Synergistic and antagonistic roles of the Sonic hedgehog N- and C-terminal lipids

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Summary

The Shh protein contains both N-terminal and C-terminal lipids. The functional redundancy of these lipid moieties is presently unclear. Here, we compare the relative roles of the N- and C-terminal lipids in early rat striatal neuronal differentiation, membrane association and multimerization, and ventralizing activity in the zebrafish forebrain. We show that these lipid act synergistically in cell tethering and the formation of a large (L) multimer (669 kDa). However, the C-terminal lipid antagonizes the rat striatal neuronal differentiation-inducing activity of the N-terminal lipid. In addition, multimerization is required but not sufficient for the differentiation-inducing activity. Based on the presence of different N- and C-lipid-containing Shh proteins in the rat embryo, and on their different activities, we propose that both N- and C-terminal lipids are required for the formation of multimers involved in long-range signaling, and that the C-terminal lipid may function in long-range signaling by reducing Shh activity until it reaches its long-range target. Comparative analysis of the ventralizing activities of different N- and C-terminal lipid-containing Shh proteins in the zebrafish forebrain shows that the presence of at least one lipid is required for signaling activity, suggesting that lipid modification of Shh is a conserved requirement for signaling in the forebrain of rodents and zebrafish.

Key words: Sonic hedgehog, Lipid modifications, Forebrain

Introduction

Patterning of the ventral forebrain depends on the combined action of the secreted signaling protein Shh (reviewed by Ho and Scott, 2002; Ingham and McMahon, 2001; Ingham, 2001; McMahon, 2000) and several homeobox-containing target genes (Anderson et al., 1997; Guillemot and Joyner, 1993; Casarosa et al., 1999; Sussel et al., 1999; Marin et al., 2000; Szucsik et al., 1997; Corbin et al., 2000; Yun et al., 2001; Torreson et al., 2000). Loss of the Shh gene in mice results in the expansion of dorsal structures at the expense of ventral structures (Chiang et al., 1996). These studies demonstrated that development of the ventral forebrain, as well as of other ventral neural structures, depends on the presence of a master secreted signaling protein.

Since its discovery as a key secreted regulatory protein, there have been many studies investigating the mechanisms used by Shh to pattern the embryo. One of the long-standing puzzles has been how Shh selectively acts in a long-range or short-range manner. Early evidence that Shh may act through long-range signaling in the vertebrate embryo resulted from comparisons of *Shh* mRNA expression with that of its target genes (Echelhard et al., 1993; Kraus et al., 1993; Riddle et al., 1993; Roelink et al., 1994). In the chick spinal cord, expression analysis showed that although *Shh* mRNA is limited to the ventral midline, direct targets of Shh can be detected several cell diameters distant from Shh-expressing cells (Marti et al., 1995a; Roelink et al., 1995; Ericson et al., 1995). In addition, concentration-dependent effects of the Shh protein suggested that the level of exposure to Shh protein regulates regional diversity (Marti et al., 1995; Roelink et al., 1995; Ericson et al., 1995; Ericson et al., 1997; Briscoe et al., 2000). Relative expression analysis combined with the ability of Shh to directly activate distant targets in a concentration-dependent manner did not rule out the possibilities that secondary relay mechanisms and/or cell migration are involved instead of the morphogenetic activity of Shh. Gritli-Linde et al. and Incardona et al. have shown that the Shh protein can be detected at a distance from its source (Gritli-Linde et al., 2001; Incardona et al., 2000), providing new evidence that long-range signaling may involve the direct action of Shh protein. In a crucial experiment, Briscoe et al. (Briscoe et al., 2001) showed that the long-range signaling effects of Shh depend upon the direct interaction of Shh with its receptor Patched (Ptc) (Marigo et al., 1996; Stone et al., 1996) in the chick spinal cord.

Taken together, these experiments clearly show that Shh can act as a morphogen in vertebrates, directly acting on targets far from its source of secretion.

The crucial issue has now become, not whether Shh acts as a morphogen, but how Shh acts as a morphogen. Several models for the mechanism of action of morphogens have been proposed (reviewed by Telemann et al., 2001). Among these are repeated cycles of endocytosis and secretion, cell growth, diffusion and cytonemes (Ramirez-Weber and Kornberg, 1999). However, it is presently not clear which, if any, of these mechanisms are used by Shh.

The unique nature of the two lipid moieties on Shh suggests that investigation of these may lead to a better understanding of the long-range signaling mechanisms used by Shh (reviewed by Ingham, 2001). Previous work showed that the addition of the C-terminal cholesterol requires an autocatalytic cleavage event (Porter et al., 1995; Porter et al., 1996a) that results in the generation of a 19 kDa active N-terminal fragment (Bumcrot et al., 1995; Marti et al., 1995b). Palmitoylation of this 19 kDa fragment generates a Shh protein that contains two lipids, an N-terminal palmitate and a C-terminal cholesterol (Pepinsky et al., 1998). The N-terminal lipid is required for activity in *Drosophila* (Lee et al., 2001) and enhances differentiation activity in both a cell line-based assay and forebrain neural explants (Pepinsky et al., 1998; Kohtz et al., 2001; Taylor et al., 2001). The C-terminal cholesterol is responsible for tethering Shh to the surface of the secreting cell (Porter et al., 1996b), but recent evidence suggests that the C-terminal cholesterol is required for long-range signaling in the mouse limb (Lewis et al., 2001), and punctate structures in the fly (Gallet et al., 2003). However, the mechanism by which the C-terminal cholesterol mediates long-range signaling remains unknown. Zeng et al. recently proposed a model suggesting that the C-terminal cholesterol is necessary for the formation of multimers that hide the hydrophobic lipid domains in a micelle structure mediating long-range signalling (Zeng et al., 2001). Taken together, these data suggest that the N- and C-terminal lipids may play different roles in Shh activity, tethering, multimerization and long-range signaling. However, the individual contributions of the N- and C-terminal lipids in these functions have yet to be defined.

In this study, Shh proteins containing different combinations of N- and C-terminal lipids were purified from stably-transfected neural cell lines and used to compare the relative roles of the N- and C-terminal lipids in ventral forebrain neuronal differentiation-inducing activity, membrane tethering, multimerization and expression profile in embryonic tissues. The data suggests that the N- and C-terminal lipids synergize during Shh multimerization and membrane tethering, but not during differentiation-inducing activity. The presence of the C-terminal lipid alone is sufficient both for multimerization and membrane tethering, whereas the N-terminal lipid alone is sufficient only for multimerization and not for membrane tethering. Contrastingly, the N-terminal lipid is required for differentiation-inducing activity, whereas the C-terminal lipid substantially reduces Shh differentiation-inducing activity. Purification of multimeric and monomeric forms of Shh proteins shows that multimerization is required but not sufficient for Shh differentiation-inducing activity. In addition, differences between Shh proteins in the embryonic brain and limb can be detected, supporting the hypothesis that expression of functionally distinct forms of Shh may be region specific. These data suggest that the N- and C-terminal lipids play both overlapping and distinct roles in Shh signaling, and that the key to understanding the morphogenic actions of Shh is a better understanding of the relative functions of its N- and C-terminal lipids.

Materials and methods

Explant culture and immunohistochemistry

These were performed as previously described (Kohtz et al., 2001), with modifications as indicated. Rat E11 (sperm plug date = 0) telencephalic explants were dissected from the region shown in Fig. 3B. An intraocular grid (Leica) was used to guide the dissection of neural explants from the embryonic telencephalon. Tungsten needles were used for fine dissections. The resulting piece measured approximately 500 μm×375 μm. The first cut was parallel to the midline and measured to be approximately 125 μm from the midline. Prior to culturing on filters (Nunc 0.02 μm), explants were oriented so that the neurectodermal layer was at the surface. The explant was bathed in medium, with 300 μl of medium inside the culture insert and 300 μl surrounding the culture insert. For the purposes of these experiments, we purified rabbit anti-*dll* using the same *dll*-homeodomain-containing protein as the immunogen originally reported by Panganiban et al. (Panganiban et al., 1995). Anti-Islet 1/2 antibody (Thor et al., 1991; Ericson et al., 1992) was obtained from the Developmental Studies Hybridoma Bank.

