Specific modulation of ectodermal cell fates in *Xenopus* embryos by glycogen synthase kinase

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**SUMMARY**

Shaggy is a downstream component of the *wingless* and *Notch* signaling pathways which operate during *Drosophila* development. To address the role of glycogen synthase kinase 3β (GSK3β), a mammalian homologue of Shaggy, in vertebrate embryogenesis, it was overexpressed in *Xenopus* embryos. Microinjection of rat GSK3β mRNA into animal ventral blastomeres of 8-cell-stage embryos triggered development of ectopic cement glands with an anterior neural tissue as evidenced by in situ hybridization with Xotx2, a fore/midbrain marker, and NCAM, a pan-neural marker. In contrast, animal dorsal injection of the same dose of GSK3β mRNA caused eye deficiencies, whereas vegetal injections had no pronounced effects on normal development. Using several mutated forms of rat GSK3β, we demonstrate that the observed phenotypes are dose-dependent and tightly correlate with GSK3β enzymatic activity. Lineage tracing experiments showed that the effects of GSK3β are cell autonomous and that ectopic cement glands and eye deficiencies arose directly from cells containing GSK3β mRNA. Molecular marker analysis of ectodermal explants overexpressing GSK3β has revealed activation of Xotx2 and of cement gland marker XAG-1, but expression of NCAM and XIF-3 was not detected. Phenotypic effects of mRNA encoding a *Xenopus* homologue of GSK3β were identical to those of rat GSK3β mRNA. We hypothesize that GSK3β mediates the initial steps of neural tissue specification and modulates anteroposterior ectodermal patterning via activation of Otx2 transcription. Our observations implicate GSK3β in signaling pathways operating during neural tissue development and during specification of anterior ectodermal cell fates.

Key words: glycogen synthase kinase, Otx2, neural induction, cement gland, eye formation

**INTRODUCTION**

Vertebrate neural tissue is induced from presumptive ectoderm and acquires anteroposterior and mediolateral pattern in response to inducing signals emitted by dorsal mesoderm (neural induction) and by the newly formed neural tissue (homeogenetic induction). The neural tube becomes subdivided into forebrain, midbrain, hindbrain and spinal cord along the anteroposterior (AP) axis. Dorsoventral regionalization of the neural tube involves differentiation of floor plate, motor neurons, commissural neurons and roof plate (see Slack and Tannahill, 1992; Placzek et al., 1991, for reviews). Cranial ectodermal placodes form next to the brain as a result of further cell-cell interactions. The optic vesicle evaginates laterally from the developing forebrain to subsequently form the optic cup; the lens is induced from apposing ectoderm (Nieuwkoop and Faber, 1967). In anuran amphibians, a mucus-secreting ectodermal organ known as the cement gland is specified during neural induction and develops anterior to forebrain (Sive et al., 1989; Drysdale and Elinson, 1993).

Anteroposterior differences in the amphibian neural tube are thought to be established during gastrulation by two major inductive processes (see Slack and Tannahill, 1992; Gilbert and Saxen, 1993, for reviews). The first process is signaling from underlying mesoderm that itself is polarized along the AP axis and that provides different patterning signals to the neural plate (‘vertical’ induction). The second process is signaling from mesoderm or from the newly induced neural tissue that occurs within the plane of the neural plate (‘planar’ induction). Several chemicals have been reported to affect AP patterning of the neural plate. Exogenous retinoic acid has been shown to abolish anterior structures including cement gland, brain and eyes (Durston et al., 1989). Ammonium salts can elicit cement gland formation (Picard, 1975), whereas phorbol myristate acetate and concanavalin A are reported to induce forebrain structures in ectodermal explants (Davids et al. 1987; Otte et al., 1988; Takata et al., 1984). Little is known, however, about the endogenous molecules involved in the processes of neural induction and anteroposterior specification of neural tissue.

Two candidates for endogenous neural inducing factors, noggin and follistatin, have emerged in recent experiments (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994). Transcripts encoding both proteins are expressed in the Spemann organizer region that possesses strong neural inducing activity (Smith and Harland, 1992; Hemmati-Brivanlou et al., 1994). Ectodermal explants (animal caps) treated with noggin, or isolated from embryos injected with follistatin express anterior neural and cement gland markers, suggesting that noggin and...
follistatin may participate in inductive interactions controlling anterior neural tissue formation (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994).

