

Gli proteins encode context-dependent positive and negative functions: implications for development and disease

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SUMMARY

Several lines of evidence implicate zinc finger proteins of the Gli family in the final steps of Hedgehog signaling in normal development and disease. C-terminally truncated mutant GLI3 proteins are also associated with human syndromes, but it is not clear whether these C-terminally truncated Gli proteins fulfil the same function as full-length ones. Here, structure-function analyses of Gli proteins have been performed using floor plate and neuronal induction assays in frog embryos, as well as induction of alkaline phosphatase (AP) in SHH-responsive mouse C3H10T1/2 (10T1/2) cells. These assays show that C-terminal sequences are required for positive inducing activity and cytoplasmic localization, whereas N-terminal sequences determine dominant negative function and nuclear localization. Analyses of nuclear targeted Gli1 and Gli2 proteins suggest that both activator and dominant negative proteins are modified forms. In embryos and COS cells, tagged *Gli* cDNAs yield C-terminally deleted forms similar to that of Ci. These results thus provide a molecular basis for the human Polydactyly type A and Pallister-Hall Syndrome phenotypes, derived from the deregulated

production of C-terminally truncated GLI3 proteins. Analyses of full-length Gli function in 10T1/2 cells suggest that nuclear localization of activating forms is a regulated event and show that only Gli1 mimics SHH in inducing AP activity. Moreover, full-length Gli3 and all C-terminally truncated forms act antagonistically whereas Gli2 is inactive in this assay. In 10T1/2 cells, protein kinase A (PKA), a known inhibitor of Hh signaling, promotes Gli3 repressor formation and inhibits Gli1 function. Together, these findings suggest a context-dependent functional divergence of Gli protein function, in which a cell represses *Gli3* and activates *Gli1/2* prevents the formation of repressor Gli forms to respond to Shh. Interpretation of Hh signals by Gli proteins therefore appears to involve a fine balance of divergent functions within each and among different Gli proteins, the misregulation of which has profound biological consequences.

Key words: Activator, Floor plate, Neuron, Gli1, Gli2, Gli3, Sonic hedgehog, Repressor

INTRODUCTION

Three *Gli* genes have been described in several vertebrate species (Kinzler et al., 1987, 1988; Ruppert et al., 1988, 1990; Walterhouse et al., 1993; Hui et al., 1994; Marigo et al., 1996; Lee et al., 1997; Marine et al., 1997; Hughes et al., 1997). The first *Gli* gene (hereafter *Gli1*) was isolated from a glioma line (Kinzler et al., 1987) and is an oncogene (Ruppert et al., 1991; Dahmane et al., 1997) possibly harboring a mutation (Hynes et al., 1997). *Gli* genes have different functions in embryonic development (Lee et al., 1997; Mo et al., 1997; Sasaki et al., 1997; Marine et al., 1997; Ruiz i Altaba, 1998; Matise et al., 1998; Ding et al., 1998) and can be transcriptionally (Marigo et al., 1996; Lee et al., 1997; Sasaki et al., 1997; Hynes et al., 1997; Ruiz i Altaba, 1998) and translationally (Jan et al., 1997) regulated. Both *Gli1* (Marigo et al., 1996; Lee et al., 1997; Hynes et al., 1997) and *Gli2* (Ruiz i Altaba, 1998) are targets of Sonic hedgehog (Shh) signaling, whereas Gli3 and Shh show a mutually repressive interaction in the limb (Masuya et

al., 1995; Marigo et al., 1996; Büscher et al., 1997) and neural tube (Ruiz i Altaba, 1998). In addition, Gli2 and Gli3 are likely to have Shh-independent functions as they are expressed in regions that require absence of SHH for proper development, such as the dorsal neural tube (Hui et al., 1993; Lee et al., 1997; Sasaki et al., 1997; Ruiz i Altaba, 1998).

In the early frog neural plate *Gli1* is expressed in midline and immediately adjacent cells close to the underlying Shh-secreting notochord, *Gli2* is expressed throughout the neural plate with the exception of the midline and *Gli3* is also absent from the midline but shows a graded distribution with highest levels laterally (Lee et al., 1997). Only Gli1 can induce ectopic floor plate differentiation (Lee et al., 1997; Sasaki et al., 1997; Hynes et al., 1997; Ruiz i Altaba, 1998), but all three Gli proteins can induce neuronal development (Brewster et al., 1998). However, each Gli protein may induce different sets of neurons (Ruiz i Altaba, 1998), and in mice normal *Gli2* is required for floor plate development (Matise et al., 1998; Ding et al., 1998). These and other results on skeletal patterning (Mo

et al., 1997) indicate that there is functional divergence but also partial redundancy in the *Gli* gene family. Indeed, whereas *Gli1* is most potent in inducing epidermal tumors (Dahmane et al., 1997), both *Gli2* and *Gli3* can mimic this effect albeit at much lower frequency (A. R. A., unpublished data). Together, these results provide support for the idea that the overall 'Gli readout' of a cell is instructive in determining its fate (Ruiz i Altaba, 1997). However, it remains unknown how Gli proteins act, especially since they, like Ci in *Drosophila*, could yield different forms with varying activities: Ci is cleaved to yield a nuclear repressor lacking most of the C-terminal sequences (Aza-Blanc et al., 1997), as the activation and cytoplasmic anchoring domains of Ci are found in the C terminus (Alexandre et al., 1996; Hepker et al., 1997; von Ohlen et al., 1997; Aza-Blanc et al., 1997).

Here, the possibility that Gli proteins encode positive and negative functions is analyzed using in vivo floor plate and neuronal induction assays as well as in vitro induction of AP activity in SHH-responsive mouse 10T1/2 cells. The results show that the N-terminal region encodes repressor function whereas C-terminal regions are required for positive inducing activity. C-terminally deleted forms are nuclear. Activating constructs, in contrast, can be mostly cytoplasmic. Analyses of transfected Gli proteins in COS-7 cells shows that these appear to be processed yielding specific shorter forms. NLS-tagged Gli proteins appear larger than the largest Gli proteins in SDS-PAGE gels, raising the possibility that mature activating Gli forms are modified. Analyses of Gli protein localization and structure in 10T1/2 cells show that *Gli2* and *Gli3* full-length or near full-length forms are stable and nuclear. Thus, whereas the behavior of endogenous proteins in different tissues remains to be determined, the results presented here show that Gli proteins can encode opposing functions. These results also suggest that modification and active nuclear accumulation of activating forms on the one hand, and formation of C-terminally truncated nuclear repressors on the other, are critical regulatory steps. Analysis of the effects of altering PKA activity, a known inhibitor of Hh signaling, shows that it promotes formation of the repressor form of *Gli3* and inhibits induction of alkaline phosphatase by *Gli1* without promoting the formation of a putative *Gli1* repressor. The importance of repressor derivatives is highlighted by their similarity with two C-terminally deleted mutant forms of human *GLI3* (Kang et al., 1997; Radhakrishna et al., 1997). The dominant nature of these mutations in heterozygous patients is proposed to derive from the ability of C-terminally truncated *GLI3* forms to inhibit activating function from the normal allele. In addition, these truncated *GLI3* genes are proposed to inhibit the activating functions of all coexpressed *GLI* genes.

MATERIALS AND METHODS

cDNA clones and synthetic RNAs

Full-length frog *Gli1*, frog *Gli2* and human *Gli3* cDNAs in pCS2-MT were as described (Lee et al., 1997; Brewster et al., 1998). pCS2-MT provides in-frame N-terminal Myc-epitope tags in the N terminus (Turner and Weintraub, 1994). Deletion constructs were as described in Fig. 2 with the enzyme in the name being used to generate the specified deletions. The constitutively active and dominant negative PKA subunits were as described (Orellana and McKnight, 1992; Clegg et al., 1987) and were subcloned into pcDNA1-Amp

(Invitrogen). Synthetic RNAs for microinjection were synthesized by linearizing the appropriate cDNA clones with *NotI* and transcribing with *SP6* RNA polymerase for pCS2-MT clones. Transcriptions were performed with the Ambion kit at 30°C with a trace of [γ - 32 P]UTP to measure incorporation.