Analysis of Shh proteins in embryonic tissues

Rat E11 forebrain, midbrain and hindbrain, or limbs from 30 embryos were solubilized in a minimum volume (100-200 μl) of denaturing lysis buffer (50 mM Tris pH 7.5, 1% SDS, 5 mM EDTA, 10 mM Dithiothreitol, 15 U/ml DNASE I, 1 mM PMSF). Glycerol, bromophenol blue and β-mercaptoethanol were added before separation by SDS-PAGE and western blotting. Visualization of bound primary antibody was performed using peroxidase-conjugated anti-rabbit antibody (1:5000, Amersham) and chemiluminesence reagent (NEN). Proteins were electrophoresed on an 18-cm long gel to obtain the optimal separation of different Shh isoforms. Rabbit anti-Shh antibody (Biogen) was used at 1:4000 dilution. In order to verify the specificity of the antibody, competitions were performed as follows: anti-Shh antibody was pre-incubated with 20 μg of uShhN or 20 μg of bovine serum albumin overnight. The pre-incubated antibodies were then compared for reactivity to bands identified in tissues and uShhN. Pre-incubation with uShhN eliminated reactivity to uShhN in the blot, as well as tissue-reactive bands, whereas pre-incubation with bovine serum albumin did not. In the pre-aborption experiment, anti-Shh antibody diluted to 1:3000 was pre-aborbed onto a column bound with uShhN or Dlx5 (a homeobox-containing transcription factor) protein. The pre-absorbed antibodies were then incubated with blots containing tissue proteins and uShhN. Anti-Shh antibody that had been pre-absorbed on the uShhN column lost reactivity to uShhN and tissue bands, whereas antibody that had been pre-absorbed on a Dlx5 column retained reactivity to uShhN and tissue bands. Membranes were prepared from stably transfected cell lines as previously described (Degtyarev et al., 1994). Membranes from rat E11 embryonic mid/hindbrain (~110 embryos) and limb (~140 embryos) tissues were isolated using the same procedure, except the tissue was homogenized using a micro-pestle in a microfuge tube prior to dispersing through a 25-gauge needle. Triton X-100 in increasing concentrations (0.1%, 0.2%, 0.4%, 0.6%, 0.8%, 1%) was incubated with 100,000 ***g*** membrane preparations, and the proteins remaining in the inextractable pellet (P) were solubilized in 2×SDS-PAGE sample buffer.

Generation of stably transfected cell lines

The C17.2 cell line was grown according to Snyder et al. (Snyder et al., 1992). Cells were transfected with human Shh cDNAs capable of producing three different N- and C-terminal lipid-containing Shh proteins (amino acids 1-197), including the signal sequence. The S4 cell line (Liu et al., 1998), a C17.2 cell line containing wild-type Shh (wtShh) was obtained from Dr A. Joyner (New York University). A point mutation changing human SHH cysteine 24 to a serine was obtained from Dr E. Garber (Biogen) (Pepinsky et al., 1998; Kohtz et al., 2001). *Shh* cDNAs (C24S-Shh, ShhN, C24S-ShhN) were subcloned into the mammalian expression vector pZeo (Invitrogen) and transfected into C17.2 cells. Selection for zeomycin (Invitrogen, 500 μg/ml)-resistant colonies was performed over a two-week period, after which 15 independent clones were recovered, as well as two groups of heterogeneous cells for each *Shh* cDNA. Stably transfected cell lines were screened by western analysis to obtain the clones that produced the highest levels of Shh proteins.

Preparation of different N- and C-terminal lipid-containing Shh proteins

Culture supernatants from C17.2 stably transfected cell lines containing soluble Shh proteins were collected over a 12-day period from 150 mm plates. Cells were fed with fresh medium every 4 days. One to two liters of cell culture supernatant was affinity purified on a 1 ml column (Pharmacia AKTA-FPLC) containing 1 mg of purified 5E1 anti-Shh antibody (Ericson et al., 1996) (a monoclonal antibody against Shh, obtained from the Developmental Studies Hybridoma Bank), cross-linked to CNBr-activated Sepharose (Pharmacia). The supernatant was applied twice to the column, the column washed with TBS-0.5% Tween 80, and proteins eluted in 100 mM glycine, pH 2.5. Shh proteins were dialyzed against PBS/0.5 mM dithiothreitol, concentrated in Centricon filters (Amicon), and quantified using uShhN as a standard by western blotting.

Determination of ratio of cell-associated and secreted Shh

In order to determine the ratio of cell-associated and secreted Shh, stably-transfected cell lines were grown in 100 mM plates for 4 days. For the secreted form: supernatants from each plate (10 ml) were immunoprecipitated with 5E1 antibody that had been pre-incubated with protein G-agarose beads (Roche). Secreted Shh bound to 5E1-protein-G agarose was recovered by centrifugation and solubilized in Laemmli sample buffer. For the cell-associated form: cells were scraped from the 100 mm plate, recovered by centrifugation and solubilized in Laemmli sample buffer. Samples were then western blotted using rabbit anti-Shh antibody as the probe.

Gel filtration of Shh proteins

Affinity-purified Shh proteins were applied to a Superdex 200 column (AKTA FPLC) and 250 μl fractions were collected. The column was standardized using the following range of proteins (Pharmacia): 669 kDa, 66 kDa, 43 kDa, 25 kDa and 13 kDa. Independently run purifications on the Superdex column resulted in standards eluting into identical fractions. Fractions were dialyzed against 100 mM ammonium carbonate (pH 7.5) and concentrated by evaporation. One-fifth of each fraction was analyzed by western blotting and probed with rabbit anti-Shh antibody. Multimeric and monomeric fractions were dialyzed against PBS/0.5 mM DTT in order to assay for their relative activities in the forebrain differentiation assay.

ShhN protein (Fig. 3A, lane 4) and wtShh protein (Fig. 3A, lane 1) were purified from the culture supernatant of stably transfected C17 cells, and separated by Superdex 200 gel filtration column (as shown in Fig. 5A,C). Fractions corresponding to M (fractions 37 and 38) and the ShhN monomeric form (59 and 61) were pooled, concentrated, and tested for their activity.

PCR primers

Sightless 1 (GenBank Accession number BC008159), 389 bp fragment: 5′-(381) gga tca aga att tga gct gga-3′ and 3′-(770) tac tcg ttc tct gtc ttg tac-5′.

Sightless 2 (GenBank Accession number AK003605): 5′-1(611-) ctt ggc aga gtg ggt ttg taa-3′; 3′-1(–1060) aca gtg ttg aca aca cca aac-5′; 5′-2(632-) cag gca cct ttg atc ttc aag-3′; and 3′-2(–1161) gat acc acg gtc gaa gtg cgt-5′.

Mouse dispatched (GenBank Accession number AY150698), 591 bp fragment: mdispatched 5′-(450) gac cca gag aaa ccc caa gaa-3′ and mdispatched 3′-(1041) ttc cac gtg acc agc ctc tgt-5′.

Zebrafish injection of RNA and in situ hybridization

mRNA encoding different Shh lipid modifiable forms was transcribed in vitro using T7 polymerase from the pT7TS plasmid linearized with *Bam*HI. Two- to four-cell stage zebrafish embryos were injected with 75 pg of mRNA encoding ShhN, C24S-Shh and C24S-ShhN, Shh (Ekker et al., 1995) and β-galactosidase. Injected embryos were fixed in 4% paraformaldehyde at the 20 somite stage, and gene expression was assayed by in situ hybridization using anti-sense digoxigenin, or fluorescein-labeled RNA probes to *nk2.2* (Barth and Wilson, 1995), *nk2.1b* (Rohr et al., 2001), *pax6* (Krauss et al., 1991) and *emx1* (Morita et al., 1995). Double in situ labeling was performed described previously (Jowett and Yan, 1996), using (1) an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) and the NBT (Roche) reaction substrate, and (2) an alkaline phosphatase-conjugated anti-fluorescein antibody (Roche) and the Fast Red (Roche) reaction substrate. After color reactions were developed, embryos were fixed overnight and cleared in 75% glycerol. Color reactions were timed and controlled for by expression in comparison with uninjected controls. For lateral views, yolks and eyes were removed. For the dorsal views, only yolks were removed. Mounted embryos were photographed using a Zeiss compound microscope with DIC optics and images were processed using Adobe Photoshop software.

Effects on gene expression were scored blind based on the degree of expansion of *nk2.1b* and *nk2.2* expression and the degree of reduction of *emx1* and *pax6* expression. Embryos were placed into four different classes: (1) no effect, (2) mild ventralization, (3) moderate ventralization and (4) major ventralization (see Fig. 5).

Results

Characterization of embryonic Shh proteins in the brain and limb

We recently reported that the N-terminal lipid enhances Shh activity in the embryonic brain (Kohtz et al., 2001), but not the limb (Lee et al., 2001). The differential activity of N-terminal lipid-containing Shh in the brain and limb raised the possibility that there may be differences between endogenous Shh proteins in these regions. In order to determine whether different forms of Shh can be detected in embryonic tissues, we solubilized E11 rat embryonic tissues from the forebrain, midbrain/hindbrain and limb regions. Tissue proteins were solubilized, separated by SDS-PAGE on 18-cm long gels, and probed with anti-Shh antibody by western analysis (Fig. 1A, lanes 7-10). The same blot was stripped and re-probed with anti-tubulin antibody as a loading control (Fig. 1A, lanes 7′-10′). The anti-Shh antibody detects four predominant bands and three minor bands in the range between 28.1 kDa and 20.4 kDa. The profile of Shh proteins obtained in brain tissues, FB (forebrain) and MB/HB (midbrain+hindbrain), are similar. However, one of the bands (marked by an asterisk) appearing in the brain tissues is not detectable in limb tissue extracts (Fig. 1, compare lane 10 with lanes 8 and 9). In addition the Shh form marked with an asterisk is not detected in mid/hindbrain extracts unless diluted fourfold in 1% SDS denaturing buffer

[compare Fig. 1, lane 7 (undiluted) and lane 8 (fourfold diluted)]. In order to verify the specificity of the anti-Shh antibody used in these experiments, soluble uShhN was pre-incubated with anti-Shh antibody prior to incubation with the blot. This pre-incubation step abolished all the bands detected in lanes 7-10 (data not shown). A second method to verify the specificity of the Shh antibody was to pre-absorb the Shh antibody on a uShhN or Dlx5 column prior to incubation with the blot. Pre-aborption of the Shh antibody with the uShhN column abolished reactivity with the bands shown in 7-10, whereas pre-absorption with the Dlx5 column did not affect binding (not shown). These data suggest that different forms of Shh proteins are differentially expressed in the embryonic brain and limb.

