and Isl$^+$ cells in induced neural plate explants. Histograms indicate mean ± SEM for 10–12 explants.

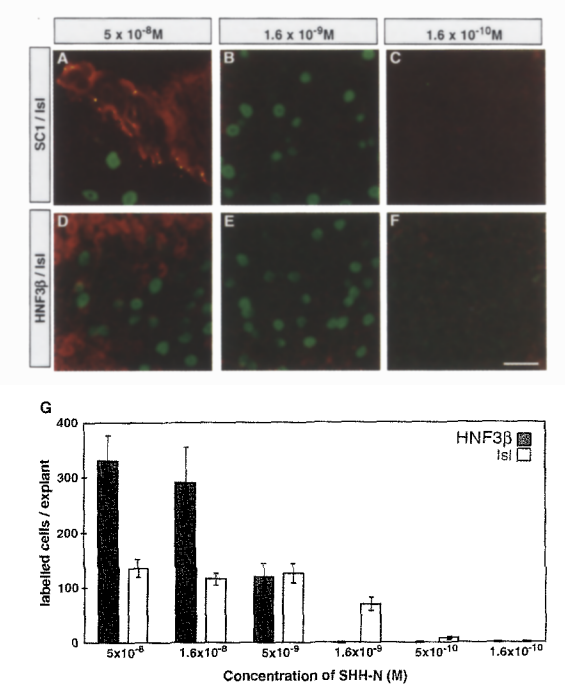

Figure 4. Induction of Floor Plate and Motor Neuron Differentiation by Conditioned Medium from COS Cells Expressing SHH-N

(A–F) Double label confocal micrographs through the equator of neural plate explants showing induction of SC1$^+$ (A–C) or HNF-3β$^+$ (D–F) cells (red) and Isl$^+$ (A–F) cells (green) in neural plate explants grown for 24 hr in media conditioned by COS cells transfected with pShh-N. (A and D) Explants exposed to 5 × 10^{-8} M SHH-N contain SC1$^+$ (A) or HNF-3β$^+$ (D) cells as well as Isl$^+$ cells (green). Isl$^+$ cells do not coexpress SC1 at early times after induction (Yamada et al., 1993). (B and E) Neural plate explants exposed for 24 hr to 1.6 × 10^{-9} M SHH-N contain Isl$^+$ cells (green) but no HNF-3β$^+$ or SC1$^+$ cells. (C and F) Neural plate explants exposed to 1.6 × 10^{-10} M SHH-N do not contain SC1$^+$, HNF-3β$^+$, or Isl$^+$ cells. Scale bar, 20 μm.

(G) Quantitation of floor plate and motor neuron induction in response to conditioned medium containing different concentrations of SHH-N. Histograms show mean ± SEM for 10–12 explants. These explants contained ~1400 cells. Similar results were obtained in four additional experiments.

explants grown in contact with COS cells expressing SHH-U_{CA} or SHH-C contained neither HNF-3β$^+$ cells nor Isl$^+$ cells (Figures 3B, 3C, and 3F). Neural plate explants grown in contact with COS cells expressing SHH-N contained both HNF-3β$^+$ and Isl$^+$ cells (Figures 3D–3F). These results indicate that the floor plate– and motor neuron–inducing activities of SHH are mediated by its amino-terminal cleavage product.

Floor Plate– and Motor Neuron–Inducing Activities of Medium Conditioned by COS Cells Expressing SHH-N

The detection of high concentrations of SHH-N in medium of COS cells transfected with pShh-N prompted us to test whether this medium has activity. HNF-3β$^+$ cells and Isl$^+$ cells were detected in neural plate explants exposed for 24 hr to medium containing SHH-N (Figures 4A, 4D, and 4G), establishing that contact by cells that synthesize SHH is not a prerequisite for the induction of floor plate differentiation. We next examined the consequences of exposing neural plate explants to different dilutions of this medium. At dilutions of medium containing 5 × 10^{-9} M or greater concentrations of SHH-N, SC1$^+$, HNF-3β$^+$, and Isl$^+$ cells were induced (Figures 4A, 4D, and 4G). At dilutions of medium containing 1.6 × 10^{-9} M and 5 × 10^{-10} M SHH-N, Isl$^+$ cells were induced in the absence of HNF-3β$^+$ cells (Figures 4B, 4E, and 4G). Medium containing 1.6 × 10^{-10} M SHH-N induced neither HNF-3β$^+$ cells nor Isl$^+$ cells (Figures 4C, 4F, and 4G). Motor neurons can, therefore, be induced in the absence of floor plate cells by medium containing low concentrations of SHH-N. Moreover, exposing neural plate explants to medium containing increasing