Microinjection, transfection and alkaline phosphatase histochemistry

To test for production of distinct Gli products and subcellular localization, plasmid DNAs were microinjected into developing frog embryos at 200 pg/embryo/10 nl in water except in coinjection experiments, in which 100 pg of each pDNA were injected. To test for function, synthetic RNAs were microinjected at 2 ng/embryo/10 nl unless otherwise stated. For coinjection experiments, each RNA was at 1 ng/embryo. Single injection controls for coinjection experiments were at 100 pg pDNA or 1 ng RNA in order to maintain the same amount of active nucleic acid. Transfection of plasmid DNA into COS-7 or C3H10T1/2 cells (ATCC) was performed with Lipofectamine (Gibco-BRL) following standard procedures. Cells were assayed 48 hours after transfection and were fed with complete medium after the first 24 hours. For alkaline phosphatase detection, transfected and control cells were fixed at room temperature for 1 minute with 3.7% formaldehyde and processed according to Katagiri et al. (1994).

Immunocytochemistry and whole-mount in situ hybridization

Transfected cells were immunolabeled with an anti-Myc monoclonal antibody (1/500-1/1000; 9E1, Santa Cruz, Inc.) followed by fluorescein-coupled secondary antibodies as described (Lee et al., 1997). Nuclei were counterstained with DAPI (Sigma). Injected embryos to be analyzed for intracellular localization of Gli proteins were processed for whole-mount immunocytochemistry using peroxidase-coupled secondary antibodies and reaction with diaminobenzidine. For viewing, the labeled embryos were squashed under a coverslip in benzyl alcohol-benzyl benzoate and photographed under Nomarski optics. All pictures were taken with an Axiophot microscope (Zeiss). Floor plate cells were identified by whole-mount immunoreactivity with polyclonal anti-HNF-3 β antibodies as previously described (Ruiz i Altaba et al., 1995; Lee et al., 1997) (1/8000). Whole-mount in situ hybridization was performed using maleic acid. Digoxigenin-labeled anti-sense RNA probes to *N-tubulin* were made by transcribing a cDNA clone with T3 after digestion with *Bam*HI. Hybridization was revealed with anti-digoxigenin alkaline-phosphatase-coupled antibodies in the presence of NBT and BCIP (BRL). After in situ hybridization, embryos were processed for whole-mount immunocytochemistry to reveal the localization of the injected Myc-tagged proteins using anti-Myc antibodies and peroxidase-coupled secondary antibodies. Embryos were not cleared before viewing and pictures were taken with an M10 binocular microscope (Leica).

Immunoprecipitation and immunoblotting

Injected embryos were harvested at early gastrula stages (stage 11-12) and homogenized (100 μ l/embryo) in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 7.5) by vortexing. 10T1/2 cells were transfected in 10 cm dishes and cells collected in RIPA buffer 24-48 hours later. RIPA buffer contained a cocktail of protease inhibitors including PMSF (Boehringer Mannheim). Insoluble material was spun out in a table top microcentrifuge at top speed for 10 minutes at 4°C. The resulting embryo supernatant was incubated with anti-Myc antibodies (0.2 μ g IgG/embryo) at 4°C for 1 hour with constant shaking. Antigen-antibody complexes were immunoprecipitated by incubating with agarose beads coupled to protein A plus protein G (Santa Cruz) for 1 hour at 4°C with constant shaking. The beads were washed four times with 1.5 ml RIPA buffer with constant shaking for 15 minutes each at 4°C. Pelleted beads were

boiled in sample buffer and the supernatant loaded onto 7.5% SDS gels for PAGE. Total cell lysates were boiled in sample buffer and directly loaded onto SDS gels for PAGE. Electrophoresed proteins were transferred to Protran nitrocellulose membranes by standard procedures. After blocking with non-fat milk, the immunoblots were probed with anti-Myc antibodies (1/1500) and binding was visualized by chemiluminescence with the ECL kit (Amersham).

RESULTS

Subcellular localization of Gli proteins in injected frog embryos: C-terminally deleted Gli proteins are nuclear

In frog gastrulae, injected Gli proteins showed both nuclear and cytoplasmic labeling within single cells (Fig. 1A,D,G; Lee et al., 1997). Gli proteins could therefore be normally modified to yield nuclear forms or selectively retained in the cytoplasm of some cell types. Because in *Drosophila* Ci is cleaved to yield a C-terminally truncated nuclear form (Aza-Blanc et al., 1997), the behavior of C-terminally deleted constructs was tested in early frog embryos.

Embryos injected with any of the Gli protein forms tested showed protein localization to both the nucleus and cytoplasm (Fig. 1A,B), with the exception of Gli1C'ΔPstI, a deletion construct terminating after conserved region 3 (Fig. 2; conserved regions are according to Ruppert et al., 1990), which was exclusively nuclear (Fig. 1C). Nuclear labeling in embryos injected with longer proteins could thus involve the production of smaller C-terminally deleted nuclear forms (Fig. 2). A full-length Gli1 protein having an SV40 NLS sequence in its N terminus also localized to the nucleus (Lee et al., 1997; Fig. 2).

For Gli2, a C-terminal deletion lacking most sequences downstream of region 6, Gli2C'ΔBgIII (Figs 1F, 2), as well as one lacking all sequences downstream of the zinc fingers, Gli2C'Δ, were exclusively nuclear (Fig. 2), like a full-length protein bearing an SV40 NLS (Figs 1E, 2). Sequences required for cytoplasmic localization are thus found closer to the C terminus in Gli2 than in Gli1.

Gli3 also contains cytoplasmic localization sequences in the C-terminal region as Gli3C'ΔClaI was nuclear (Fig. 1I) but Gli3C'ΔSphI showed both cytoplasmic and nuclear labeling (Fig. 2). Nuclear localization of Gli3 required N-terminal sequences as their removal yielded Gli3N'ΔStuI, which was strictly cytoplasmic (Figs 1H, 2).

Floor plate induction by Gli proteins

The functional significance of Gli proteins derived from the engineered constructs was tested in established *in vivo* floor plate and neuronal induction

assays (see Lee et al., 1997; Ruiz i Altaba, 1998; Brewster et al., 1998). Ectopic expression of Gli1 leads to the ectopic differentiation of floor plate cells expressing HNF-3β (Lee et al., 1997; Figs 2, 3, 4B), not observed in control sibling embryos (Figs 2, 3, 4A). Injection of NLSGli1 also resulted in ectopic HNF-3β expression (Lee et al.; Figs 2, 3), as did a C-terminal deletion lacking region 7 (Gli1C'ΔBsaBI, Figs 2, 3). However, floor plate-inducing activity was lost in proteins lacking C-terminal sequences downstream of region 4 (Figs 2, 3). Gli1N'Δ also lacked floor plate-inducing activity (Figs 2, 3). Thus, both N- and C-terminal Gli1 sequences appear to be required for floor plate induction, although an N-terminal deletion of the human glioma protein was cytoplasmic and active in floor plate induction (not shown), possibly due to a putative mutation in the C terminus (Hynes et al., 1997). Neither full-length nor any deletion proteins of Gli2 or Gli3 had ectopic floor plate-inducing activity (Fig. 3; Lee et al., 1997; Ruiz i Altaba, 1998).

Repression of floor plate differentiation

To test whether deletion of C-terminal sequences of Gli1 turned an activator into a dominant negative form of floor plate differentiation, full-length Gli1 was coexpressed with different truncated forms at identical concentrations, and the injected