In the search for other molecules that may control neural tissue formation in vertebrate embryos, we focused our attention on the Drosophila shaggy product (also known as zeste-white 3), which plays a critical role during Drosophila neurogenesis. Decisions between epidermal and neural cell fates on the fly thorax depend on proneural genes, which encode helix-loop-helix transcription regulators known as the achaete-scute complex (AS-C) (Campuzano and Modolell, 1992). AS-C gene expression is normally restricted by a lateral inhibition signaling pathway involving the neurogenic genes Notch and Delta, which encode transmembrane proteins with EGF-like repeats (Ghysen et al., 1993). Mutations in neurogenic genes as well as in shaggy eliminate this inhibition leading to neuralization of a large number of ectodermal cells (Ruel et al., 1993). Shaggy mutant phenotypes are not affected by a dominant gain-of-function allele of Notch, indicating that Shaggy is required downstream of Notch to repress AS-C genes (Ruel et al., 1993). In addition to its role in neurogenesis, shaggy functions during transduction of a Wingless (Wg) signal (Siegfried et al., 1992; Perrimon, 1994). It has been shown to suppress the Wg effects on Drosophila segmentation in a cell-autonomous manner and to function downstream of dishevelled (dsh), another Drosophila segment polarity gene (Perrimon, 1994). Interestingly, Wnt1 (a Wg homologue in mice) has been implicated in development of vertebrate brain (McMahon and Bradley, 1990). The connection between the role of Shaggy in Notch signaling pathway and its function in the Wingless/Wnt signaling pathway remains to be clarified.

Homologues of the Shaggy protein have been found in many eukaryotes, including yeast and slime mold (Plyte et al., 1992). Glycogen synthase kinase β (GSK3β), a mammalian homologue of shaggy, was originally identified as a serine/threonine kinase regulating metabolism of glycogen (Plyte, et al., 1992). Interestingly, a cDNA encoding GSK3β, but not a related kinase GSK3α, can rescue the shaggy mutant phenotype (Ruel et al., 1993). GSK3β activity is regulated by phosphorylation of serine, threonine and tyrosine residues (Hughes et al., 1993; Wang et al. 1994); its substrates are phosphorylation of serine, threonine and tyrosine residues but not a related kinase GSK3α. Shaggy homologues have been found in many eukaryotes, including yeast and slime mold (Plyte et al., 1992). The role of Shaggy in the Wingless/Wnt signaling pathway remains to be clarified. Homologues of the Shaggy protein have been found in many eukaryotes, including yeast and slime mold (Plyte et al., 1992). Glycogen synthase kinase β (GSK3β), a mammalian homologue of shaggy, was originally identified as a serine/threonine kinase regulating metabolism of glycogen (Plyte, et al., 1992). Interestingly, a cDNA encoding GSK3β, but not a related kinase GSK3α, can rescue the shaggy mutant phenotype (Ruel et al., 1993). GSK3β activity is regulated by phosphorylation of serine, threonine and tyrosine residues (Hughes et al., 1993; Wang et al. 1994); its substrates are phosphorylation of serine, threonine and tyrosine residues but not a related kinase GSK3α. Homologues of the Shaggy protein have been found in many eukaryotes, including yeast and slime mold (Plyte et al., 1992).

Materials and Methods

DNA constructs

GSK3β cDNA was amplified from rat heart mRNA using Mo-MLV reverse transcriptase and Vent DNA polymerase (RT-PCR) with two oligonucleotide primers: 5'-GGTGAATCGAGAAGAGGCCCATTGATAGTCTACTCTCTA-3' and 5'-TGGCACCACCTCCGAACTGCTTTC-3' based on the published sequence (Woodgett, 1990). The PCR product encoding full-length GSK3β protein was cloned into the EcoRI site of pBluescript SK (Stratagene) to produce the plasmid pGSK-BSSK, and its identity was verified by sequencing. For RNA microinjection experiments, the Smal-HincII DNA fragment containing the full-length GSK3β cDNA was subcloned into the blunt-ended EcoRI site of pXT7 (Dominguez et al., 1995) to give pGSK-XT7T. To generate a Y216F mutation in GSK3β, the Xhol-EcoRI fragment which includes the codon for tyrosine was subcloned into pSP73 (Promega), and PCR was carried out on the generated plasmid with SP6 primer and a specific oligonucleotide 5'-GAGAGCCCGAATTTTCATT-TATCTG-3' that encompasses the mutated nucleotide (underlined). The PCR product was digested with BanII and EcoRI and cloned into BanII/EcoRI-digested pGSK-pSP73. The point mutation was verified by sequencing. The Xhol-EcoRI fragment from pSP73 including the Y216F mutation was subcloned back into Xhol/EcoRI digested pGSK-XT7 yielding the pF216-XT7 derivative. The ΔΔβ mutant was constructed by deleting the Accl-BstXI fragment of GSK3β cDNA and religating blunted ends. To introduce a myc epitope at the N terminus of GSK3β, the Smal-HincII fragment of GSK3β cDNA from pGSK-BSSK was subcloned into the Smal site of pJ3M (a gift of J. Chernoff, Patriots et al., 1994). The myc-GSK3β cDNA was excised by SalI and BglII from the pJ3M vector and cloned into Xhol/BglII-digested pXT7. The myc epitope was attached to the Y216F GSK3β construct by replacing the SacI fragment of myc-GSK3β in pXT7 by the SacI fragment including the Y-F point mutation from pF216-XT7. Deletions of the HindIII (ΔH) and EcoRI (ΔE) fragments of the GSK3β cDNA were made to generate control myc-tagged forms of GSK3β (ΔH and ΔE) which lack C-terminal non-catalytic region plus 10 and 53 amino acids of the GSK3β catalytic domain, respectively.