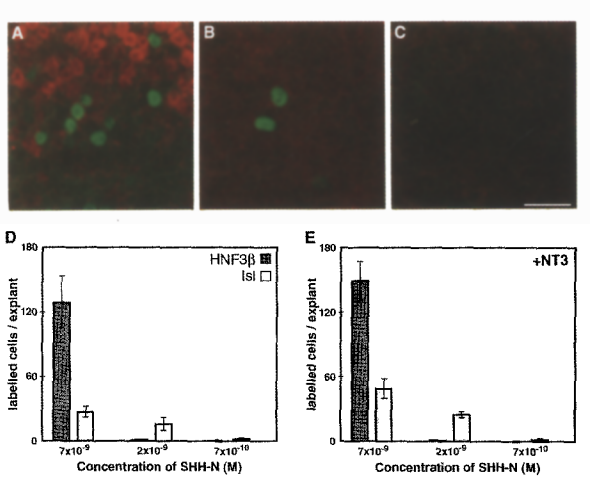

Figure 5. Floor Plate and Motor Neuron Induction by Purified SHH-N Derived from E. coli

(A) Induction of HNF-3β$^+$ (red) and Isl$^+$ (green) cells in neural plate explants exposed for 24 hr to 7 × 10^{-9} M SHH-N. The section is obtained through the equator of the explant.

(B) Induction of Isl$^+$ cells (green) in the absence of HNF-3β$^+$ cells in neural plate explants exposed for 24 hr to 2 × 10^{-9} M SHH-N.

(C) No HNF-3β$^+$ or Isl$^+$ cells are induced in neural plate explants exposed for 24 hr to 7 × 10^{-10} M SHH-N. Scale bar, 20 μm.

(D and E) Quantitation of HNF-3β$^+$ and Isl$^+$ cells induced in neural plate explants exposed to different concentrations of SHH-N in the absence (D) or presence (E) of NT-3 (10 ng/ml). Histograms indicate mean ± SEM of 10–12 explants.

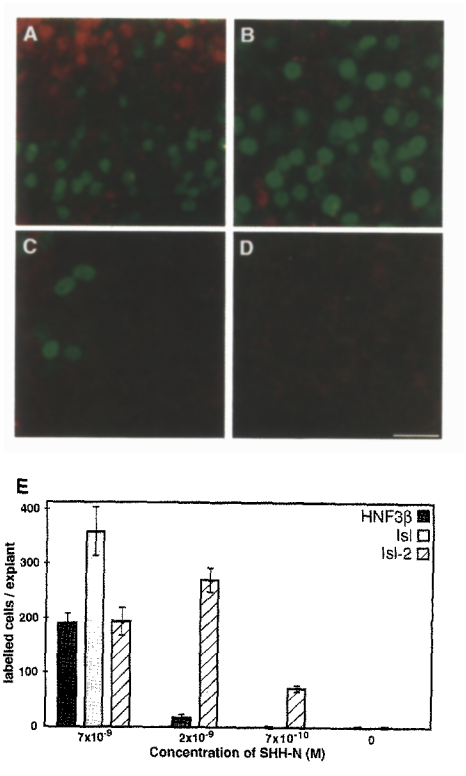

Figure 6. Enhanced Motor Neuron Differentiation after Prolonged Exposure to Purified SHH-N

Induction of HNF-3β$^+$ (red) and Isl-2$^+$ (green) cells in neural plate explants exposed for 44 hr to purified SHH-N in the presence of NT-3 (10 ng/ml).

(A) HNF-3β$^+$ and Isl-2$^+$ cells are present in explants exposed to 7 × 10^{-9} M SHH-N. The section is through the equator of the explant, with the edge shown at the top.

(B) Many Isl-2$^+$ cells but few HNF-3β$^+$ cells are present in neural plate explants exposed to 2 × 10^{-9} M SHH-N.

(C) Isl-2$^+$ cells but not HNF-3β$^+$ cells are present in neural plate explants exposed to 7 × 10^{-10} M SHH-N.

(D) Absence of HNF-3β$^+$ and Isl$^+$ cells in neural plate explants grown alone. Scale bar, 20 μm.

(E) Quantitation of HNF-3β$^+$, Isl$^+$, and Isl-2$^+$ cells in neural plate explants exposed to SHH-N. Histograms indicate mean ± SEM of 10–12 explants.

concentrations of SHH-N produces a progressive increase in the ratio of floor plate cells to motor neurons.