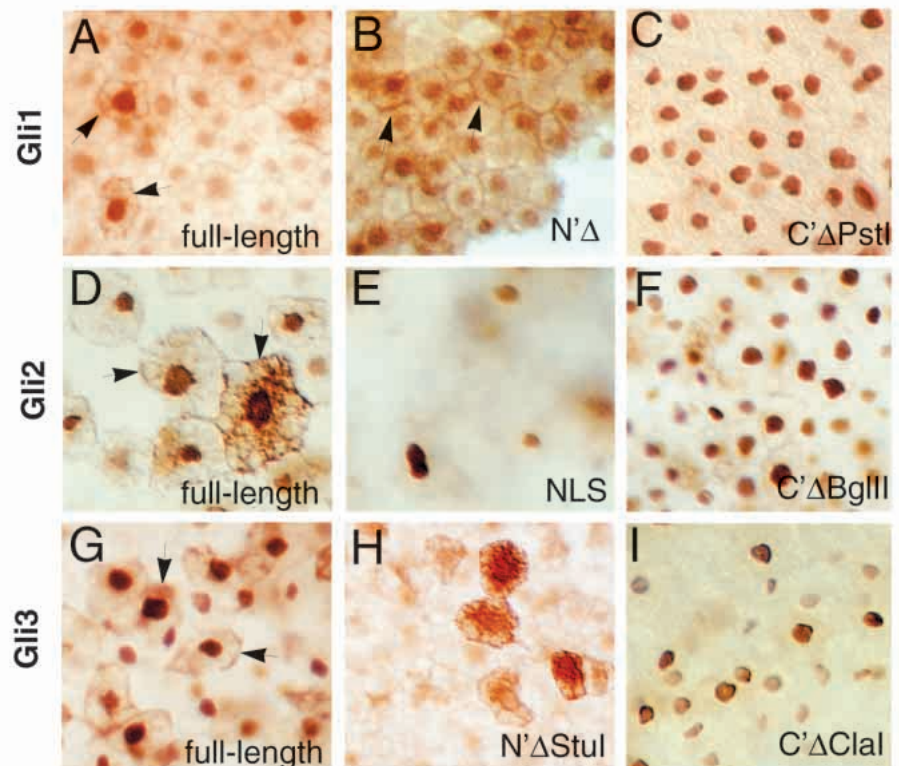
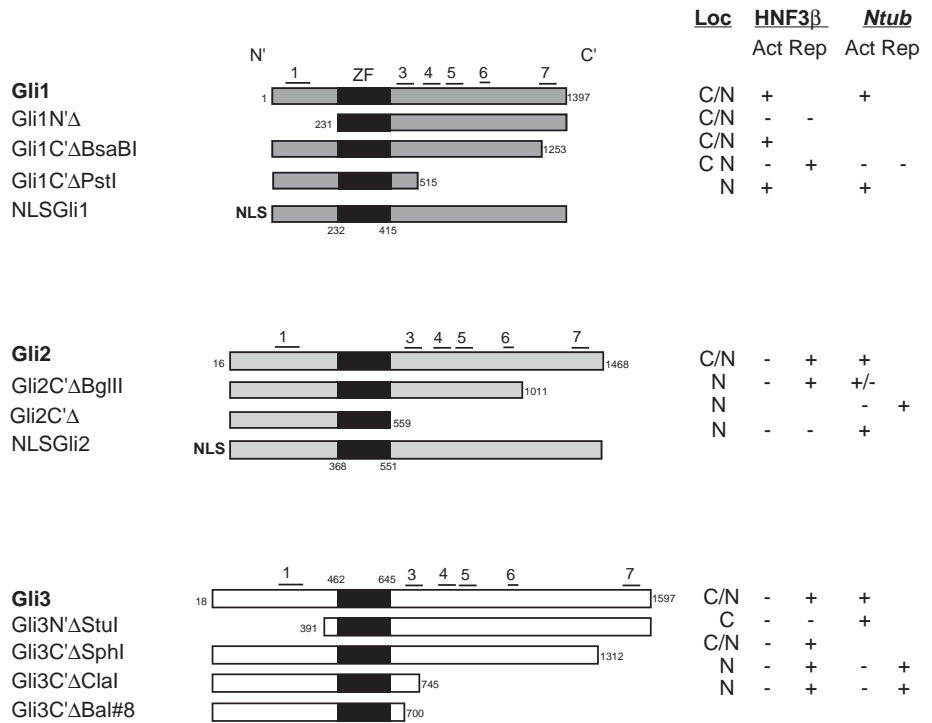


Fig. 1. Intracellular localization of Gli proteins and C-terminally deleted forms.

(A,D,G) Expression and intracellular localization of myc-tagged proteins produced from full-length *Gli1* (A), *Gli2* (D) or *Gli3* (G) cDNAs in injected frog embryos. (B,C) Intracellular localization of N- (B) and C-terminal (C) deletions of Gli1. (E,F) Nuclear localization of an NLS-tagged Gli2 protein (E) and a C-terminal deletion of Gli2 (F). (H,I) Intracellular localization of N- (H) and C-terminal (I) deletions of Gli3. The deletion constructs used are noted in each panel and are according to Fig. 2. Arrows denote the cytoskeletal-like distribution of Gli proteins in the cytoplasm or membrane. Labeling is in ectodermal cells.

Fig. 2. Diagram of full-length and deletion forms of Gli proteins and summary of intracellular localization and functional assays. The name of the constructs is given to the left. The bar diagrams show the structure of the different Gli proteins in the middle. The zinc finger domain is shown in black and all proteins are aligned according to this domain. Sizes and sites of truncation are indicated by amino acid numbers. Conserved domains are depicted above the protein bar diagrams as dashes with their appropriate number according to Ruppert et al. (1990). Region 2 (not shown) includes the zinc finger domain. Localization (Loc) of protein products is also indicated as nuclear (N) and/or cytoplasmic (C). Presence (+) or absence (-) of activity in activation (Act) or repression (Rep) of HNF-3 β or *N-tubulin* (*N-tub*) of the different constructs is also shown in the right columns. Absence of a symbol means that the experiments were not done.



tadpoles assayed for repression of ectopic HNF-3 β expression within the neural tube. Expression of Gli1C' Δ PstI inhibited ectopic HNF-3 β expression by coexpressed full-length Gli1 (Figs 2, 3). Because Gli1N' Δ failed to inhibit ectopic floor plate induction by coexpressed full-length Gli1 (Figs 2, 3), the N terminus of Gli1 is required for dominant negative activity. Gli1C' Δ PstI, however, was unable to inhibit endogenous floor plate differentiation when targeted alone to the ventral midline of early embryos (not shown).

Full-length Gli2 or Gli3 repressed floor plate induction by full-length Gli1 coexpressed at the same concentrations (Figs 2, 3; Ruiz i Altaba, 1998). Coexpression of the C-terminal deletion Gli2C' Δ BgIII also repressed Gli1 function (Figs 2, 3). However, a nuclear targeted NLSGli2 protein failed to inhibit Gli1 function (Figs 2, 3). Analysis of Gli3 also showed that repressor function is encoded in the N terminus. All C-terminal deletions, but not an N-terminal deletion, of Gli3 repressed floor-plate-inducing function of Gli1 (Figs 2, 3).

Neuronal induction by Gli proteins

Induction of *N-tubulin*⁺ primary neurons by Gli proteins (Fig. 4C,E; Brewster et al., 1998) was then used as an additional test to dissect positive and dominant negative functions in Gli2 and Gli3. Like full-length Gli2 (Figs 2, 3, 4C), NLSGli2 induced ectopic neuronal differentiation (Figs 2, 3, 4D). Expression of Gli2C' Δ BgIII also resulted in neuronal induction (Figs 2, 3). A Gli2 protein lacking all C-terminal sequences, Gli2C' Δ , lacked neuronal-inducing function (Figs 2, 3). Analyses of Gli3 clearly showed also that C-terminal sequences are required for neuronal induction as Gli3C' Δ ClaI and Gli3C' Δ Bal#8 failed to induce neuronal differentiation (Figs 2, 3). Moreover, N-terminal sequences, which are required for repression of floor plate induction by coinjected Gli1, are not required for neuronal induction, as an N-terminal deletion of Gli3,

Gli3N' Δ StuI, induced neuronal differentiation (Figs 2, 3, 4F). As with Gli2 or Gli3, deletion of C-terminal sequences of Gli1, yielding Gli1C' Δ PstI, also resulted in the loss of neuronal-inducing activity (Figs 2, 3, 4G). Induction of neuronal differentiation by all three Gli proteins, thus appears to involve proteins containing C-terminal sequences.

Neuronal repression by C-terminally truncated forms of Gli2 and Gli3

To test for the existence of a dominant negative effect on neuronal differentiation, Gli proteins lacking all or most of the C terminus were used, as these approximate the structure of Gli1C' Δ PstI as well as the repressor form of Ci and the human GLI3 mutant proteins (Fig. 2). Gli1C' Δ PstI lacked dominant repressor activity of ectopic neuronal induction by coexpressed Gli3 (Figs 2, 3). In contrast, a similar Gli3 C-terminal truncation, Gli3C' Δ ClaI, acted as a dominant repressor of ectopic neuronal induction by full-length Gli3 or Gli2 coexpressed at the same concentrations (Figs 2, 3, 4H). The same result was obtained for a Gli3 protein lacking almost all C-terminal sequences, Gli3C' Δ Bal#8, and a Gli2 protein lacking all C-terminal sequences downstream of the zinc finger domain, Gli2C' Δ (Figs 2, 3). Moreover, Gli1C' Δ PstI did not interfere with endogenous neuronal differentiation (Figs 2, 3, 4G), whereas Gli3C' Δ ClaI completely inhibited endogenous and ectopic primary neuronal differentiation (Figs 2, 3, 4H). Gli3 and Gli2 thus can encode dominant negative forms for neuronal differentiation.