Capped synthetic RNAs were generated as described (Krieg and Melton, 1984) by in vitro transcription of different cDNAs in pXT7 vector with T7 RNA polymerase. Noggin mRNA was synthesized with SP6 RNA polymerase from a corresponding plasmid (a gift of R. Harland, Smith and Harland, 1992).

Embryos, microinjections and explant culture

Eggs obtained from Xenopus females injected with 700 units of human chorionic gonadotropin were fertilized as described (Newport and Kirschner, 1982). The fertilized eggs were cultured in 0.1× MMR (Newport and Kirschner, 1982). Embryonic stages were determined according to Nieuwkoop and Faber (1967). For microinjection, the embryos were transferred to 3% Ficoll in 0.5× MMR. Embryos were injected into one blastomere at the 8- and 16-cell stages with 10 nl of a solution containing 0.5-2 ng of RNA. At the 8- to 16-cell stages, animal dorsal blastomeres are less pigmented than animal ventral blastomeres (Nieuwkoop and Faber, 1967). For ectodermal explant (animal cap) isolation, injections were performed into the animal pole region of both blastomeres at the 2-cell stage. Embryos injected with mRNA were cultured until midblastula stages, after which they were transferred into 0.1× MMR or used for isolation of animal caps. Animal caps were excised at stage 8-8.5 and cultured in 0.6× MMR until stage 11 or stage 28 when RNA was extracted.

Lineage tracing

For lineage tracing, mRNA encoding β-galactosidase was prepared by in vitro transcription from the corresponding template (a gift of R. Harland) and injected alone or with GSK3β mRNA into one blastomere at the 8- to 16-cell stage. When control sibling embryos had developed to stage 36, injected embryos were fixed in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM MgSO4 and 3.7% formaldehyde) (Hemmati-Brivanlou and Harland, 1989) for 15 minutes. After washing with PBS, β-galactosidase activity was visualized by inu-
bating embryos in a solution containing 1 mg/ml X-Gal, 5 mM K$_3$Fe(CN)$_6$, 5 mM K$_4$Fe(CN)$_6$.3H$_2$O, 2 mM MgCl$_2$ in PBS.

**Immunoprecipitation and kinase assay**

Six embryos injected with myc-tagged constructs were lysed in 600 µl of lysis buffer B (1% Triton, 25 mM Tris-HCl at pH 8.0, 0.1 M KCl, 1 mM EDTA) when sibling embryos had developed to stage 9 (late blastula). Lysates were cleared by centrifugation at 10000 g for 5 minutes. Anti-myc monoclonal antibody (30 µl of 9E10 hybridoma supernatant) was added to 500 µl of lysate and incubated for 1.5 hours at room temperature, and 20 µl of protein A agarose (Sigma) was added and incubated for an additional 30 minutes. The immunoprecipitates were washed three times with buffer B and twice with 30 mM Tris-HCl, pH 7.5, 100 mM MgCl$_2$. Immune complex kinase assays were carried out using γ$^3$P-ATP and myelin basic protein as substrate according to Wang et al. (1994). The reaction products (one embryo equivalent per lane) were subjected to 12.5% SDS-polyacrylamide gel electrophoresis followed by autoradiography.