Next, we tested whether these embryonic Shh forms correspond to different N- and C-terminal lipid-containing Shh proteins. Because it is not possible to purify sufficient amounts of Shh protein from embryonic extracts for mass spectroscopy,

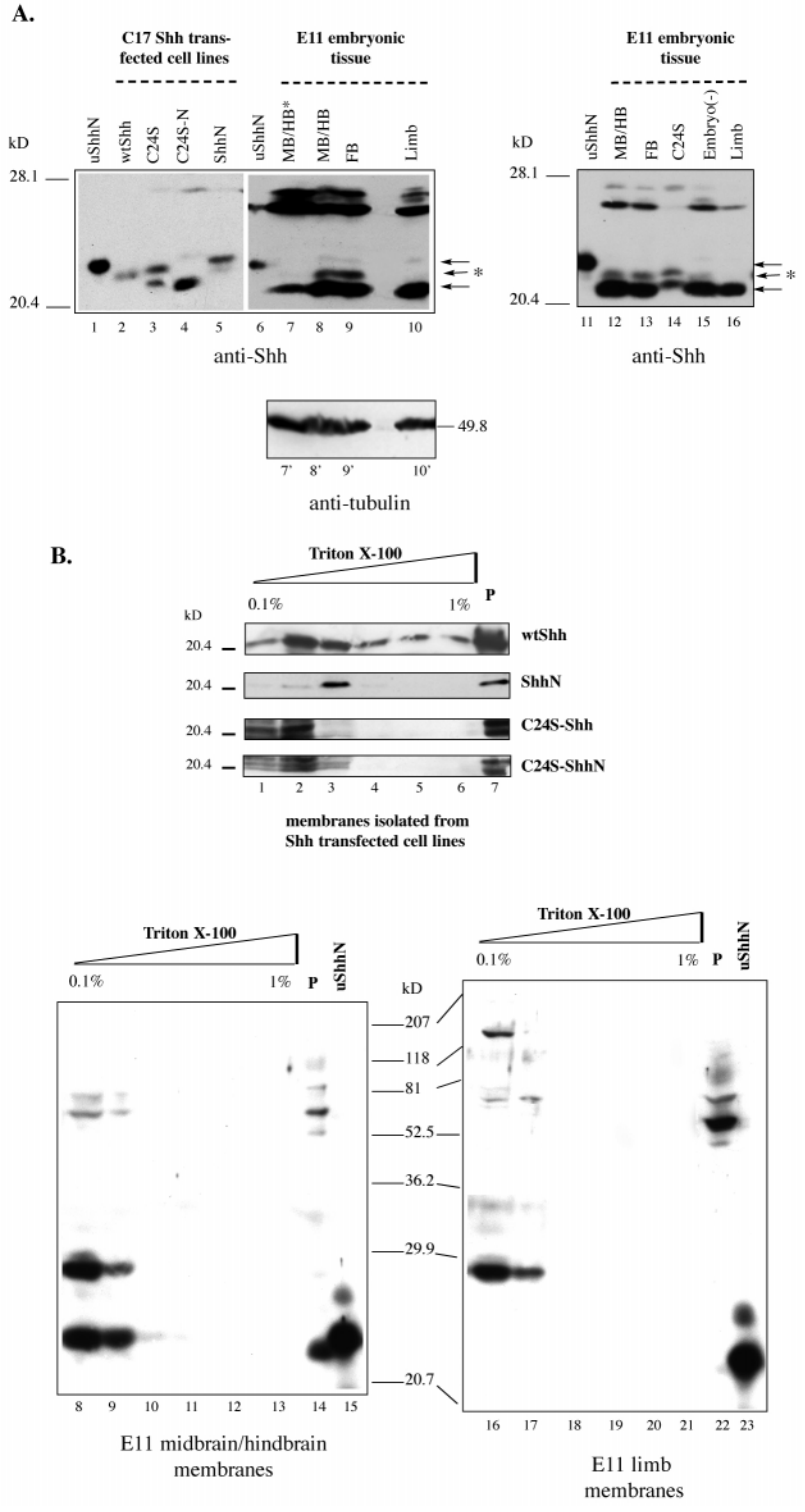

Fig. 1. Characterization of different Shh proteins in rat E11 brain and limb tissue. (A) Comparison of different N- and C-terminal lipid-containing Shh proteins expressed by stably transfected cell lines and embryonic tissue. The neural cell line C17 was transfected with different Shh constructs in order to obtain stable cell lines expressing different N- and C-terminal lipids. Whole-cell lysates from stably transfected cells were separated by SDS-PAGE and used to compare the migration of Shh proteins present in E11 embryonic tissues. Western blot analysis using anti-Shh (1-10, 11-16) or anti-tubulin (7′-10′ antibodies). (Lane 1) uShhN (7 ng; Shh made in *E. coli* containing no N- or C-terminal lipids). Lanes 2-5 show lysates of C17 cells stably expressing different lipid-containing Shh proteins (described in Table 1). (Lane 2) wtShh (N+C lipid); (lane 3) C24S-Shh (C lipid alone); (lane 4,) C24S-ShhN (no lipid, 10-fold more cell lysate was loaded in order to detect cell-associated protein); (lane 5) ShhN (N lipid alone). (Lane 6) uShhN (5 ng). (Lane 7) Rat E11 midbrain/hindbrain (MB/HB) tissue lysate. (Lane 8) Rat E11 MB/HB lysate diluted 4-fold in denaturing lysis buffer containing 1% SDS. (Lane 9) Rat E11 forebrain (FB) tissue lysate. (Lane 10) Rat E11 limb tissue lysate. (Lanes 7′-10′) Lanes 7-10 were stripped and re-probed with anti-tubulin antibody as a loading control. (Lane 11) uShhN (10 ng). (Lane 12) E11 rat MB/HB tissue lysate. (Lane 13) E11 rat FB tissue lysate. (Lane 14) Lysate of C17 cells stably expressing C24S-Shh protein. (Lane 15) Rat E11 embryo tissue lysate with brain, limbs and branchial arches removed. (Lane 16) Rat E11 limb tissue lysate. Lanes 11-16 were exposed for a shorter amount of time. Size standards are shown on the left. The three arrows on the right highlight the migration of different Shh proteins in the range of those detected in C17 transfected lysates. The asterisk marks a form of Shh that is present in the brain and absent from limb tissue. (B) A comparison of the profile of Triton X-100 extractable membrane Shh proteins containing different N- and C-lipids from Shh stably transfected cells, and from embryonic midbrain/hindbrain and limb tissues. Triton X-100 extracted membranes were analyzed by western analysis using anti-Shh antibody. Membranes isolated from Shh stably transfected cell lines (lanes 1-7), E11 rat embryonic midbrain/hindbrain (lanes 8-14) or E11 rat embryonic limb (lanes 16-22) were treated with increasing amounts of Triton X-100: 0.1% (lanes 1,8,16), 0.2% (lanes 2,9,17), 0.4% (lanes 3,10,18), 0.6% (lanes 4,11,19), 0.8% (lanes 5,12,20), 1% (lanes 6,13,21). (Lanes 7,14,22) Pellet; (lanes 15,23) uShhN, 10 ng.

we compared the migration of known N- and C-terminal lipid-containing Shh forms during SDS-PAGE with those found in the embryonic tissues. It has previously been reported that the C-terminal lipid increases the migration of the Shh protein during SDS-PAGE (Porter et al., 1996a). Therefore, we investigated whether N- and C-terminal lipid-containing proteins can be distinguished during SDS-PAGE, in a similar way to that reported for C-terminal lipid-containing Shh. In order to generate different N- and C-terminal lipid-containing proteins, Shh cDNA encoding different combinations of N- and C-terminal lipids was transfected into the neural cell line C17.2 (Snyder et al., 1992), and cells selected for the stable expression of Shh proteins. Table 1 lists the different combinations of N- and C-terminal Shh proteins generated by stable transfection into C17 cells (1-4). The S4 cell line, stably transfected with a full-length Shh cDNA construct (wtShh, N+C lipid), was previously generated by Liu et al. (Liu et al., 1998). The C24S point mutation (C24S-Shh, C lipid alone, 4. C24S-ShhN, no lipid) results in the conversion of cysteine at position 24 to serine, generating Shh proteins that lack the N-terminal lipid (Pepinsky et al., 1998; Kohtz et al., 2001). It has previously been shown that the addition of cholesterol to the C terminus occurs through an autocatalytic cleavage event requiring residues C-terminal to Gly197 (Porter et al., 1996a). Thus, C-lipid-lacking forms were generated by using Shh cDNA constructs truncated at Gly197 (ShhN, N lipid alone,

Table 1. Summary of the properties of different N- and C-terminal lipid-containing Shh proteins