Floor Plate- and Motor Neuron-Inducing Activities of Purified E. coli Derived SHH-N

To examine whether SHH is sufficient to induce both floor plate cells and motor neurons, we added purified E. coli derived SHH-N to neural plate explants for 24 or 44 hr. Addition of 7 × 10^{-9} M SHH-N for 24 hr induced HNF-3β$^+$ and Isl$^+$ cells (Figures 5A and 5D); addition of 2 × 10^{-9} M SHH-N induced Isl$^+$ cells but no HNF-3β$^+$ cells (Figures 5B and 5D), and addition of SHH-N below 10^{-9} M induced only a few Isl$^+$ cells (Figures 5C and 5D). The number of motor neurons induced by SHH-N was enhanced in the presence of COS cell–conditioned medium (compare Figures 4 and 5; data not shown), suggesting that other factors increase the final number of motor neurons generated in response to SHH-N. Neurotrophin-3 (NT-3) enhances the number of motor neurons in dissociated neural tube cultures (Averbuch-Heller et al., 1994) but has no direct floor plate– or motor neuron–inducing activity (Tanabe et al., 1995; Figure 5E). Addition of NT-3 together with SHH-N produced an ~1.5-fold increase in the number of Isl$^+$ cells (compare Figures 5D and 5E). NT-3, therefore, increases the number of motor neurons generated in response to SHH-N, but not to the number obtained by addition of COS cell–conditioned medium containing SHH-N. Neural plate explants exposed for 44 hr to 7 × 10^{-9} M SHH-N contained 1.4-fold the number of HNF-3β$^+$ cells found at 24 hr but 10-fold the number of Isl$^+$ cells (Figures 6A and 6E). Neural plate explants exposed to 2 × 10^{-9} M and 7 × 10^{-10} M SHH-N contained numerous Isl-2$^+$ cells, often in the absence of HNF-3β$^+$ cells (Figures 6B and 6E; data not shown).

Purified SHH-N is, therefore, able to induce motor neurons and floor plate cells independently and at distinct concentration thresholds. The threshold concentrations of E. coli derived SHH-N sufficient for the induction of motor neurons and floor plate cells were similar to the concentrations of SHH-N in dilutions of conditioned medium effective in induction of these two ventral cell types (see Figure 4). The floor plate– and motor neuron–inducing activity in medium conditioned by cells transfected with pShh-N is, therefore, attributable to SHH-N itself.

The Fate of Neural Plate Cells Is Sensitive to the Concentration of SHH-N

Low concentrations of SHH-N induced motor neurons but

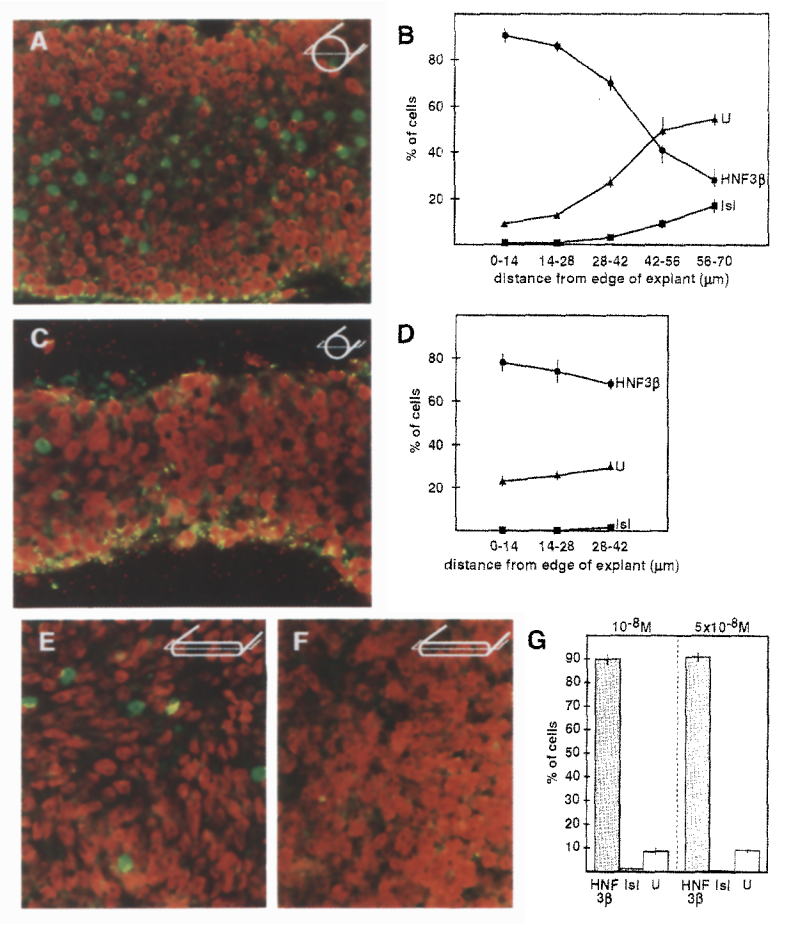

Figure 7. The Fate of Neural Plate Cells Exposed to SHH-N Is Influenced by Explant Size