**Western blotting**

Injected embryos were lysed in buffer B. One tenth embryo equivalent of the lysate was electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto PVDF membranes (Millipore) at 100 V for 1 hour. The membrane was blocked with 5% skim milk for 1 hour and incubated with anti-myc monoclonal antibody for 1 hour. After washing three times with PBS plus 0.05% Tween 20, it was incubated with HRP-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Labs) for 1 hour. Membranes were washed three times with PBS-Tween and peroxidase activity was visualized by enhanced chemiluminescence (ECL; Amersham).

**Northern blot analysis**

For RNA extraction, embryos or explants were treated with lysis buffer A (50 mM Tris, pH 8.0, 10 mM EDTA, 0.5% SDS, 0.1 M NaCl, 200 µg/ml Proteinase K) for 1 hour at 55°C. Total RNA was isolated by phenol/chloroform treatment, precipitated with ethanol and separated in a 1% formaldehyde/agarose gel using standard techniques (Sambrook et al., 1989). RNA from ten animal caps or from two embryos was loaded per lane. The RNA was transferred to GeneScreen nylon membrane with 20× SSPE and hybridized with radiolabeled DNA or RNA probes as described (Sambrook et al., 1989). Antisense RNA probes were prepared by in vitro transcription from plasmids containing Xotx2 (Pannese et al., 1995), NCAM (Kintner and Melton, 1987), muscle actin (Dworkin-Rastl et al., 1986), XIF3 (Sharpe et al., 1989), fibronectin (Krieg and Melton, 1985), Xbra (Smith et al., 1991), Xwnt8 (Christian et al., 1991), goosecoid (Blumberg et al., 1991) using SP6 or T7 RNA polymerase. DNA probe from XAG1 (Sive et al., 1989) was radiolabeled using random hexamer priming method (Sambrook et al., 1989).

**Histology**

Embryos injected with GSK3β or with the deleted form of GSK3β mRNA were fixed with MEMFA for 1 hour, dehydrated with ethanol and xylene and embedded in paraffin. Serial sections were cut at 8 µm and stained with hematoxylin/eosin.

**Whole-mount in situ hybridization and sectioning**

Whole-mount in situ hybridization was performed according to Harland (1991). Digoxigenin-labeled antisense RNA probes were generated from plasmids containing XAG1, Xotx2, NCAM and muscle actin using T3 or SP6 RNA polymerase. For histological examination, stained embryos were refixed in MEMFA, embedded in paraffin and sectioned at 14 µm.

**RESULTS**

**Overexpression of GSK3β causes specific ectodermal abnormalities in Xenopus embryos**

To assess the effects of GSK3β overexpression on embryonic development, in vitro synthesized capped mRNAs encoding wild-type and mutated forms of GSK3β were microinjected into Xenopus embryos at the 8-cell stage. Since it has been reported that the kinase activity of GSK3β requires phosphorylation of tyrosine 216 (Hughes et al., 1993), a point mutation Y216F was generated, in which tyrosine 216 of GSK3β was replaced with phenylalanine. Other controls included deletions in different regions of the protein (ΔAB, ΔH and ΔE; see Materials and Methods).

The effects of GSK3β overexpression on embryogenesis were strictly dependent on the site of injection. When 0.5-2 ng of GSK3β mRNA was injected into an animal-ventral blastomere, ectopic cement glands developed (Fig. 1A, Table 1). In contrast, animal dorsal injections of GSK3β mRNA caused eye deficiencies. Typically, one eye was missing or underdeveloped (Fig. 1B, Table 1). We observed that an ectopic cement gland frequently formed next to the deficient eye (Table 1). Vegetal injections of GSK3β mRNA had less pronounced effect on embryogenesis, occasionally one eye deficiencies were observed when a dorsoventral blastomere was injected (Table 1). Whereas embryos injected with control mRNAs from the deletion mutants developed normally, overexpression of

**Fig. 1.** Developmental effects of microinjected GSK3β mRNA. In vitro synthesized GSK3β mRNA was injected into a single blastomere of the 8-cell-stage embryos. The injected embryos were photographed at stage 36. (A) Animal-ventral blastomere injected. The arrow indicates an ectopic cement gland. (B) Animal-dorsal blastomere injected. Defective eyes are apparent (compare with A or C). (C) Animal ventral blastomere was injected with mRNA encoding ΔAB deletion mutant of GSK3β.
containing the Y216F substitution unexpectedly caused the same effect as the wild-type GSK3β (Fig. 1, Table 1).