Shh signaling proteins	Source	Construct length	Differentiation activity	Membrane tethering	Multimerization		
			3 nM Shh (Dlx density $\times 10^6$)	% Membrane bound	>669 kDa %	669-69 kDa %	Monomer %
1. palmitate/myristate; wtShh (N+C lipid); C 24 G 197 – chol	C17$^{\zeta}$	F*	0.01±0.02	98	4	87	9
2. C24S-Shh (C lipid); S 24 G 197 – chol	C17$^{\zeta}$	F*	0	54	0	99.9	0.1
3. palmitate/myristate; ShhN (N lipid); C 24 G 197	C17$^{\zeta}$	N$^{\tau}$	44±10	1	0	52	48
4. C24S-ShhN (no lipid); S 24 G 197	C17$^{\zeta}$	N$^{\tau}$	0	0.1	0	0	100
5. myristate; mShhN (N lipid); C 24 G 197	*E. coli*	N$^{\tau}$	0.3±0.1				
6. uShhN (no lipid); C 24 G 197	*E. coli*	N$^{\tau}$	0				

* F, encoded by a full-length construct
$^{\tau}$ N, encoded by an N-terminal fragment construct
$^{\zeta}$ secreted soluble Shh protein purified from stably transfected C17 cell lines

wtShh contains both N-and C-terminal lipids, C24S-Shh contains only the C-terminal lipid, ShhN contains only the N-terminal lipid, C24S-ShhN contains no lipid and mShhN contains only the N-terminal lipid. The difference between ShhN and mShhN is that ShhN is affinity purified from the culture supernatant of a stably-transfected C17 neural cell line, whereas mShhN was purified from *E. coli* and myristoylated in vitro (Kohtz et al., 2001). uShhN was made as previously described (Williams et al., 1999).

C24S-ShhN, no lipid). Lysates from these four cell lines were blotted adjacent to uShhN (Shh produced in *E. coli* with no lipid moieties). The migration of these proteins was used as a standard (Fig. 1A, lanes 2-5) to compare the Shh forms detected in tissue extracts of brain and limb. The bottom of the three arrows in Fig. 1 points to Shh migrating very close to the N-lipid-lacking forms of Shh (C24S-ShhN and C24S-Shh). This band is common to all three tissues. The top band (faintest) migrates in the same position as ShhN and is also common to all three tissues. The middle band (asterisk) of Shh migrates in the same position as wtShh; this band is detectable in the brain extracts (forebrain and mid-hindbrain), but is below the level of detection in the limb. Because transfected Shh proteins in the 28.1 kDa range are expressed at low levels, it is difficult to determine which of the bands in this region may correspond with N- and/or C-terminal lipid-containing proteins. Fig. 1A (lanes 11-16) shows the relative migration of the brain Shh* form and the C24S-Shh protein doublet, in a blot that has been exposed for less time, confirming that Shh* migrates between the C24S-Shh doublet. These co-migration data raise the possibility that different N- and C-terminal lipid-containing Shh proteins may be co-expressed within the same tissue, and that different ratios of lipid-containing Shh proteins may be expressed in the embryonic brain and limb.

Midbrain/hindbrain tissues were diluted fourfold (Fig. 1A, lane 8) in order to detect Shh forms co-migrating with N- and N+C-terminal lipid-containing Shh proteins. Thus, Shh* and the faint band migrating above it are clearly more difficult to solubilize than the fastest migrating form, compare lanes 7 and 8 (Fig. 1A). Dilution of limb tissues (lane 10) did not reveal these slower migrating forms of Shh. Further support that N+C and N-terminal lipid-containing Shh proteins require higher concentrations of detergent in order to be solubilized is shown in Fig. 1B. We previously showed that N-terminal lipid-containing Shh is detected in membranes prepared from a C17 cell line stably transfected with wtShh (Kohtz et al., 2001). Membranes from different N- and C-terminal lipid-containing containing Shh transfected cell lines were similarly treated with increasing amounts of Triton X-100 (0.1%-1%), and subjected to western analysis with anti-Shh antibody (Fig. 1B, lanes 1-6). The Shh proteins remaining in the 1% Triton X-100 inextractable pellet are shown in Fig. 1B, lane 7. Although a large fraction of all Shh proteins remain in the pellet fraction, comparison of the Triton X-100 solubilized Shh proteins shows that N-terminal lipid-containing Shh proteins are more difficult to solubilize than those lacking N-terminal lipids (Fig. 1B, compare lanes 3-6). An analysis of Triton X-100 extractable Shh proteins from embryonic mid/hindbrain (Fig. 1B, lanes 8-14) and limb (Fig. 1B, lanes 16-22) membranes reveals that the majority of Shh protein in these embryonic tissues is either extracted by 0.1% and 0.2% Triton X-100 or remains in the inextractable pellet, similar to the Shh proteins lacking N-terminal lipids in transfected cells (compare Fig. 1B, lanes 8,9,16 and 17 with lanes 1 and 2). Major differences between Shh protein forms in the mid/hindbrain and limb membranes are observed. The proportion of SDS-PAGE-resistant multimers is significantly higher in limb membranes, in both the soluble and insoluble fractions. In addition, unlike mid/hindbrain membranes, limb membranes contain an ~120 kDa multimer, whereas only a minute fraction of 21 kDa Shh is detectable in the pellet (Fig. 1B, lane 22, upon overexposure; data not shown). Given the difficulty of solubilizing the N-terminal lipid co-migrating forms in mid/hindbrain extracts, even in 1% SDS (Fig. 1A, lane 7, top two arrows), it would be expected that these forms are not extractable from the membrane by non-ionic detergent (Triton X-100). Taken together, these data suggest that different Shh protein forms exhibiting properties similar to different N- and C-terminal lipid-containing Shh proteins (by migration during SDS-PAGE and membrane extractability) are present in the embryonic brain and limb, supporting the hypothesis that Shh proteins containing different N- and C-lipids may function in these tissues.

The N- and C-terminal lipids synergize to increase membrane tethering

It has previously been shown that the C-terminal cholesterol tethers Shh to the membrane of the secreting cell (Porter et al., 1996a). However, the contribution of the N-terminal lipid to membrane tethering has not been defined. Fig. 2A shows that wtShh (N+C lipid) exhibits the highest cell-associated:secreted protein ratio, whereas C24S-ShhN (no lipid) exhibits the lowest cell-associated:secreted protein ratio. Comparison of the ratios of cell-associated and secreted Shh proteins in cell lines stably transfected with different lipid-containing Shh cDNA are shown in Table 1. Cell association and secretion are inversely related (as would be expected). That the majority of cell-associated wtShh is membrane bound and not cytosolic was previously determined by subcellular fractionation (Kohtz et al., 2001). Ninety-eight percent of wtShh (N+C lipid) is cell associated. Fifty-four percent of C24S-Shh (C lipid alone) remains cell associated, whereas only 1% of ShhN (N lipid alone) remains cell associated. These data suggest that the N-

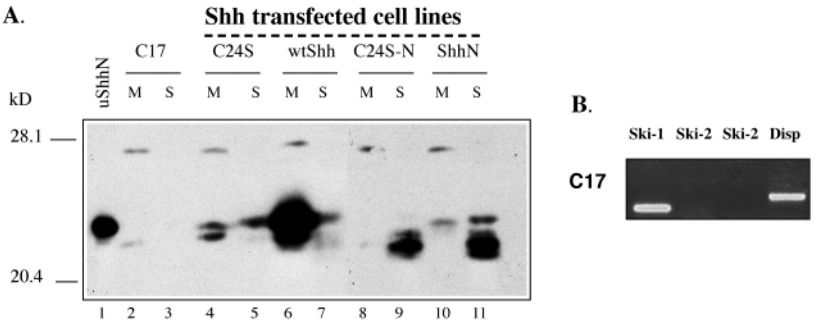

Fig. 2. The N and C-terminal lipids act synergistically to restrict Shh to the membrane. (A) Anti-Shh western analysis of lysates derived from C17 cells expressing different lipid-containing Shh proteins. M, membrane bound (whole cell lysates); S, secreted (cell culture supernatants immunoprecipitated using 5E1 monoclonal antibody). (Lane 1) uShhN (7 ng; Shh made in *E. coli* containing no N- or C-terminal lipids); (lanes 2,3) C17 (non-transfected parental cell line); (lanes 4,5) C24S-Shh (C lipid alone); (lanes 6,7) wtShh (N+C lipid); (lanes 8,9) C24S-ShhN (no lipid); (lanes 10,11) ShhN (N lipid alone). Size standards are shown on the left. (B) The C17 neural cell lines expresses Ski1 and mouse dispatched, but not Ski2. cDNA isolated from C17 cells was subjected to PCR using primers against Ski1, Ski2 and dispatched (Disp), as indicated, and loaded on a 2% agarose gel stained with ethidium bromide to visualize PCR products.

and C-terminal lipids synergize during membrane tethering. In addition, the C-terminal lipid alone can tether Shh to the membrane, whereas the N-terminal lipid cannot.

Proteins required for fly *hh* palmitoylation and long-range signaling [*ski* and *sightless* (Chamoun et al., 2001; Lee and Treisman, 2001), *rasp* (Michelli et al., 2002) and *disp* (Burke et al., 1999)] have been reported. In mice, two forms of Ski have been identified. In addition, mouse Disp has been shown to be required for the release of Shh from secreting cells (Ma et al., 2002). Consistent with the ability of Ski to palmitoylate and Disp to release Shh from cells, we find that the C17 neural cell line expresses both mouse Ski1 and mouse Disp (Fig. 2B).