(A) Confocal micrograph taken through the equator of a large neural plate explant (final diameter, ~140–150 μm) that had been exposed to 7 × 10^{-9} M SHH-N for 24 hr. HNF-3β$^+$ cells are concentrated at the periphery, whereas Isl$^+$ cells and unlabeled cells are concentrated at the core. Diagrams at top right in (A), (C), (D), and (E) show shapes of explant and planes of section.

(B) Quantitative analysis of HNF-3β$^+$, Isl$^+$, and unlabeled cells in an ~140 μm diameter neural plate explant exposed for 24 hr to 7 × 10^{-9} M E. coli derived SHH-N, as a function of distance from the edge of the explant. Bars show range of values derived from analysis of a single explant. A similar spatial distribution of labeled cells has been observed in over 50 explants of diameters of 120–150 μm.

(C) Confocal micrograph taken through the equator of smaller neural plate explant (final diameter, 80–85 μm) that had been exposed to 7 × 10^{-9} M SHH-N for 24 hr. Over 80% of cells express HNF-3β (red), and there are very few Isl$^+$ (green) cells. This image is representative of analyses performed on 10–12 explants of diameters of less than 90 μm. Small diameter explants were selected as extremes of the normal range of explant diameters.

(D) Quantitative analysis of HNF-3β$^+$, Isl$^+$, and unlabeled cells in an ~80–85 μm diameter neural plate explant exposed to 7 × 10^{-9} M E. coli derived SHH-N for 24 hr, as a function of a distance from the edge of the explant. Bars show range of values derived from analysis of a single explant. A similar spatial distribution of labeled cells has been observed in 10–12 explants with diameters of less than 90 μm.

(E–F) Confocal micrographs taken through two large neural explants, originally equivalent in size to that shown in (A), which had been grown on a tissue culture plastic substrate such that they flattened to a final thickness of approximately two cells. Explants exposed to 10^{-8} M are comprised almost exclusively of HNF-3β$^+$ cells with few (E) or no (F) Isl$^+$ cells.

(G) Quantatative analysis of HNF-3β$^+$, Isl$^+$, and unlabeled cells (U) in flattened neural plate explants exposed for 24 hr to 10^{-8} M or 5 × 10^{-8} M SHH-N.

Scale bars, 30 μm.

not floor plate cells, whereas higher concentrations were not able to induce floor plate cells in the absence of motor neurons (see Figures 4–6). One possible reason for this is a limitation to the penetration of SHH-N into neural plate explants such that only cells in the periphery of explants are exposed to high concentrations. In support of this idea, we found that in explants exposed to high concentrations of SHH-N, HNF-3β$^+$ cells were concentrated at the periphery, whereas Isl$^+$ cells were restricted almost exclusively to the core (Figures 7A and 7B; see also Figures 3E, 5A, and 6A). About 90% of cells at the periphery expressed HNF-3β, whereas Isl$^+$ cells comprised only about 20% of cells at the core (Figures 7A and 7B). In addition, many cells at the core did not express HNF-3β or Isl proteins.

If the segregation of HNF-3β$^+$ and Isl$^+$ cells resulted from exposure of cells within explants to varying concentrations of SHH-N, we reasoned that HNF-3β$^+$ cells should be induced at the expense of Isl$^+$ cells if all cells were exposed to high concentrations of SHH-N. We tested this idea in two ways. First, we monitored the number of HNF-3β$^+$ and Isl$^+$ cells in neural plate explants with diameters about half that of explants used routinely in assays described above. Addition of 7 × 10^{-9} M SHH-N for 24 hr to these small explants induced about 80% of cells to express HNF-3β (Figures 7C and 7D). Importantly, labeled cells were detected throughout the explant, including its core, and very few Isl$^+$ cells were detected (Figures 7C and 7D). Second, we plated normal-sized explants on a substrate that promoted spreading to form a layer approximately two cells thick. Addition of 10^{-8} M and 5 × 10^{-8} M SHH-N to these flat explants for 24 hr induced HNF-3β expression in about 90% of cells (Figures 7E–7G). Very few Isl$^+$ cells were detected in flat explants exposed to 10^{-8} M SHH-N, and these were scattered uniformly (Figures 7E and 7G). No Isl$^+$ cells were detected in flat explants exposed to 5 × 10^{-8} M SHH-N (Figure 7G). These two results show that SHH-N can induce HNF-3β expression in the vast majority of neural plate cells under conditions in which all cells are likely to be exposed to SHH-N. Moreover, under these conditions the differentiation of Isl$^+$ cells was almost completely suppressed. These results support strongly the idea that neural plate cells differentiate into floor plate