Gli proteins from injected full-length cDNAs in frog embryos

The nuclear localization of C-terminally deleted Gli repressors raised the possibility that the nuclear localization of Gli proteins in injected embryos (Fig. 1) could be due to

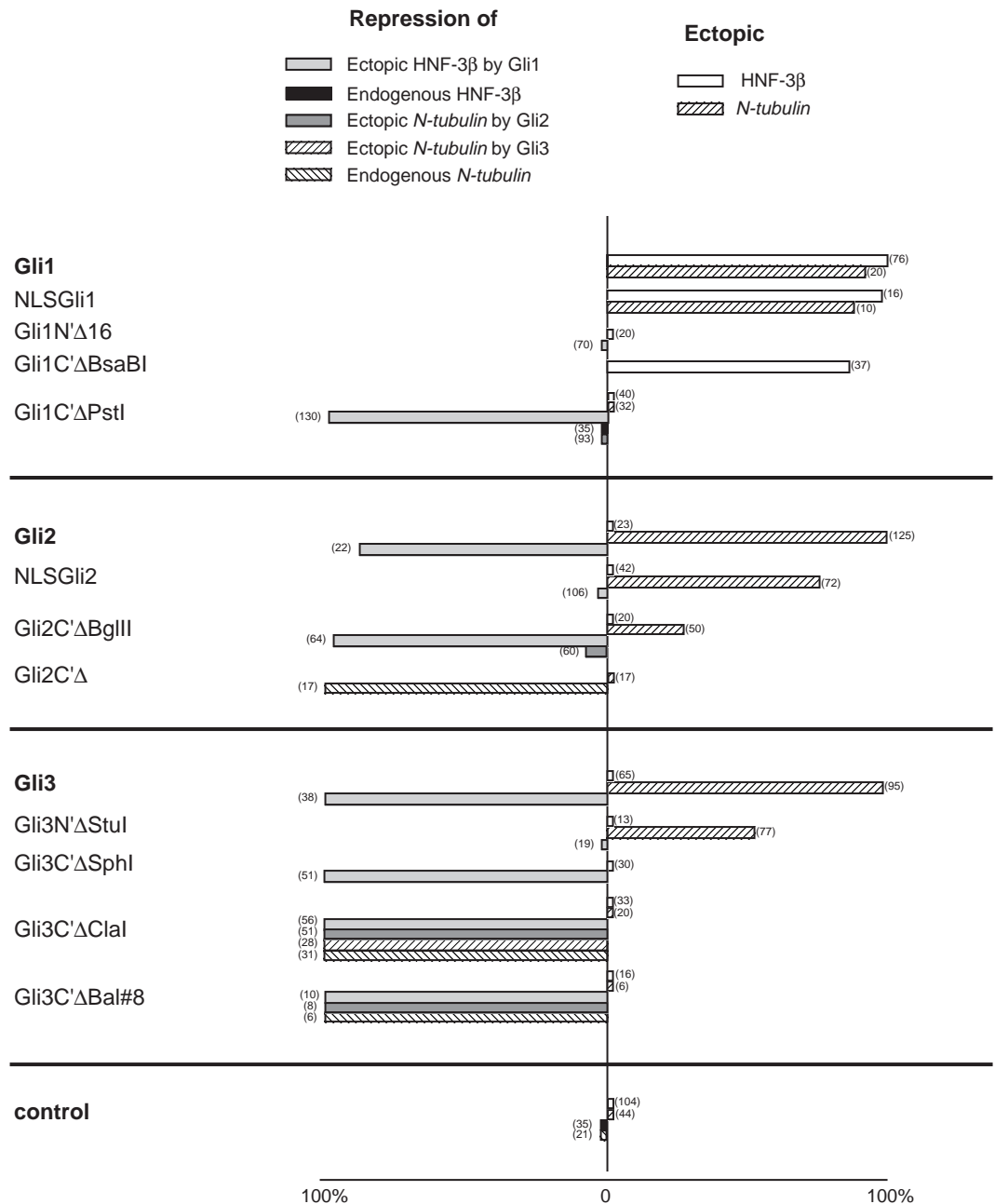


Fig. 3. Quantitation of results of functional assays. Injected embryos analyzed in assays depicted on the top were scored and the results are presented as percentages in horizontal histograms. The total number of embryos assayed in each case is given in brackets next to the histogram. Activation assays are shown to the right and repression assays are shown to the left. The injected RNAs are shown on the left column. Control embryos are uninjected embryos.

the production of C-terminally truncated dominant negative forms in vivo. The proteins made from the injected full-length cDNAs were then analyzed by SDS-PAGE after immunoprecipitation (IP) and visualized by immunoblotting (IB), using antibodies against the N-terminal Myc tags. Injection of full-length Gli1, Gli2 or Gli3 yielded full-length products at gastrula stages that migrated in the predicted anomalous manner (Fig. 5), for example, Gli3 is approximately 1597 aa in length but migrates at >190 kDa (Fig. 5; Ruppert et al., 1990). Discrete smaller bands were also consistently observed that do not arise from differential splicing as northern analyses detected only one or two transcripts for each gene (A. Chokas and A. R. A., not shown; Kinzler et al., 1987; Ruppert et al., 1990; Hui et al., 1993). Measuring the length of these products from the N-terminal

tag determined the approximate site of their C-terminal end, taking into account the tags themselves. However, due to the abnormal migration of Gli proteins, C-terminally deleted constructs of known size with identical tags were used to measure the approximate length of the forms produced (Fig. 2). In addition to proteins considered to be full length, smaller bands were observed for Gli1, Gli2 and Gli3. Gli1C'ΔBsaBI resembles a Gli1 form of approx. 1100 aa, suggesting that this could have activating function. Gli1C'ΔPstI resembles a Gli1 form of approx. 540 aa and Gli3C'ΔClaI resembles a Gli3 form of approx. 760 aa, suggesting that these small forms of Gli1 and Gli3 could have dominant negative activity. A Gli2 form of approx. 860 aa is smaller than Gli2'ΔBglII but larger than Gli2C'Δ, suggesting that it could also act in a dominant negative manner.