The C-terminal lipid reduces the N-terminal lipid-mediated increase in early striatal neuronal differentiation-inducing activity

We recently reported that the Shh N-terminal lipid significantly enhances the ability of Shh to induce the differentiation of rat ventral forebrain neurons (Kohtz et al., 2001). These experiments were performed using soluble recombinant proteins in vitro, and virally produced proteins in vivo. In vivo, the inactivity of Shh lacking the N-terminal lipid supported a requirement for the N-lipid in Shh signaling in the forebrain. This inactivity also suggested that the C-terminal cholesterol could not substitute for the N-terminal lipid to rescue activity in the forebrain. Recent experiments suggest that the cholesterol group may function in long-range signaling in the mouse limb (Lewis et al., 2001), and that this may result from the formation of multimeric complexes (Zeng et al., 2001). We observed that ectopic Dlx expression occurs at a distance from Shh virally infected clusters of cells, consistent with the ability of wtShh (N+C lipid) to signal in a long-range manner in the forebrain (Kohtz et al., 2001). These results raise the issue of whether the inactivity of C24S-Shh to induce Dlx expression in vivo results from its inability to signal once it reaches its target cell, or from its inability to form multimeric complexes that would enable it to signal in a long-range manner, or both.

Using in vitro neural explant differentiation assays, it is possible to study this issue because cells within the explant are exposed to soluble Shh and do not require Shh transportation. Thus, if C24S-Shh is inactive in vivo primarily because it fails to participate in long-range signaling, but can signal once it reaches the cell, then the C24S-Shh protein should be active in vitro. If, however, C24S-Shh is inactive independent of its long-range or short-range signaling properties, then it should be inactive in vitro and in vivo. In order to distinguish between these possibilities, we purified different N- and C-terminal lipid-containing Shh proteins from cell culture supernatants of stably transfected C17 cell lines (see Table 1 and Fig. 3A, lanes 1-4). Purified proteins were assayed for their differentiation-inducing activities at three different concentrations (1 nM, 3 nM and 12 nM) in rat E11 telencephalic explants (Fig. 3C-N). Fig. 3B shows a diagram of the dissection used to generate neural explants for the telencephalic differentiation assay [modified from a method described previously (Kohtz et al., 1998)]. Fig. 3C-N shows that the ShhN protein has the highest differentiation-inducing activity, as indicated by the number of Dlx and/or Islet1/2 expressing early striatal neurons (Fig. 3E,I,M). ShhN is active at concentrations as low as 1 nM (Fig. 3M). Shh proteins lacking the N-terminal lipid either failed to induce Dlx or Islet1/2 expression (Fig. 3H,L,F,J,N), or did so at very low levels (Fig. 3D). This supports the hypothesis that C24S-Shh is inactive in vivo because it is unable to signal, independent of its ability to be transported. Thus, the C-terminal lipid cannot substitute for the activity-enhancing properties of the N-terminal lipid either in vitro or in vivo.

These experiments also indicate that wtShh containing both N- and C-terminal lipids is significantly less active than ShhN containing only the N-terminal lipid. This suggests that the C-terminal lipid actually reduces the differentiation-inducing activity of N-terminal lipid-containing Shh. Fig. 3O quantifies the Dlx expression data presented in Fig. 3C-N, and adds data obtained with in vitro N-terminal fatty-acylated recombinant Shh protein (mShhN) for comparison (Kohtz et al., 2001). At 1 and 3 nM, significant activity is only detected for ShhN. At 12 nM concentrations, differentiation-inducing activity is detected for wtShh, ShhN and mShhN.

The question remains as to whether Shh can signal in its membrane-bound form, or whether it requires secretion for activity. Although the majority of wtShh is cell associated (Fig. 2), the ability to extract soluble wtShh and ShhN from membranes using relatively low concentrations of Triton X-100 (Fig. 1B) suggested that it may be possible to compare the differentiation-inducing activities of membrane-extracted and secreted Shh proteins. However, affinity purification of Triton X-100 membrane-extracted Shh proteins failed repeatedly (data not shown). Triton X-100 (0.1%) membrane-extracted wtShh fails to bind a column containing 5E1 (Fig. 3A, lanes 9-11), a function-blocking monoclonal anti-Shh antibody (Ericson et al., 1996). However, the same 5E1 column purifies secreted Shh forms (Fig. 3A, lanes 1-4). These data suggest that membrane extraction of wtShh alters its conformation so that it is no longer recognized by the 5E1 monoclonal antibody.

Both N- and C-terminal lipids are required for the formation of the L multimer, but not the M multimer

Although a role for the C-lipid in multimerization has been proposed, the role of the N-terminal lipid in this process remains unclear. In addition, the relationship of multimerization to activity has not been defined. In order to characterize large multimeric complexes of Shh, different N- and C-terminal lipid-containing Shh proteins (Fig. 3A, lanes 1-4) were analyzed by Superdex 200 gel-filtration (Pharmacia, AKTA-FPLC). Fractions collected after gel-filtration were western blotted (Fig. 4A-D). Shh proteins were primarily found in three peaks: fractions 24 and 25, >669 kDa (L, large); fractions 30-39, 669-66 kDa (M, medium); and fractions 59-61 (monomers). Comparison of these profiles indicates that only wtShh forms the largest multimeric Shh complex L (compare fraction 24 in Fig. 4A-D). Loss of either lipid eliminates the ability to form L (C24S-Shh or ShhN, Fig. 4B and C, respectively), and the loss of both lipids eliminates the ability to form L or M (C24S-ShhN, Fig. 4D).

In addition to L and M multimers, monomeric forms of Shh are detected in the presence or absence of N- and C-terminal lipids (Fig. 4A-D, fraction 60). However, the ratio of monomeric and multimeric forms differs, depending on the lipid content. Table 1 summarizes the results of the gel filtration analysis. In the absence of either N- or C-terminal lipids, nearly 100% of the Shh protein is in a monomeric form (C24S-ShhN). The presence of the N-terminal lipid results in 52% of the M form (ShhN), whereas the presence of the

C-terminal lipid results in 99.9% of the M form. wtShh containing both N- and C-terminal lipids consists of 4% L, 87% M, and 9% monomer.

The presence of either the N- or C-terminal lipid results in the formation of medium sized multimeric complexes (Fig. 4A-C, fractions 36 and 39). Within M-containing fractions 36 and 39, SDS-PAGE stable multimers are present (Fig. 4A,C). In the presence of both N- and C-terminal lipids, SDS-PAGE stable multimers are detected at ~30 kDa, ~50 kDa and ~90 kDa. The predominant SDS-PAGE stable multimer migrates at approximately 30 kDa, and can be detected in the presence of the N-terminal lipid either alone or together with the C-terminal lipid. In the absence of both lipids, SDS-PAGE stable multimers are not detected (Fig. 4D). The significance of these SDS-PAGE stable multimers is presently unknown; however, Fig. 1B shows that differences in membrane extractability, as well as in relative ratios of SDS-PAGE stable multimers, occurs in the embryonic brain and limb tissues.

ShhN M multimers induce early striatal neuronal differentiation

Our results show that the N- and C-terminal lipids synergize in the formation of the L multimer. However, the C-terminal lipid reduces the differentiation-inducing activity of Shh, suggesting that the relative roles of the N- and C-terminal lipids in differentiation-inducing activity differ from multimerization. One possible explanation for the C-terminal lipid-mediated decrease in activity may be that multimerization occurs at the expense of activity. Another is that some fraction of the monomeric form must be present in order for Shh activity to occur.