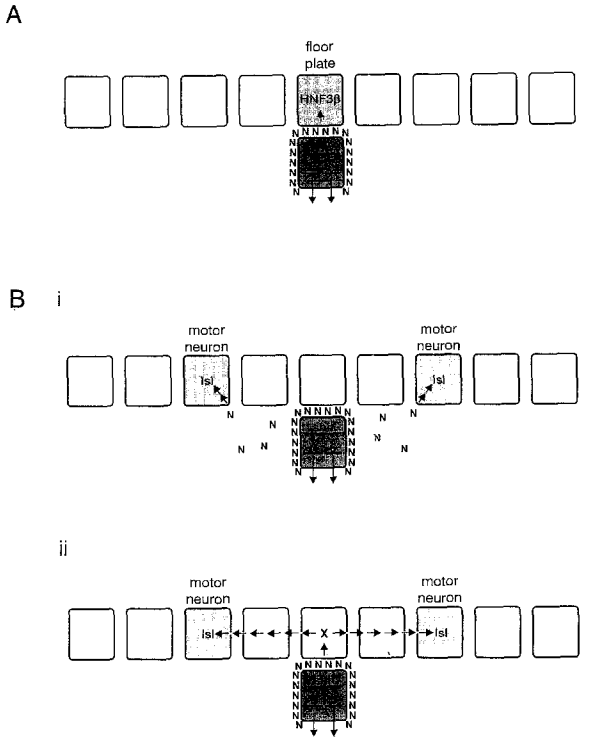

Figure 8. Pathways of Floor Plate and Motor Neuron Differentiation in Response to the Amino-Terminal Autoproteolytic Product of SHH

(A) Floor plate induction by the amino-terminal cleavage product of SHH. In this scheme, SHH synthesized by the notochord undergoes autoproteolytic cleavage to generate amino (N)- and carboxy (C)-terminal cleavage products. N is secreted, and the vast majority of the protein is retained on the surface of notochord cells. The surface association of N generates a high local concentration that is sufficient to induce floor plate differentiation in overlying neural plate cells.

(B) Two possible pathways by which SHH could induce motor neuron differentiation.

(i) Motor neuron induction by diffusible N. This scheme shows that a small proportion of N diffuses away from the notochord, generating a low concentration that is sufficient to induce motor neuron differentiation in lateral neural plate cells. The steps involved in the commitment of neural plate cells to a motor neuron fate are not known. The important element in this scheme is that N itself exerts both local and long-range inductive actions.

(ii) Motor neuron induction by N-dependent synthesis of a distinct secreted factor (X) that acts out over a long range to trigger motor neuron differentiation in lateral neural plate cells. The critical difference between this scheme and that shown in (i) is that N would exert its entire spectrum of inductive activities through local signaling. For simplicity, the extracellular location of C has not been shown. The differentiation of distinct cell types generated in the region that is interposed between the floor plate and notochord could be induced by a concentration of the amino-terminal cleavage product (or of X) greater than that required to initiate motor neuron differentation.

cells or motor neurons in response to the concentration of SHH-N to which they are exposed.

Discussion

This study addresses how a single inducing molecule, SHH, can mediate the differentiation of two distinct cell types at different positions in the ventral half of the neural tube. Our results establish three main points about the activities of SHH: SHH synthesized by the notochord is processed into an amino-terminal cleavage product that mediates both the floor plate– and motor neuron–inducing activities of SHH; the vast majority of the amino-terminal cleavage product generated from SHH is retained on the cell surface, although small amounts diffuse into the medium; the concentration of the amino-terminal cleavage product of SHH appears to determine whether neural plate cells differentiate into motor neurons or floor plate cells.

These observations suggest a model that might explain how SHH can induce distinct cell types in the ventral half of the neural tube. In this model (Figure 8), the synthesis of SHH by the notochord generates amino- and carboxy-terminal products through autoproteolytic cleavage. Once cleaved, the carboxy-terminal product is free to diffuse but has no intrinsic inductive activity, whereas most of the active amino-terminal product remains attached to the surface of notochord cells. Cells at the midline of the neural plate that are contacted by the notochord are exposed to a high concentration of the amino-terminal cleavage product sufficient to initiate floor plate differentiation. Small amounts of the amino-terminal product might diffuse from the notochord and initiate motor neuron differentiation in more lateral neural plate cells (Figure 8B, part i).