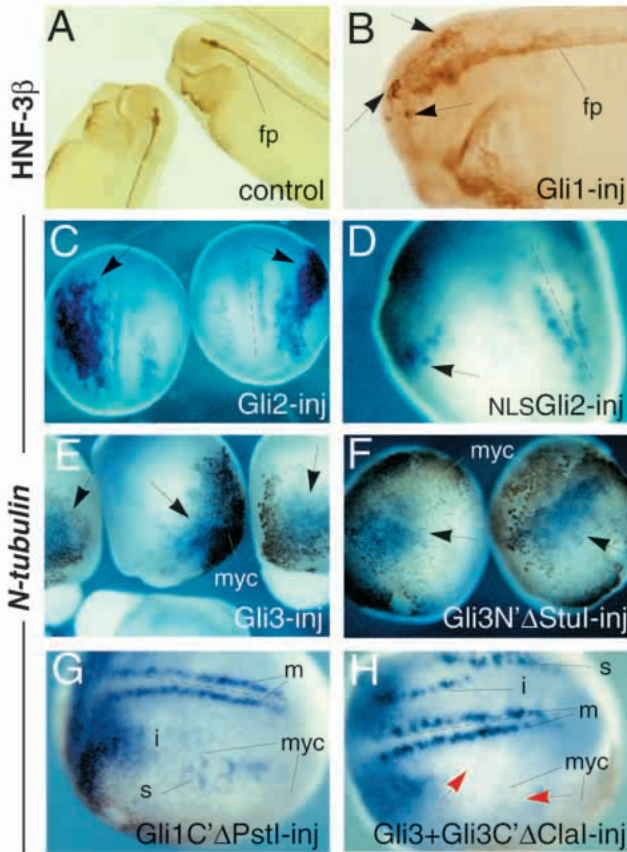


Fig. 4. Floor plate and neuronal induction assays. Normal (A) and ectopic (B) expression of HNF-3 β protein in control uninjected (A) and Gli1-injected (B) approx. stage 34 tadpoles. The embryo shown in B is an example of the floor-plate-induction assay used in this study (see Lee et al., 1997; Ruiz i Altaba, 1998). Arrows in B point to sites of ectopic expression anterior and dorsal to the floor plate (fp). The panels show side views of cleared whole-mount-labeled specimens with anterior to the top in A and to the left in B. (C-F) Ectopic neurogenesis marked by the ectopic expression of *N-tubulin* in neurulae (approx. stage 14) injected unilaterally with Gli2 (C), NLSGli2 (D), Gli3 (E) of an N-terminal deletion of Gli3. In (F), *N-tub* expression is restricted ventrally. (C) Dorsal view with anterior end to the top. Endogenous labeling in primary neuronal stripes is evident in the non-injected sides. (D,E) Dorso-lateral views with anterior end to the top. (F) Ventral views. Arrows point to sites of ectopic expression and the myc label depicts the localization of Myc-tagged Gli proteins, coincident with the sites of ectopic *N-tub* expression. (G,H) Gli3C' Δ Cla (H), but not Gli1C' Δ PstI (G), inhibits ectopic and endogenous neurogenesis. The region inheriting the injected protein is labeled (myc) and arrows in H point to absence of *N-tub* expression in the injected side. The motor neuron (m), interneuron (i) and sensory neuron (s) stripes are identified. Dashed lines depict axes of bilateral symmetry.

Injection of NLSGli2 into developing embryos resulted in the production of a protein that migrated slower in SDS-PAGE than the largest protein detected in Gli2-injected embryos. All shorter forms were rare and non-specific and appeared to be degradation products. This result was also seen in transfected 10T1/2 and COS (see below) cells, suggesting that it was not due to an artifact in injected embryos. Moreover, NLSGli2 did not yield a prominent band of approx. 860 aa similar to that

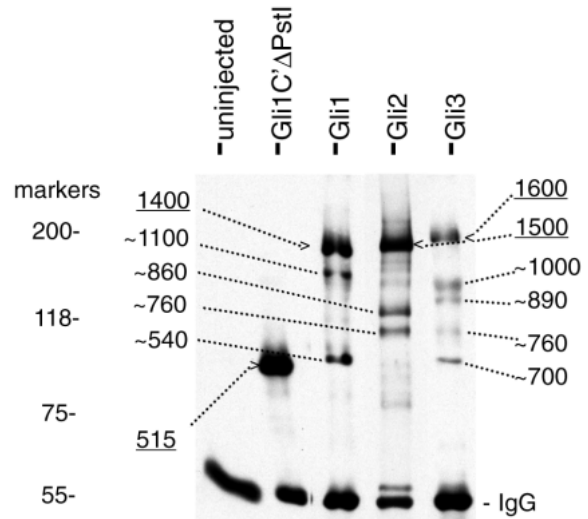
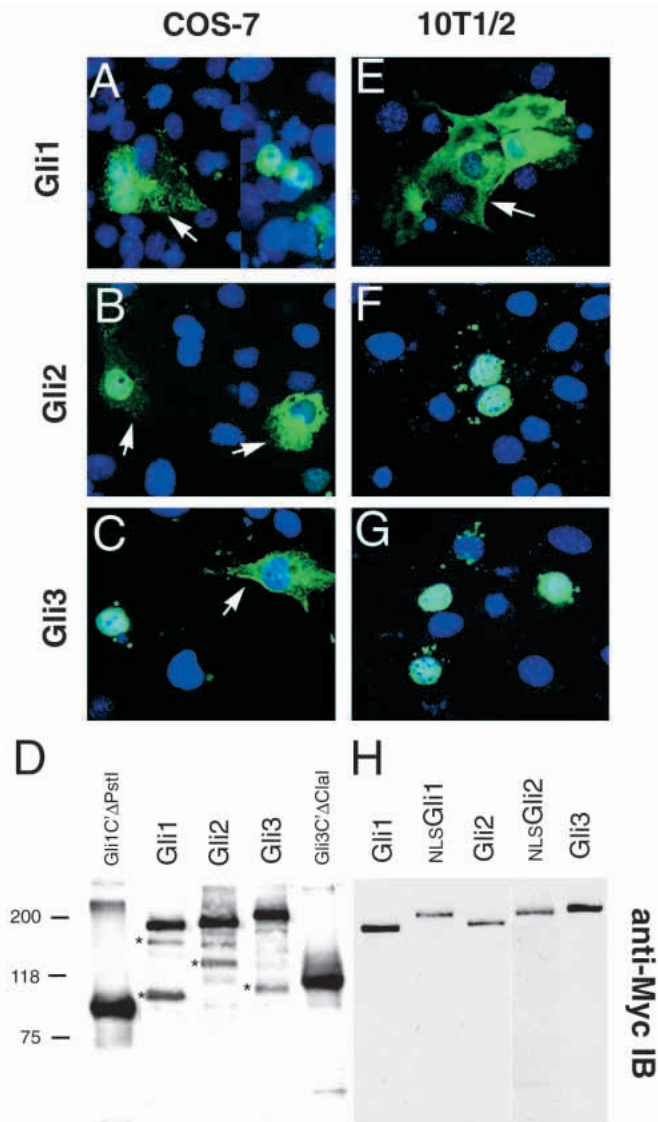


Fig. 5. IP analysis of Gli proteins in injected frog embryos. Myc-tagged products of Gli1, Gli2 and Gli3 proteins produced in injected frog cells. Full-length Gli products from a variety of injected plasmid DNAs are depicted by arrows with dashed lines and their length, given in amino acids, is underlined. These include the full-length Gli proteins as well as a product from the truncated cDNA Gli1C' Δ PstI. The known sizes of several other truncated products were used to determine the approximate sizes of shorter bands (not shown). For Gli1, smaller bands were approx. 1100 aa and a smaller form of approx. 540 aa. For Gli2, a major band was seen of approx. 860 aa and a minor one at approx. 760 aa (Fig. 5). For Gli3, bands were seen of approx. 1000, 890, 760 and 700 aa in length at similar abundance. All sizes are approximate to ± 30 aa. Each lane is labeled on top with the injected pDNA. The sizes of full-length and of smaller products (depicted by dashed lines and their length, given in amino acids) are shown on the sides. The positions of standard markers are also shown to the left. Immunoglobulins (IgG) are also indicated. All lanes in each panel were loaded with immunoprecipitated proteins from the same number of embryos. Deletions are as in Fig. 2.

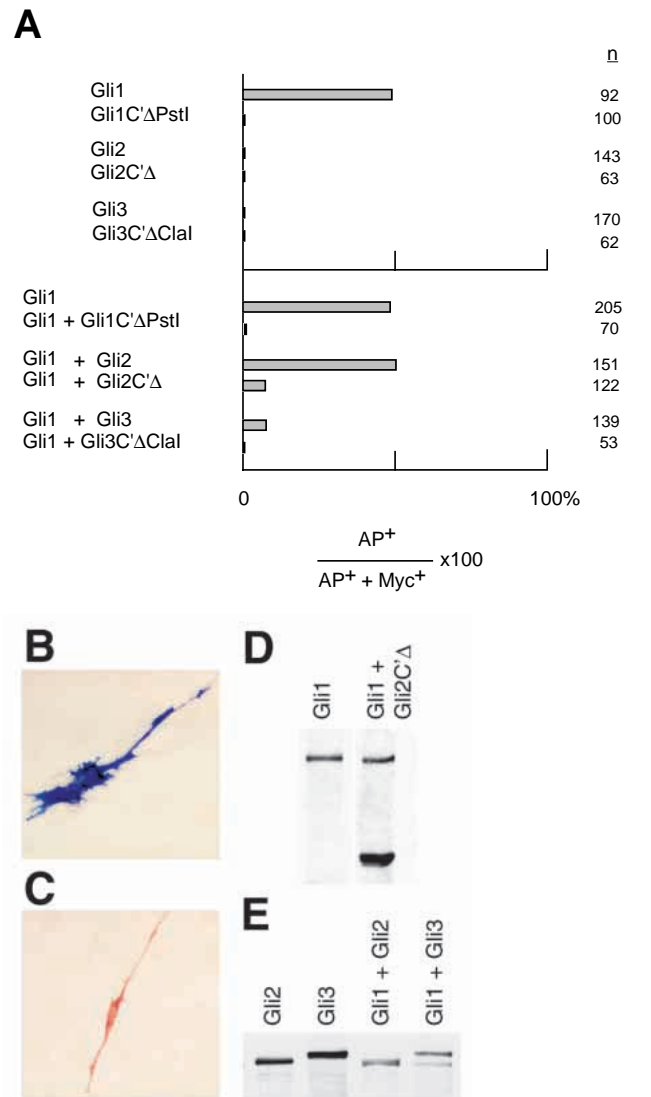
seen for Gli2 (not shown). However, since NLSGli1 and NLSGli2 yield forms that appear to be larger than Gli1 or Gli2, it is not clear how to interpret the absence of shorter forms in NLSGli expressing cells.