In order to test directly the relationship between

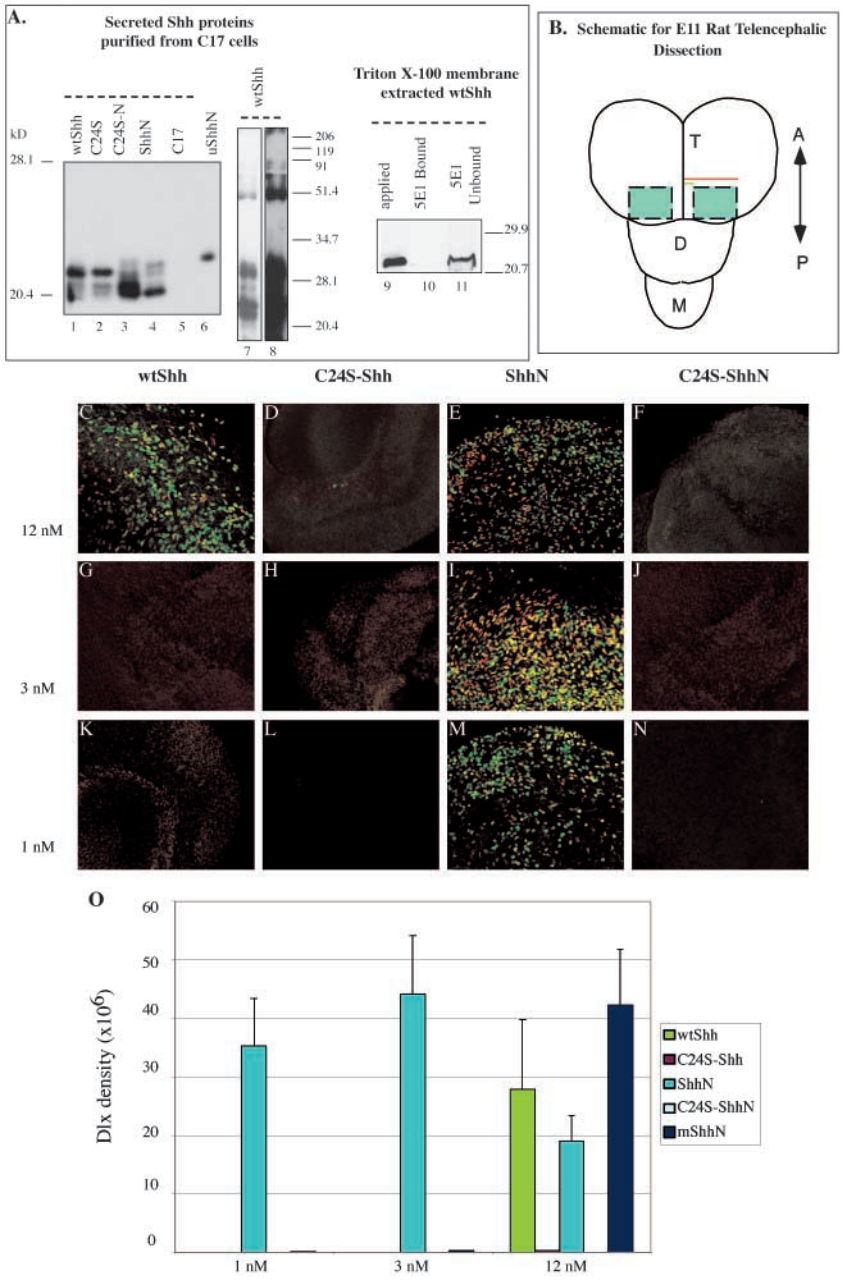

Fig. 3. The C-terminal lipid diminishes the early striatal neuronal differentiation-inducing activity of N-terminal lipid-containing Shh. (A) Secreted Shh proteins containing different N-and C- terminal lipids were purified by anti-Shh (5E1) affinity chromatography and probed with anti-Shh. (Lane 1) wtShh; (lane 2) C24S-Shh; (lane 3) C24S-ShhN; (lane 4) ShhN; (lane 5) untransfected culture supernatant control from the parental C17 cell line; (lane 6) uShhN (7 ng); (lanes 7,8) overexposures of purified wtShh protein showing slower migrating forms; (lane 9) membrane-extracted wtShh before application to 5E1 affinity column; (lane 10) 0.2 M glycine-eluted protein from 5E1 column; (lane 11) membranes extracted wtShh protein in the flow through after application to the 5E1 column. For lanes 9 and 10, wtShh protein was extracted with 0.1% Triton X-100 from wtShh stably transfected C17 cell membranes. (B) E11 rat telencephalic pieces were dissected from the blue region as indicated. The distances from the midline (red and green lines) were measured using a custom-made grid for the Leica MZ-12.5 microscope, placed in the eyepiece. The proximal cut is 125 μm from the midline, as measured by the green line; the distal cut is 625 μm from the midline, as measured by the red line. The resulting explant (blue) averages 500 μm×375 μm. T, telencephalon; D, diencephalon; M, mesencephalon; A, anterior; P, posterior. (C-N) E11 rat telencephalic explants (*n*=8) were cultured in the presence of different concentrations (1 nM, 3 nM and 12 nM) of N- and C-terminal lipid-containing proteins and stained for the early striatal neuronal markers Dlx (green) and Islet1/2 (red). wtShh (C,G,K), C24S-Shh (D,H,L), ShhN (E,I,M), C24S-ShhN (F,J,N). No Dlx- or Islet1/2-expressing cells were detected in the absence of Shh (data not shown). (O) Quantitative representation of Dlx induction in early striatal neurons by different N- and C-terminal lipid-containing Shh proteins. Explants shown in C-N, and treated with myristoylated ShhN (mShhN, not shown), were measured for Dlx density (fluorescent intensity×area of fluorescent signal×10^6) using Open Lab software. Standard deviation measurements are shown above each bar.

multimerization and differentiation-inducing activity, we compared the differentiation-inducing activities of the multimeric forms of ShhN, wtShh and C24S-Shh with monomeric ShhN. M multimeric ShhN is highly active at 6 nM (G), whereas M multimeric wtShh (E), M multimeric C24S-Shh (F) and monomeric ShhN (G) activities are not detected (Fig. 4). These data show that the presence of monomers is not required for activity. In addition, these data suggest that multimerization may be required for differentiation-inducing activity. However, in the absence of the N-lipid, multimerization is not sufficient for differentiation-inducing activity.

Shh containing the N- or C-terminal lipid ventralizes the zebrafish forebrain in vivo

We have previously determined that the N-lipid is required for ventralizing the mouse forebrain in vivo (Kohtz et al., 2001), at the same time showing that the C-lipid cannot substitute for the N-lipid in this context. In this paper, we show that the inability of the C-lipid to substitute for the N-lipid results from protein inactivity in rat forebrain explants assays. In contrast to the mouse forebrain, in the absence of the N-lipid, the C-terminal lipid-containing Shh is sufficient to induce mouse digit duplications, but the C-lipid does not substitute for the N-lipid in the fly wing (Lee et al., 2001). Taken together, these data suggest that the relative roles of the N- and C-lipids are both context and species dependent. In order to determine the degree of conservation of the relative roles of the N- and C-lipids in the developing forebrain, we compared the activities of different N- and C-terminal lipid-containing Shh proteins in the zebrafish forebrain (Fig. 5). As previously shown, the expression of the ventrally expressed Shh-responsive genes *nk2.2* and *nk2.1b* was expanded dorsally in the zebrafish