Structure and Biological Activities of Hh Proteins

The structural requirements for SHH activity on neural plate explants defined in the present studies are in agreement with analyses of the activity of HH during Drosophila development. The patterning activities exhibited by HH (Ingham, 1993; Ma et al., 1993; Heberlein et al., 1993; Heemskerk and DiNardo, 1994; Basler and Struhl, 1994; Tabata and Kornberg, 1994) can be reproduced by expression of a HH-N fragment, whereas HH-U_{CA} and HH-C have little or no activity (Lee et al., 1994; Porter et al., 1995). In zebrafish embryos, ectopic expression of *Shh* and a related gene, *Twhh*, results in a change in the pattern of *Pax* gene expression in the diencephalon, and this activity can be mimicked by the TWHH amino-terminal cleavage product (Ekker et al., submitted). Similarly, the ability of a diffusible form of SHH to ventralize paraxial mesoderm is also mediated by SHH-N (Fan and Tessier-Lavigne, 1994; Fan et al., 1995 [this issue of *Cell*]).

Although the carboxy-terminal product of SHH does not appear to possess floor plate– or motor neuron–inducing activity, SHH-C is not secreted from COS cells (Figure 2C). Evidence for the lack of activity of the carboxy-terminal cleavage product therefore derives primarily from the absence of activity in medium conditioned by COS cells transfected with pShh (Roelink et al., 1994; data not shown), even though this medium contains high levels of the carboxy-terminal cleavage product (Figure 2C). Similarly, the lack of activity of SHH-U_{CA} could be the consequence of its retention within cells. The carboxy-terminal domain of HH appears to have an essential role in tethering the amino-terminal cleavage product to the surface membrane (Porter et al., 1995). In our studies, the amino-terminal cleavage product generated from SHH is detectable on the cell surface and in the medium is found at

a concentration that is about 10^3-fold lower than that in medium conditioned by COS cells expressing SHH-N. A mechanism similar to that operating in Drosophila (Porter et al., 1995) might, therefore, govern association of the amino-terminal cleavage product of SHH with the cell surface.

Direct Nature of Floor Plate and Motor Neuron Induction by SHH

The present results, together with previous studies (Tanabe et al., 1995), show that SHH can induce floor plate cells and motor neurons independently. One important issue is whether the differentiation of these two ventral cell types is triggered directly by SHH or by the induction in neural plate cells of a distinct secreted factor.

The induction of floor plate differentiation by SHH appears not to require an intermediary secreted factor. The expression of *HNF-3β* in neural plate explants in response to factors derived from the notochord, or to SHH itself, is maintained in the absence of protein synthesis (Ruiz i Altaba et al., 1995b; our unpublished data) and thus does not appear to depend on the de novo synthesis of additional secreted factors. In addition, widespread expression of *HNF-3β* and related winged-helix genes in neural tube cells is sufficient to trigger ectopic floor plate differentiation (Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1993a, 1995a; Hynes et al., 1995). Neural expression of HNF-3β therefore provides a reliable marker of floor plate cells. Exposure of neural plate cells to SHH thus appears to lead directly to the induction of HNF-3β and in turn to the activation of other floor plate–specific genes. Short-range signaling by *hh* in Drosophila is also thought to involve the direct action of the HH protein (Ingham, 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994; Capdevila and Guerrero, 1994) and thus resembles the local SHH-mediated induction of floor plate differentiation.

Whether the induction of motor neurons results from a direct action of SHH is unclear. Neural plate cells exposed to notochord-derived signals appear to undergo several rounds of division before differentiating into motor neurons (Leber et al., 1990; Yamada et al., 1993). Because the earliest marker of motor neuron differentiation, *Isl-1*, appears to be expressed as progenitors undergo their final cell division (Ericson et al., 1992), it has not been possible to resolve whether SHH acts directly to commit cells to a motor neuron fate. Direct induction of motor neurons in vivo would require a long-range action of SHH. In Drosophila, many of the longer-range organizing effects of HH on cell pattern appear to be mediated by the induction of intermediary factors, in particular the transforming growth factor β (TGFβ)-related protein decapentaplegic (dpp) (Ma et al., 1993; Heberlein et al., 1993; Basler and Struhl, 1994; Tabata and Kornberg, 1994; Capdevila and Guerrero, 1994; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995) and possibly wingless (wg) (Struhl and Basler, 1993). Members of the TGFβ and Wnt families could act as mediators of the patterning activities of SHH in vertebrate development. However, this seems unlikely in the case of motor neurons since members of TGFβ family suppress motor neuron differentiation (Basler et al., 1993) and vertebrate Wnt proteins do not induce motor neurons (Dickinson et al., 1994; our unpublished data).