Structure and subcellular localization of Gli proteins in transfected COS cells

Previous analyses of the subcellular distribution of tagged Gli proteins transfected into COS cells showed that these are predominantly cytoplasmic (Lee et al., 1997). To analyze the structure of transfected Gli proteins in COS cells, these were transfected by standard procedures but instead of analyzing the transfected cells 24 after transfections, the cells were fed with complete medium 24 hours after transfection and then cultured for another 24 hours. Under these conditions, Gli proteins were found to be localized both in the cytoplasm and in the nucleus (Fig. 6A-C). 16% of cells expressing Gli1 had nuclear label, 46% had cytoplasmic label and 38% had both cytoplasmic and nuclear label ($n=63$ cells counted). For Gli2, 19% of cells had nuclear label, 54% had cytoplasmic label and 27% had both cytoplasmic and nuclear label ($n=123$ cells counted). For Gli3, 15% of cells had nuclear label, 55% had cytoplasmic label and



30% of cells had both cytoplasmic and nuclear label ($n=88$ cells counted). As expected, transfection of NLSGli1 (Lee et al., 1997) and NLSGli2 showed nuclear labeling (not shown, 75-77% of cells with exclusive nuclear label; $n=80$ and 78 cells



counted, respectively). The finding that COS cells could have nuclear Gli proteins raised the possibility that these could be processed to yield C-terminally truncated nuclear forms. IB analyses of transfected COS cell lysates showed that the three *Gli* cDNAs were able to yield full-length forms as well as one or two major shorter forms per protein (marked by asterisks in Fig. 6D). For Gli1, two forms were detected that were similar to those seen in injected frog embryos, of approx. 1100 and 540 aa. This smaller form was just slightly larger than Gli1C' Δ PstI.

For Gli2, a single major form was seen similar to that detected in injected frog embryos of approx. 860 aa. For Gli3, a single major form was detected and this appeared to be similar to the approx. 760 aa form seen in injected embryos, and migrated close to Gli3C' Δ Clal (Fig. 6D). To test whether the appearance of these forms required cytoplasmic events, the proteins made in cells transfected with NLSGli1 or NLSGli2 constructs were analyzed. Cells expressing NLSGli1 or NLSGli2 showed a single large band in SDS-PAGE and a series of faint degradation products (not shown). However, as in the case of injected embryos, the larger size of the NLSGli proteins makes interpretation of the absence of C-terminally truncated products difficult.

Structure and subcellular localization of Gli proteins in transfected C3H10T1/2 cells

To test the behavior of Gli proteins in a homogeneous cell population that may be more relevant to Gli protein behavior than COS cells, *Gli* constructs were transfected into 10T1/2 cells, as these are known to be able to respond to SHH (Kinto et al., 1997) and to express the three *Gli* genes (A. Chokas and A. R. A., not shown). Tagged Gli1 was found in both the nucleus and the cytoplasm (100% of cells) of transfected 10T1/2 cells. (Fig. 6E; $n=50$ cells counted). In contrast, both Gli2 (86% nuclear, 14% nuclear and cytoplasmic; $n=70$ cells counted) and Gli3 (85% nuclear, 15% nuclear and cytoplasmic; $n=50$ cells counted) were mostly nuclear (Fig. 6F,G). IB analyses of transfected cell extracts showed the abundant presence of full-length Gli1, Gli2 and Gli3 proteins but not significant amounts of any C-terminally deleted forms (Fig. 6H). Analysis of the NLSGli1 (66% of cells with exclusive nuclear label, $n=56$ cells counted) and NLSGli2 (100% of cells with nuclear label, $n=26$ cells counted) proteins in these cells showed that the proteins produced are apparently larger than Gli1 or Gli2 (Fig. 6H), suggesting that as in COS cells and embryos, mature and stable positively acting Gli proteins may be modified.

Induction of alkaline phosphatase activity in 10T1/2 cells by transfected Gli proteins

Because 10T1/2 cells respond to SHH by expressing AP activity (Kinto et al., 1997; Murone et al., 1999), the ability of the different Gli proteins to mimic SHH induction was tested in this assay. For quantification, cells were processed for AP histochemistry (Fig. 7B) and then assayed for Myc immunoreactivity in order to identify transfected cells expressing Myc-tagged Gli proteins (Fig. 7C). Because the AP product can hide the red-brown DAB reaction used to visualize Myc-tagged proteins, all AP⁺ cells were considered also Myc⁺ in transfections with Myc bearing constructs. Transfection of single cDNAs showed that only full-length *Gli1* could induce AP activity (Fig. 7A). Full-length *Gli2* and *Gli3*, as well as all C-terminal deletions, were ineffective.

Since full-length *Gli* cDNAs transfected into 10T1/2 cells do not produce significant levels of C-terminal truncated forms (Fig. 6H), this cotransfection assay allowed the testing of the activity of full-length *Gli2* and *Gli3* relative to that of *Gli1*. Previous studies showed that in vivo, misexpression of Gli2 or Gli3 in the ventral neural tube of frog embryos resulted in an inhibition of floor plate differentiation, a cell type that can be induced by SHH and Gli1 but not by Gli2 or Gli3 (Ruiz i Altaba, 1998). Coexpression of Gli1 and Gli2 in

10T1/2 cells gave a result similar to that obtained after transfecting Gli1 alone (Fig. 7A), showing that full-length Gli2 does not have dominant negative activity over Gli1 in this assay. In contrast, full-length Gli3 did decrease the number of AP⁺ cells induced by Gli1 (Fig. 7A), showing that Gli3 has dominant negative activity. As expected, all C-terminally deleted Gli proteins shown to have dominant negative activity inhibited AP induction by Gli1 in cotransfection assays (Fig. 7A).

Analyses of the transfected proteins by SDS-PAGE showed that coexpressed proteins did not affect the structure of each other. As in single transfections, only full-length (or near full-length) Gli1, Gli2 and Gli3 proteins were detected in cotransfected 10T1/2 cells (Fig. 7D,E). Similarly, as seen in frog embryos, C-terminally truncated proteins were more stable and thus more abundant than full-length ones (Fig. 7D and not shown). The activities detected in 10T1/2 cells transfected with full-length cDNAs can thus be assigned to full-length or near full-length proteins.

Protein kinase A promotes repressor formation of Gli3, but not of Gli1 or Gli2, and inhibits Gli1 function in 10T1/2 cells

PKA is a known negative regulator of Hh signaling (e.g. Ingham, 1998). Previous studies in vertebrate embryos using mutant PKA dominant negative subunits have shown a requirement of PKA to repress Shh signaling (Hammerschmidt et al., 1996; Concordet et al., 1996). Increasing PKA activity through an increase of cAMP levels by forskolin administration also results in inhibition of Shh signaling (e.g. Fan et al., 1995; Dahmane and Ruiz i Altaba, 1999). Effects of PKA activity on Gli protein structure and function were then assayed in 10T1/2 cells using dominant negative or constitutively active subunits of PKA (Clegg et al., 1987; Orellana and McKnight, 1992). Gli1, Gli2 or Gli3 were cotransfected into 10T1/2 cells with either constitutively active (PKA-ca) or dominant negative (PKA-dn) PKA subunits (Fig. 8A). Neither subunit affected the structure of Gli1 or Gli2 proteins as assayed in IBs of total cell lysates. In contrast, the constitutively active subunit induced the appearance of a shorter protein of similar size to that of approx. 760 aa seen in injected blastomeres and transfected COS cells, representing the putative repressor derivative of Gli3 (Fig. 8A). The dominant negative PKA subunit had no effect (not shown).