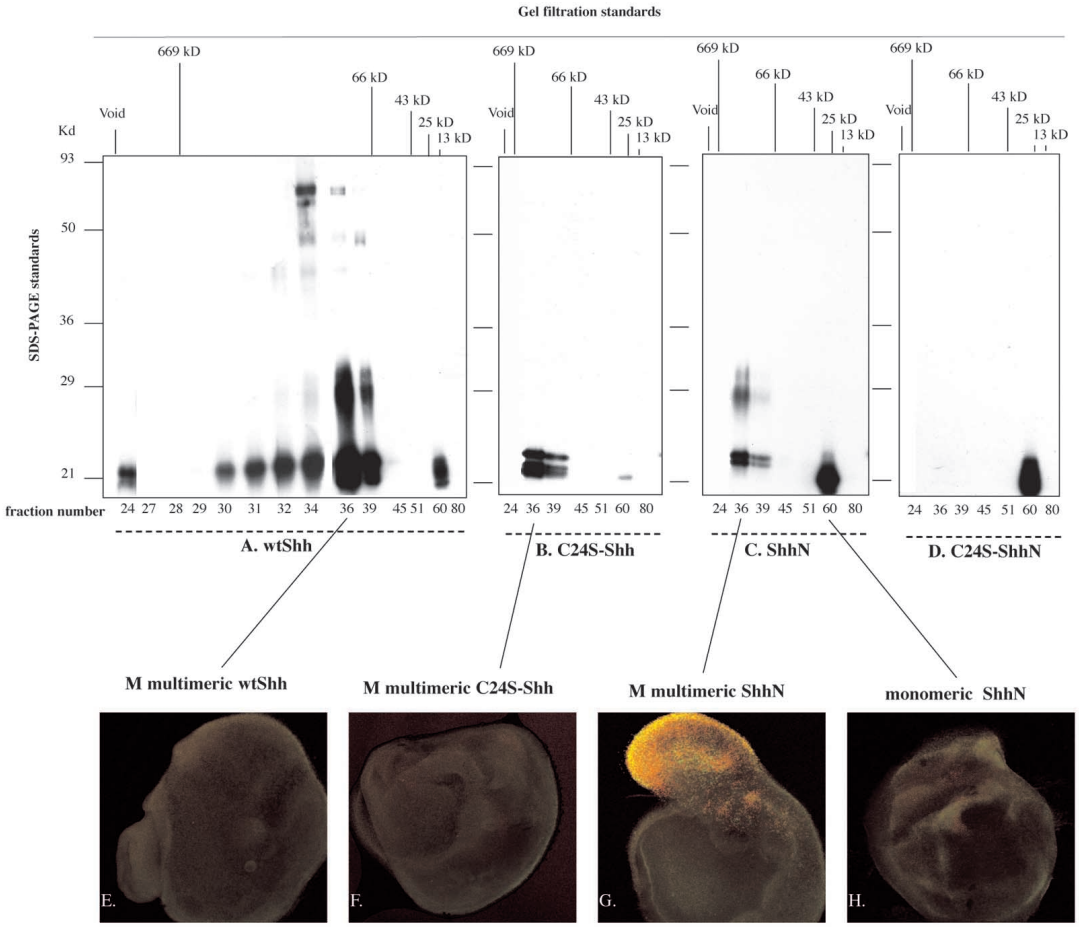

Fig. 4. Multimerization is required but not sufficient for Shh early striatal neuronal differentiation-inducing activity. Gel filtration of Shh allows the classification of Shh multimers into three distinct size groups: >669 kDa (L, large), 669-69 kDa (M, medium), and monomers. Different N- and C-terminal lipid-containing proteins from cell culture supernatants derived from C17 cell lines (Fig. 3A) were affinity purified on a 5E1 anti-Shh antibody column. Affinity-purified proteins were separated by Superdex 200 gel filtration column chromatography (AKTA FPLC). Fractions eluted from the column were separated by SDS-PAGE, and probed with anti-Shh antibody by western analysis. (A) wtShh (N+C lipid), (B) C24S-Shh (C lipid alone), (C) ShhN (N lipid alone), (D) C24S-ShhN (no lipid). The elution profile of standard proteins on the Superdex column (Pharmacia) is indicated at the top of each gel. The fraction number is indicated at the bottom of each lane. SDS-PAGE standards are indicated on the left. (E-H) A comparison of the early striatal neuronal differentiation-inducing activities of multimeric wtShh, multimeric C24S-Shh, multimeric ShhN and monomeric ShhN. Rat E11 telencephalic explants were treated with multimeric wtShh (E; fractions 37 and 38; 8/8 explants lacked Dlx- or Islet1/2-expressing cells), multimeric C24S-Shh (F; fractions 37 and 38; 7/8 lacked Dlx- or Islet1/2-expressing cells), multimeric ShhN (G; fractions 37 and 38; 6/7 explants contained Dlx- and Islet1/2-expressing cells) or monomeric ShhN (H; fractions 59 and 61; 6/7 explants lacked Dlx- or Islet1/2-expressing cells). Anti-Dlx was detected with an anti-rabbit Cy2-conjugated antibody (green), and anti-Islet1/2 was detected with an anti-mouse Cy3-conjugated antibody (red).

forebrain upon injection of RNA encoding wild-type Shh (Fig. 5) (Karlstrom et al., 2003; Rohr et al., 2001). Consistent with ventralization of the CNS, the dorsally expressed genes *emx1* and *pax6* were reduced in injected embryos (Fig. 5) (Macdonald et al., 1994; Morita et al., 1995). The ventralizing activities of constructs encoding the different N- and C-lipid Shh proteins are classified as mild, moderate or major, based on (1) the expansion of the ventral forebrain markers *nk2.1b* (Fig. 5A,D,G) and *nk2.2* (Fig. 5B,E,H), and (2) the reduction of the dorsal markers *pax6* and *emx1* (Fig. 5C,F,I). A comparison of the percentage of the embryos exhibiting different ventralizing activities reveals that N+C-terminal lipid-containing Shh and Shh containing N-terminal lipid alone are similar in activity (Fig. 5M). Although Shh containing the C-lipid alone is less active than N+C or N-lipid Shh, it retains significant ventralizing activity when compared with Shh lacking both lipids (C24S-ShhN) or uninjected controls. These data suggest that either the N- or the C-terminal lipid is necessary for zebrafish ventralizing activity (Fig. 5M).

raises the possibility that L multimers may be crucial to long-range signaling. In addition, although multimerization has been proposed as a mechanism for long-range signaling (Zeng et al., 2001), we show that it is required for soluble ShhN activity in vitro, and therefore for activity in forebrain neural explant assays.

Our results confirm previous reports that the C-terminal lipid tethers Shh protein to the surface of the secreting cell (Porter et al., 1996b). Comparison of the relative levels of cell-associated and secreted C-terminal lipid, and N+C-terminal lipid-containing Shh proteins, suggests that the N-terminal lipid increases cell association by 44% in the presence of the C-terminal lipid. Thus, the N- and C-terminal lipids act synergistically to maintain cell association. This synergistic interaction is slightly different from that involved in multimerization. Formation of the L multimer requires the presence of both N- and C-lipids. However, neither lipid alone is sufficient for the formation of the L multimer. The formation of the M multimer is not synergistic and occurs in the presence of either the N- or the C-lipid alone. Finally, the antagonistic

Discussion

In this paper, we compare the relative roles of the N- and C-terminal lipids in Shh tethering, activity and multimerization, using different N- and C-terminal lipid-containing Shh proteins. Multiple lines of evidence show that different proteins result from the different transfected Shh cDNA constructs. Specifically, the four proteins differ in the following characteristics: (1) migration during SDS-PAGE; (2) membrane association; (3) activity in the E11 forebrain neural explant assay and zebrafish forebrain; and (4) profiles of multimerization. These data suggest that the N- and C-terminal lipids play synergistic and antagonistic roles in the structure and function of the Shh protein.

Significance of multimerization and synergism between the N- and C-terminal lipids

It is known that multimerization is involved in regulating the action of diverse groups of proteins both in the extracellular and intracellular compartments of the cell. In this study, we find that Shh forms different sized multimers depending upon the presence of N- and/or C-terminal lipids. The ability of wtShh, but not ShhN to participate in long-range signaling in the limb (Lewis et al., 2001), and the ability of the former, but not the latter, to participate in L multimer formation,

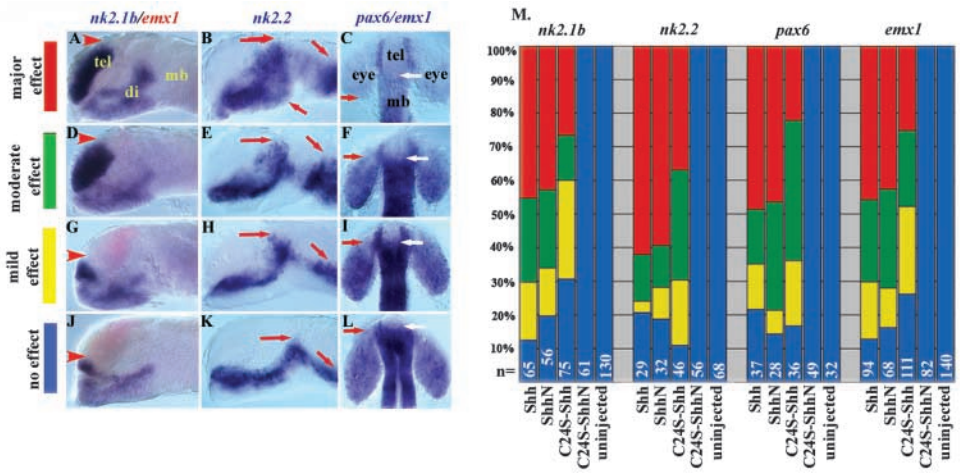

Fig. 5. Shh ventralizing activity in the zebrafish forebrain requires the N- or C-terminal lipid. Zebrafish embryos were injected at the two- to four-cell stage with 75 pg of in vitro synthesized mRNA encoding different lipid modifiable forms of Shh. Changes in expression of different Shh responsive genes were assayed at 20 hours by in situ hybridization. (A,D,G,J) *nk2.1b* (blue) and *emx1* (red) double labeling, lateral views of brain, eyes removed. (A,D,G) Expression of different N- and C-terminal lipid-containing forms of Shh leads to variable expansion of *nk2.1b* into the dorsal telencephalon and the simultaneous reduction of dorsal *emx1* expression (arrowheads show the border of *nk2.1b* and *emx1* expression). The effects of injections were categorized as mild, moderate or major, based on the degree of *nk2.1b* expansion and *emx1* reduction (red arrowheads). (J) In uninjected embryos, *nk2.1b* is expressed in a small band in the ventral telencephalon (red arrow), as well as in the underlying diencephalon. *Emx1* expression in the dorsal telencephalon is complementary to that of *nk2.1b*. (B,E,H,K) *nk2.2* expression, lateral views of brain, eyes removed. (B,E,H) Examples of variable expansion of *nk2.2* expression (arrowheads indicate the extent of expansion of the *nk2.2* expression domain). (K) In uninjected embryos, *nk2.2* is expressed in the dorsal diencephalon and in the ventral midbrain, hindbrain and spinal cord (data not shown). (C,F,I,L) *pax6* and *emx1* expression, dorsal views of the head. (C,F,I) Examples of reduced *pax6* (red arrows) and *emx1* (white arrows) expression. (L) In uninjected embryos, *pax6* is expressed in the eyes, while *emx1* expression is seen in the telencephalon between the eyes. (M) Graph showing the percentage of embryos with differently affected gene expression patterns after expression of different Shh N- and C-terminal lipid-containing forms. Gene expression is shown at the top, numbers of embryos assayed in each experiment is shown in each bar. Blue, normal gene expression; yellow, mild ventralization; green, moderate ventralization; red, major ventralization; Shh, wild-type Sonic Hedgehog; ShhN, Shh lacking the C-terminal lipid; C24S-Shh, Shh lacking the N-terminal lipid; C24S-ShhN; Shh lacking both lipids; di, diencephalon; mb, midbrain; tel, telencephalon.

effect of the C-terminal lipid on the N-terminal lipid-mediated enhancement of differentiation-inducing activity clearly identifies a distinct role for the C-lipid.

Significance of the C-terminal lipid-mediated reduction in early striatal neuronal differentiation-inducing activity

The lack of activity of C24S-ShhN (no lipids) and the high activity of ShhN (N-terminal lipid alone) confirms our previous data that the N-terminal lipid enhances early striatal neuronal differentiation-inducing activity. However, we obtained a surprising result in that the presence of the C-terminal lipid reduces the differentiation-inducing activity of N-terminal lipid-containing Shh. This differs from the findings of Zeng et al. who report that the C-lipid increases Shh activity by 15-fold in a C3H10T1/2 cell line-based assay (Zeng et al., 2001). Based on their data, Zeng et al. propose that a highly active, large multimeric form of Shh is secreted and functions in long-range signaling (Zeng et al., 2001). A long-range signaling form that is more active than the short-range signaling form suggests that some mechanism of inactivation must be present in order to prevent activity during transport. This inactivation mechanism would presumably not affect the short-range signaling form of Shh. The data in this paper suggests that the long-range signaling form of Shh is likely to be less active than the short-range signaling form. Therefore, some mechanism of activation may be present once the long-range signaling form has reached its target. It will be important to determine whether activation/inactivation mechanisms are involved during this process.