Three lines of evidence suggest that the amino-terminal cleavage product is indeed able to diffuse away from the notochord and initiate directly or through an intermediary factor, the differentiation of motor neurons. First, low levels of the amino-terminal cleavage product are detectable in the medium of COS cells transfected with pShh. Second, the notochord is the source of a diffusible factor that can induce motor neuron differentiation (Yamada et al., 1993; Tanabe et al., 1995). Third, the actions of this notochord-derived diffusible factor can be mimicked by COS cells expressing SHH (Tanabe et al., 1995). The simplest explanation of these observations is that low levels of the amino-terminal cleavage product of SHH diffuse from the notochord in vivo to initiate motor neuron differentiation (Figure 8B, part i). Nevertheless, SHH synthesized by the notochord could initiate motor neuron differentiation solely through its local action on overlying midline neural plate cells by inducing, independently of floor plate differentiation, the synthesis of a distinct secreted factor (Figure 8B, part ii).

Responses of Neural Plate Cells to Different Concentrations of SHH

In Drosophila, patterning of the dorsal cuticle appears to depend on the concentration-dependent inductive activities of HH (Heemskerk and DiNardo, 1994). Our results provide evidence that the fate of neural plate cells is similarly determined by the concentration of SHH to which they are exposed, although they do not exclude that there is normally some heterogeneity in the sensitivity of response of cells to SHH. High concentrations of SHH-N can induce floor plate differentiation in over 90% of neural plate cells, and under conditions in which access of SHH-N to cells in neural plate explants is not limited, few (if any) motor neurons are induced. At concentrations of SHH-N below the threshold for floor plate induction, only about 10% of neural plate cells are converted into motor neurons. This probably reflects the asymmetric division of progenitors such that motor neurons always represent a minority of clonally related progeny (Leber et al., 1990), together with the dominance of floor plate differentiation at high concentrations of SHH.

Relatively small differences in the threshold concentration of the amino-terminal cleavage product of SHH appear to be capable of specifying the fates of distinct classes of ventral cells in vitro. It remains uncertain whether equivalent concentration thresholds operate to control ventral fates in vivo. In transfected COS cells and in vivo, the vast majority of the active amino-terminal cleavage product of SHH appears to be retained on the cell surface, and the local concentration is likely, therefore, to exceed greatly that achieved through the diffusion of amino-terminal cleavage product away from the notochord. The proportion of the amino-terminal cleavage product that is released from cells in vivo might, however, be subject to forms of regulation not detected in vitro. It also remains possible that, in vivo, additional factors modify the concentration thresholds of SHH that we detected in vitro. Indeed,

the narrow concentration range of SHH-N over which motor neuron differentiation can be elicited in the absence of floor plate differentiation was not observed in previous studies using floor plate–conditioned medium (Yamada et al., 1993; Placzek et al., 1993). Floor plate–conditioned medium itself may, therefore, contain factors that modify the threshold of neural plate explants for induction of floor plate or motor neuron differentiation by SHH.

Our studies have focused on the induction by SHH of only two cell types generated in the ventral region of the neural tube. The differentiation of ventral neurons at prospective forebrain levels is also induced by SHH (Ericson et al., 1995). Moreover, serotonergic and dopaminergic neurons are generated adjacent to the floor plate and can be induced in response to factors from the notochord and floor plate, possibly SHH (Yamada et al., 1991; Hynes et al., 1995). The differentiation of neurons that are generated close to the floor plate might require a concentration of SHH intermediate between that sufficient to induce floor plate cells and motor neurons. SHH may, therefore, act at different concentration thresholds to control distinct ventral neuronal fates.

Experimental Procedures

Antibodies

Antibodies directed against specific regions of mouse (Chang et al., 1994) and rat SHH proteins were generated by immunization of rabbits with PCR-generated, His_6-tagged fusions (in the vector pTrcHis [InVitrogen] or pQE-31 [Qiagen]) to amino-terminal domain residues 25–159 (mouse SHH) or 27–185 (rat SHH) and to carboxy-terminal domain residues 202–389 (mouse SHH). HNF-3β was detected with rabbit antibodies (Ruiz i Altaba et al., 1995b), Isl-1 and Isl-2 with rabbit antibodies (Thor et al., 1991) and MAb 4D5 (Tsuchida et al., 1994), Isl-2 with MAb 4H9 (Tanabe et al., 1995), and SC1 with MAb SC1 (Tanaka and Obata, 1984). The HA epitope was detected with MAb 12CA5 (Wilson et al., 1984).