The functional consequences of altering PKA function in 10T1/2 cells was measured by testing for induction of AP activity by Gli proteins in transfected cells. As shown above, only Gli1 was able to induce AP activity in 10T1/2 cells (Fig. 8B) and this was not altered by coexpression of a dominant negative subunit (Fig. 8B). Gli2 or Gli3 transfected cells also did not acquire AP activity upon coexpression of this dominant negative subunit or a constitutively active one. In contrast, constitutive PKA activity repressed AP induction by Gli1 (Fig. 8B), showing that PKA inhibits Gli1 function.

DISCUSSION

Gli proteins are versatile transcription factors that are tightly regulated. *Gli1* and *Gli2* mRNAs are usually detected close to

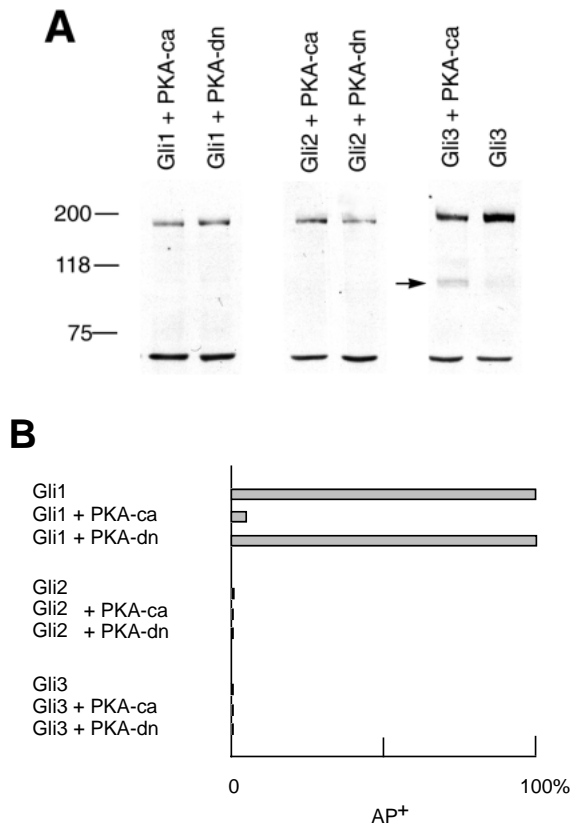


Fig. 8. Effects of PKA on Gli protein structure and function in 10T1/2 cells. (A) IB analysis of Gli1, Gli2 and Gli3 proteins transfected into 10T1/2 cells along with constitutively active (PKA-ca) or dominant negative (PKA-dn) subunits of PKA. No effect is detected for Gli1 or Gli2. For Gli3, only PKA-ca induces the appearance of a short form of similar size to that seen in transfected COS cells and injected blastomeres as well as Gli3C Δ Cl Δ I, which acts as a repressor. PKA-dn has no effect on Gli3 (not shown). Compare the relative levels of the full-length and the shorter forms in Gli3+PKA-ca versus Gli3 lanes. Twice the amount of Gli3 plasmid was transfected in cells that received Gli3 alone versus Gli3+PKA-ca. (B) Quantification of the number of AP⁺ cells detected in different transfection experiments shown as percentage relative to the number observed in Gli1-transfections. Note that only Gli1+PKA-ca shows a difference from single transfections with the *Gli* genes.

sources of Hh signals whereas *Gli3* mRNA is usually found at a distance from these (Marigo et al., 1996; Lee et al., 1997; Platt et al., 1997; Sasaki et al., 1997; Dahmane and Ruiz i Altaba, 1999). The results presented here suggest that Gli function is also regulated post-transcriptionally, like Ci in flies (Aza-Blanc et al., 1997). Different parts of the Gli proteins encode distinct functions, with dominant negative activity found in the N terminus and activating function in the C terminus. Moreover, C-terminal sequences are involved in cytoplasmic retention. In their absence, C-terminally truncated proteins are nuclear. These results, together with the finding that the repressor activity of Gli3 is more prevalent than that of Gli1, suggest that Gli proteins may have evolved to differentially utilize their activator and repressor potentials, which nevertheless appear to be regulated in a context-dependent manner.

Positive functions of Gli proteins are context-dependent

Gli1 has been shown to positively regulate the target gene *HNF-3 β* (Lee et al., 1997; Sasaki et al., 1997) and thus it must act in the nucleus. In this context, the finding that full-length or near full-length activating Gli/Ci forms are abundant in the cytoplasm is intriguing. It has been suggested that very small amounts of Ci translocate to the nucleus and that these are sufficient for positive activating function (Kelsey Motzny and Holmgren, 1995), acting in cooperation with CBP (Akimaru et al., 1997). Here it is shown that near full-length or full-length Gli2 and Gli3, but not Gli1, proteins can accumulate in the nuclei of transfected 10T1/2 cells, suggesting that nuclear localization of positive acting Gli forms is a regulated event and that Gli proteins are regulated differently. Availability of activator forms in the nucleus may therefore be part of the mechanism that regulates Gli function.

The positive activating function of Gli proteins is context-dependent. For example, Gli1, but not Gli2 or Gli3, can induce ectopic floor plate differentiation in frog embryos (Lee et al., 1997; Ruiz i Altaba, 1998) and AP reactivity in mouse 10T1/2 cells. However, all three Gli proteins can induce primary neurogenesis in frog embryos (Brewster et al., 1998) and there is some redundancy of Gli2 and Gli3 in skeletal patterning (Mo et al., 1997). Nevertheless, a closer inspection may reveal non-redundancy in many cases. Indeed, all Gli proteins do not induce the same classes of neurons, suggesting that combinatorial Gli function is involved in pattern formation (Ruiz i Altaba, 1997, 1998), which would, nevertheless, appear to be context-dependent.

Gli genes encode dominant negative activity

All three Gli proteins harbor dominant negative potential. In the assays used, full-length Gli1 or Gli2 do not have dominant negative or repressor activity and this is only found in C-terminally truncated forms. Full-length Gli3, in contrast, can have either activating function (in neuronal induction in vivo) or repressive function (repressing Gli1 activity in 10T1/2 cells). This suggests that dominant negative activity of full-length Gli3 is context-dependent. In contrast, C-terminally truncated Gli3 proteins have strong constitutive dominant negative activity, inhibiting the positive function of positive Gli functions in all cases, including induction of neurogenesis by full-length Gli3.

The inability of Gli2 to inhibit Gli1 function in 10T1/2 cells is evidence that dominant negative activity of Gli2 in other assays, such as ectopic floor plate induction in vivo by coexpressed Gli1, derives from a modified form. This is predicted to be a C-terminally deleted product: C-terminally truncated Gli2 proteins are detected in injected frog embryos and transfected COS cells but not in transfected 10T1/2 cells. Moreover, the inability of NLSGli2 to inhibit floor plate differentiation by coinjected Gli1 is also consistent with this possibility. The molecular basis of the dominant negative function of C-terminally truncated forms is likely to involve transcriptional repression, as a Gli3 protein lacking the DNA-binding zinc finger domain (Gli3ZFD Δ) failed to inhibit Gli1 function (Ruiz i Altaba, 1998), Gli3 can act as a transcriptional repressor (Marine et al., 1997) and the C-terminally truncated form of Ci is a nuclear DNA-binding repressor (Aza-Blanc et al., 1997).

The presence of both full-length or near full-length as well as C-terminally deleted Gli proteins in embryos injected with *Gli* cDNAs could account for the observed cytoplasmic and nuclear localization of Gli proteins within single cells. Full-length forms could be both nuclear and cytoplasmic but C-terminally truncated forms are predicted to be nuclear. In this case, however, the function of full-length forms in floor plate or neuronal induction assays would not appear to be inhibited by C-terminally truncated forms made in vivo from the injected full-length cDNAs. One possibility to explain this discrepancy may be that not all cells process Gli proteins. Indeed, in transfected COS cells, some cells displayed nuclear Gli protein while others showed exclusive cytoplasmic protein. In injected embryos, those that become floor plate or neurons may not efficiently produce dominant negative forms from injected cDNAs.