Support for the fact that ShhN is more potent in vivo than wtShh has been reported by Lewis et al. (Lewis et al., 2001), who show that the short-range signaling activities in the limb of N-Shh/Shh^{n} mice are enhanced compared with wtShh/+. Our data suggests that the increased short-range signaling activity of N-Shh/Shh^{n} results not only from greater activity, but also from increased release from the membrane. Lewis et al. also report that expression of ShhN in a wild-type background (N-Shh/+) results in expanded long-range Shh signaling that is inconsistent with the loss of long-range activity in the absence of the C-lipid (Lewis et al., 2001). Given our multimerization and activity data indicating that both wtShh and ShhN are able to form multimers, it is possible that in the N-Shh/+ mice, multimers consisting of both wtShh and ShhN are formed. Thus, it is possible that such hybrid multimers would not only be able to be transported, but also exhibit greater activity once at their target. Our data showing that wtShh (N+C lipid) is less potent than ShhN (N-lipid) in differentiation-inducing activity in vitro, together with the in vivo data of Lewis et al. (Lewis et al., 2001), support the intriguing possibility that the C-terminal lipid may function to keep Shh in an inactive state during long-range signaling. We propose a model in which long-range signaling of Shh is mediated by inactive N+C-terminal lipid-containing Shh multimers that are converted to an active form upon reaching their long-range target cell (see Fig. 6). Because of the small fraction of L-multimeric wtShh, we were unable to purify sufficient amounts to assay in the neural explant assay. Therefore, we cannot say whether the L-multimer is active or inactive. This is reflected in the model, where a question mark

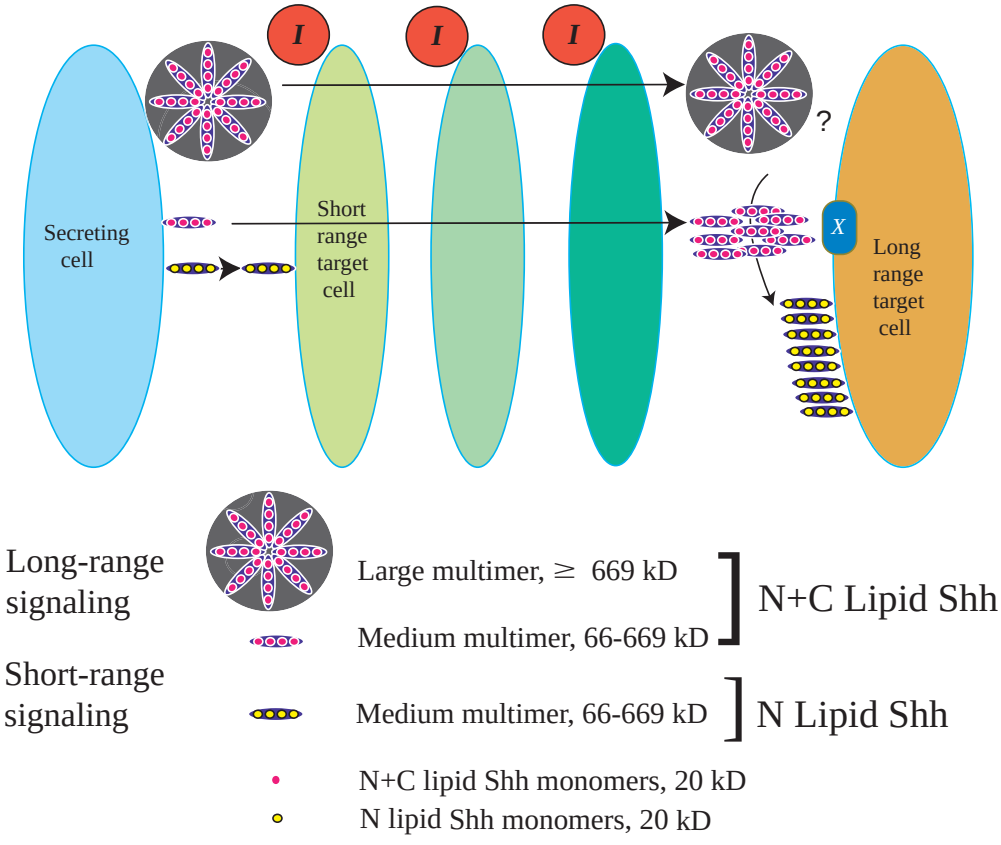

Fig. 6. Model proposing that the C-lipid mediates long-range signaling of wtShh by facilitating both multimerization and inactivity. Different N- and C-terminal lipid-containing Shh multimers are secreted in the ventral forebrain. The C-terminal lipid-containing multimers exhibit very little differentiation-inducing activity during short-range signaling. During long-range signaling, we propose that the C-lipid enables Shh to form L multimers required for transport, and at the same time maintains Shh in an inactive state, either by rendering it susceptible to an inhibitor (I) or by necessitating activation by an activator (X). One possible mechanism of activation is shown, in which removal of the C-lipid from N+C lipid-containing L and/or M multimers results in the generation of highly active N-lipid M multimers.

has been placed next to the L-multimer. It will be important to determine whether the L-multimer undergoes an activation or inhibition mechanism.

Do different N- and C-terminal lipid-containing Shh proteins function in the embryo?

Our data suggests that the N- and C-terminal lipids have distinct effects on Shh structure, and can act either synergistically or antagonistically to modify Shh function. However, how these different N- and C-terminal lipid-containing forms of Shh participate in long and short-range signaling within a specific region of the embryo, or whether all the functions of Shh are performed by fully modified Shh (N+C lipid containing) requires further investigation. Mass spectroscopic analysis by Pepinsky et al. suggested that mammalian 293 cells transfected with full-length *Shh* cDNA (wtShh) produce three different lipid-containing Shh proteins: no lipid (9%), C lipid (61%), and N+C lipid (30%) (Pepinsky et al., 1998). Given the fact that Shh proteins produced in insect cells (Pepinsky et al., 1998), mammalian non-neural 293 cells (Pepinsky et al., 1998; Zeng et al., 2001) and mammalian neural cells (this paper) all have different properties, it is possible that Shh function could be regulated by changes in the ratios of Shh isoforms in a context-dependent manner in vivo.

In this paper, we clearly show that Shh proteins with different biochemical properties are present in embryonic brain and limb tissues. Differences in migration in SDS-PAGE, in the formation of SDS-PAGE-resistant multimers, and in solubility strongly support the hypothesis that, within different tissues, Shh proteins contain different N- and/or C-terminal lipids. The difference in the proportions of Shh protein forms in the brain and limb may influence the distance that Shh is transported across embryonic tissue, and/or may regulate the type of extracellular matrix components through which Shh travels in mesenchymal versus epithelial tissues. The relatively normal patterning observed in hair, whiskers, teeth and lung development in mice lacking the C-terminal lipid (Lewis et al., 2001) contrasts with the severe defects in the limb and brain, further supporting the idea that context determines the requirement for different lipid-modified Shh proteins.

Another factor that may influence the ratio of different lipid-containing Shh proteins is the accessibility of the target tissue to secreted Shh. For instance, although the prechordal plate supplies Shh in the forebrain initially, as development proceeds, neural sources of Shh arise. Whether these neural sources arise in conjunction with a loss of the initial prechordal plate source remains to be determined. As the source of Shh changes from non-neural to neural tissue, the need for a long-range multimerized form of Shh may also change. Therefore, it is possible that the requirement for different ratios of lipid-containing Shh proteins may depend on the physical and structural relationship between any given source of Shh and its target. Clearly, our techniques for determining the ratios of Shh proteins do not distinguish between those Shh proteins that remain close to the source in secreting cells and those that travel further from the source. Understanding the precise locations of the different Shh forms may also be especially important in the limb, where the source of Shh expands with the outgrowth of the progress zone. This change may require a different complement of Shh proteins than those needed in regions where this relationship is static.

A major question raised by these experiments is do different N- and C-terminal lipid-containing Shh proteins result from the removal of existing lipids or the failure to add a lipid? The discovery of an enzyme responsible for the addition of the N-terminal lipid to fly *hh* (Lee et al., 2001; Chamoun et al., 2001; Micchelli et al., 2002) makes it possible to study whether regulation of the addition of the N-terminal lipid occurs during development. Although enzymes capable of removing a thiol-linked N-terminal palmitate have been described (Camp and Hofmann, 1993; Camp et al., 1994), enzymes capable of removing an amide-linked palmitate have not. Because the N-terminal palmitate on Shh is thought to be amide-linked (Pepinsky et al., 1998), study of the regulation of the removal of the N-terminal lipid will depend on the discovery of such enzymes. As the data in this paper suggests, equally important will be the study of whether regulation of the addition or removal of the C-terminal lipid occurs.

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