Localization of SHH Protein In Situ

For immunofluorescence localization of SHH, rat, mouse, or chick embryos were fixed with 4% paraformaldehyde containing 10% acetic acid or in Bouin's fixative at 4°C and processed essentially as described (Yamada et al., 1991; Roelink et al., 1994).

Expression of SHH in Cultured Cells

The pShh-U_{CA} construct was generated using the trimmed open reading frame of murine *Shh* (described as *Hhg-1* in Chang et al., 1994) in pBluescript (Stratagene) as the template for PCR-mediated mutagenesis with an oligonucleotide that changed the Cys-199 codon to Ala. The pShh-C construct was generated by deletion of codons for residues 25–198. The pShh-N construct was generated by PCR amplification of *Shh* sequences encoding amino acids 1–198, with a chain termination codon incorporated following codon 198. Constructs were inserted into the pCIS vector (Chang et al., 1994; Gorman, 1985). The pShh-HA construct was generated by PCR-based addition of an HA epitope (Wilson et al., 1984) to the carboxyl terminus of the rat *Shh* cDNA (*vhh-1* in Roelink et al., 1994) and inserted into pcDNA1-Neo (InVitrogen).

Expression of constructs in COS cells was performed as described previously (Roelink et al., 1994). HEK-293 cells were transfected with pShh-HA, and stable lines were selected in G418 (0.5 mg/ml).

Throughout the text, SHH-N and SHH-C are used to indicate proteins generated from the truncated pShh-N and pShh-C constructs. The terms amino- and carboxy-terminal cleavage product are used to designate proteins generated from the full-length SHH precursor.

Purification of Recombinant SHH-N Product

A cDNA encoding the amino-terminal domain of mouse SHH (residues 25–198) was amplified by PCR and inserted into a glutathione transferase fusion vector carrying a thrombin cleavage site (pGEX2T; Pharmacia). E. coli strain BL21 was transformed and induced with IPTG. Cells were lysed (Ausubel et al., 1994) in the absence of detergent. Cleared lysates were bound to glutathione-conjugated agarose beads (Sigma), and bound fusion protein was cleaved with a mass of thrombin (Boehringer Mannheim) equivalent to 1% the mass of fusion protein in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2.5 mM $CaCl_2$. The protein released from the beads was batch adsorbed to anti-thrombin–agarose (Sigma). SHH-N was purified from this supernatant by adsorption to heparin–agarose (Sigma) in PBS containing 1 mM DTT and 0.2 mM PMSF followed by elution with 650 mM NaCl. Protein species other than the SHH-N product could not be detected by Coomassie blue staining of heavily loaded SDS–polyacylamide gels, indicating a purity greater than 95%. Concentrations of SHH-N were determined by the Coomassie blue dye binding assay.

Immunodetection of SHH Protein

For detection of SHH by immunoblotting, samples from dissected stage 10 chick notochord, transfected and untransfected COS cells, and culture supernatants were suspended and boiled in sample loading buffer and electrophoresed in SDS–polyacrylamide (12%) gels (Laemmli, 1970). For chick notochords, integral membrane and membrane-associated proteins were enriched by Triton X-114 extraction (Bordier, 1981) and batch adsorbed to heparin–agarose. The heparin beads were washed in PBS and eluted with SDS sample buffer prior to SDS–PAGE. Proteins were transferred to nitrocellulose (Burnette, 1981) and detected by chemiluminescence (ECL, Amersham) with affinity-purified anti-SHH antibodies (1:500) and HRP-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) (1:10,000).

Estimates of the concentration of SHH-N in medium derived from transfected COS cells were determined by ELISA using purified E. coli derived SHH-N as a standard with rabbit antibodies directed against the amino-terminal domain of rat SHH (1:3000 dilution). These ELISAs assume that the adsorption of SHH proteins from different sources was similar.

In Vitro Induction Assays

Neural plate explants were dissected from stage 10 chick embryos (Hamburger and Hamilton, 1951), and induction assays were performed as described (Yamada et al., 1993; Roelink et al., 1994; Tanabe et al., 1995). Labeled explants were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics. Confocal images of labeled explants were obtained using a Bio-Rad MRC-500 confocal microscope.

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