The mechanisms of regulation of formation of dominant negative Gli forms remain unclear. It is worth noting that in the absence of stimulation by SHH, transfected 10T1/2 cells do not constitutively yield appreciable amounts of C-terminally truncated forms. Since COS cells do, this cannot be due to a simple saturation effect by the transfected material. This observation therefore suggests two possibilities. (1) 10T1/2 cells have tonic signaling that prevents formation of C-terminally truncated forms, which nevertheless is insufficient to induce AP activity. In this case, Glis and Ci would behave similarly, as Ci is processed to yield a constitutive C-terminally truncated repressor form in the absence of Hh signaling (Aza-Blanc et al., 1997). (2) The regulation of Glis and Ci is partially different. Aspects of the regulation of product formation of Ci and Glis must indeed be partially distinct. Since SHH represses the transcription of the *Gli3* gene (Marigo et al., 1996; Ruiz i Altaba, 1998), this would appear to preclude maintained posttranscriptional regulation of Gli3 forms by SHH signaling over a prolonged period. However, Gli3, but not Gli1 or Gli2, is regulated by PKA activity in the sense that PKA promotes the formation of repressor formation, making a parallel with Ci (Chen et al., 1998). Moreover, while this paper was being reviewed, Dai et al. (1999) have reported the detection of shorter forms of Gli3 in mouse embryo extracts similar to Gli3C'ΔC1aI and the approx. 760 aa forms detected here. Their production in transfected cells is promoted by PKA and this is antagonized by SHH signaling (Dai et al., 1999). However, PKA may affect Ci function at different levels and in opposite ways (Chen et al., 1998; Ohlmeyer and Kalderon, 1997), making a simple interpretation of these results difficult. Indeed, PKA could inhibit Gli1 function in 10T1/2 cells by inducing the formation of repressor forms from the endogenous Gli3 protein. However, it remains possible that PKA directly affects Gli1 function in other ways.

Patterning implications of dominant negative Gli function

The dominant negative activity of the three Gli proteins could be partially distinct. The dominant negative function or production of C-terminally deleted forms of Gli1 may be ineffective, as Gli1C'ΔPstI is unable to repress endogenous floor plate or neuronal differentiation as well as ectopic neurogenesis by coexpressed Glis. Alternatively, endogenous Gli1 activating proteins may be modified in ways that render them unable to be inhibited by dominant negative Gli1 forms.

Gli2 and Gli3, in contrast, encode strong dominant repressor functions. Full-length and C-terminally truncated Gli3 forms inhibit Gli1 in 10T1/2 cells but only a C-terminally truncated form of Gli2 has dominant negative activity in 10T1/2 cells. Thus, the regulation of Gli2/3 repressor formation and *Gli3* expression is critical for the type of response to SHH. Pivotal steps of cells responding to SHH may thus be the regulation of activator or repressor forms of Gli2 and Gli3 and the long-term repression of *Gli3* transcription. Initial activation of Gli1 transcription by Shh, however, may require a factor already present. One possibility is that low levels of Shh-independent transcription of *Gli* genes, such as that of *Gli1* and *Gli3* in the animal cap before midline development (Lee et al., 1997), accounts for the initial mediation of the Shh signal, which then gets amplified by upregulation of Gli1.

Misexpression studies in frogs and loss of function analyses in mice appear to show a discrepant role of Gli1 and Gli2 in floor plate development. In frog embryos Gli1 can induce floor plate cells and Gli2 cannot (Lee et al., 1997; Ruiz i Altaba, 1998). In contrast, in mice *Gli1* appears to be non-essential and loss of *Gli2* leads to the absence of a mature floor plate (Matise et al., 1998; Ding et al., 1998). Beyond possible explanations relating to species-specific assays or different cDNAs, and the fact that all three *Gli* genes are coexpressed in the mouse, but not in the frog, early neural plate (Lee et al., 1997; Sasaki et al., 1997), these results raise three questions. (1) Is *Gli1* important? In addition to the results in frog embryos summarized above, *Gli1* is the only *Gli* gene known to be expressed in midline cells that become floor plate in frog embryos (Lee et al., 1997). In mouse 10T1/2 cells, Gli1 also appears to be the only Gli protein that can mimic SHH function, providing further evidence for the primacy of Gli1 in mediating Hh signals. The importance of *Gli1* is also suggested by its consistent expression in mouse and human cancers derived from ectopic activation of the Shh pathway (Dahmane et al., 1997; Goodrich et al., 1997; Hahn et al., 1998; Reifenberger et al., 1998). (2) Is *Gli1* required? There may be parallel pathways to that involving Gli proteins (Krishnan et al., 1997; Lessing and Nusse, 1998). Alternatively, Gli2 could be modified so that it cooperates with or mimics Gli1 in floor plate induction. (3) Is *Gli2* important? The absence of floor plate differentiation in *Gli2* KO mice suggests a critical function of Gli2 in the process of induction or maintenance of these cells. Gli2 could act in parallel with or before Gli1. However, the possibility still remains that there is an abnormal C-terminally truncated protein made from the *Gli2* KO locus as this gene was disrupted only downstream of the second zinc finger. If protein were made from this allele, it could have unpredicted effects on other coexpressed Gli proteins, giving the impression that Gli2 is required for positive induction of floor plate cells. This explanation, however, cannot account for the apparent normal development of motor neurons in *Gli2* KO mice (Matise et al., 1998; Ding et al., 1998). Experiments in chick and mouse embryos show that early motor neurons are induced by SHH (Martí et al., 1995; Chiang et al., 1996; Ericson et al., 1996), and in frog embryos Gli2, but not Gli3, can induce spinal motor neurons, without inducing *Gli1* or *Shh* (Ruiz i Altaba, 1998). Thus, these results suggest the existence of parallel pathways or an unnoticed plurality of early motor neurons, all of which express the markers (*Isl1/2* and *HB9*) used in these assays. It remains possible, however, that under

the abnormal conditions of absence of Gli2, Gli3 is modified in a way that becomes active in motor neuron induction.

Notwithstanding these differences, the use of Gli2 and Gli3 dominant negative forms further supports a requirement of activating Gli function in primary neurogenesis in frog embryos (Brewster et al., 1998) and raises the possibility that pattern formation by SHH involves the utilization of repressor forms in a temporal and/or spatially regulated manner. For example, *Gli2* and *Gli3* are expressed throughout the frog neural plate with the exception of the midline (Lee et al., 1997), but primary neurons differentiate only in three bilateral stripes. In this case, a regional production/action of repressor Gli proteins in areas in which neurogenesis is absent could have a complementary or additional function to other anti-neurogenic prepattern factors such as *Zic2* (Brewster et al., 1998). Similarly, Gli repressors may control the spatial or temporal activity of activating forms in the development of other tissues and organs such as the limb, brain, hair and face. The multiplicity of Gli forms is also indicated by the finding that the human *GLI2* gene produces four differentially spliced variants (Tanimura et al., 1998) although the functional significance in development has not been yet tested. Together, these results show that Gli proteins are versatile and that their functional repertoire is complex. Understanding how cells respond to Hh signals thus requires an understanding of how positive or negative Gli functions are utilized. Moreover, C-terminally truncated Gli proteins may have different functions, as suggested by the finding that such forms of Gli3, but not Gli1, bind Smads (Liu et al., 1998). Ongoing work is designed to address where and when repressor forms are active.

Deregulated production of repressor forms as a molecular basis of C-terminally truncated GLI3-related human syndromes

Two dominant mutations in human *GLI3* have been recently found in the C terminus. One is predicted to produce a C-terminally truncated protein terminating before conserved region 3 and produces Pallister-Hall Syndrome (PHS), which includes malformations of the neural tube, face and limbs (Kang et al., 1997). The second one is also predicted to yield a C-terminally truncated protein, but terminating after region 3, and produces Polydactyly type A (PAP-A), with a sixth well-formed post-axial toe and finger (Radhakrishna et al., 1997). In the present assays, differences between the Gli3C' Δ Claf and Gli3C' Δ Bal#8 proteins, which mimic the PAP-A and PHS forms, respectively, were not detected. The PAP-A form could be a weaker allele of the PHS form or it could be functional only in limbs. Notwithstanding this difference, the results of in vivo and in vitro assays presented here suggest that C-terminally truncated protein made from the mutant allele acts as a dominant repressor of all activating GLI3 function. These C-terminally truncated GLI3 proteins are also predicted to inhibit the activating function of all other coexpressed GLI proteins. Consistent with this, while this paper was under review Shin et al. (1999) showed that Gli3 forms that mimic the PAP-A and PHS mutant proteins act as transcriptional repressors. Dominant repressor activity could thus provide the basis for the phenotypic differences between Greig's Cephalopolysyndactyly Syndrome, which results from loss of GLI3 function (Vortkamp et al., 1991), and PHS. Together,

these findings show that deregulation of dominant negative Gli forms has severe biological consequences.

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