The ectopic cement glands in GSK3β mRNA-injected embryos (usually one per embryo) appeared at neurula stages concomitant with the appearance of normal cement glands. After mRNA injection into a single ventral blastomere at the 4-cell stage and the embryo was left to develop until stage 21. The arrow indicates a large band of ectopic cement gland in the ventrolateral region. (B) Control sibling embryo.

Table 1. Ectodermal abnormalities caused by GSK3β overexpression

<table>
<thead>
<tr>
<th>Injected RNAs</th>
<th>Injected site</th>
<th>Total number of injected embryos</th>
<th>One eye deficient</th>
<th>One deficient eye plus ectopic cement gland</th>
<th>Ectopic cement gland</th>
<th>Normal</th>
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<tr>
<td>GSK</td>
<td>AV</td>
<td>173</td>
<td>12 (7%)</td>
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<td>83 (48%)</td>
<td>17 (10%)</td>
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<td></td>
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<td>98</td>
<td>62 (63%)</td>
<td>19 (19%)</td>
<td>6 (6%)</td>
<td>11 (11%)</td>
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<tr>
<td></td>
<td>VV</td>
<td>135</td>
<td>10 (7%)</td>
<td>3 (2%)</td>
<td>3 (2%)</td>
<td>119 (88%)</td>
</tr>
<tr>
<td></td>
<td>VD</td>
<td>63</td>
<td>18 (28%)</td>
<td>0</td>
<td>2 (3%)</td>
<td>43 (68%)</td>
</tr>
<tr>
<td>Y216F</td>
<td>AV</td>
<td>100</td>
<td>9 (9%)</td>
<td>37 (37%)</td>
<td>54 (54%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>AD</td>
<td>88</td>
<td>45 (51%)</td>
<td>21 (24%)</td>
<td>13 (15%)</td>
<td>9 (10%)</td>
</tr>
<tr>
<td>ΔAB</td>
<td>AV</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12 (100%)</td>
</tr>
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<td>14 (100%)</td>
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</tbody>
</table>

Capped RNAs were transcribed in vitro from cDNAs encoding wild-type GSK3β, Y216F mutated form, and ΔAB deletion mutant. The mRNAs were injected into animal-ventral (AV), animal-dorsal (AD), vegetal-ventral (VV) and vegetal-dorsal (VD) blastomeres of 8-cell-stage embryos. The phenotypes of the injected embryos were scored morphologically at stage 36-40 by eye deficiencies and ectopic cement gland formation.

Fig. 2. Cement gland formation at neurula stage caused by GSK3β overexpression. (A) GSK3β mRNA was injected into a single ventral blastomere at the 4-cell stage and the embryo was left to develop until stage 21. The arrow indicates a large band of ectopic cement gland in the ventrolateral region. (B) Control sibling embryo.

Fig. 3. Kinase activity and expression of different myc-GSK3β constructs in injected embryos. Both blastomeres of 2-cell-stage embryos were injected with 2 ng of mRNAs encoding wild-type GSK3β (GSK), Y216F point mutant (F²¹⁶), ΔE and ΔH deletion mutants. The injected embryos were lysed at stage 9 for immunoprecipitation and for western blotting with anti-myc 9E10 antibodies. (A) Immunoprecipitation and kinase activity analysis using myelin basic protein (MBP) as a substrate. (B) Western blotting with anti-myc antibodies. Uninjected embryos (lane C) were used as a negative control in both experiments.

GSK3β cement glands are induced by GSK3β or by the Y216F mRNA injections quite randomly along the entire anteroposterior axis. They usually appear ventrally or laterally, being excluded from the most dorsal region. Ectopic cement glands that form in the ventral part of the head region are often fused with the endogenous cement glands to form an enlarged cement gland and are accompanied by an eye deficiency.
To further confirm the specificity of the developmental effects of GSK3β, different doses of GSK3β RNA were injected into one animal ventral blastomere at the 8-cell stage. Ectopic cement glands were scored when sibling control embryos developed to the tail bud stage (stage 30). The injection of GSK3β RNA at a dose of 500 pg, 100 pg or 20 pg gave rise to ectopic cement glands in 90% of embryos (a total of 88 embryos were injected), 40% of embryos (n=103) or 2% of embryos (n=52), respectively. These studies indicate that rat GSK3β causes ectodermal defects in a dose-dependent manner. In contrast, microinjection of mRNAs encoding two other serine/threonine kinases, Xenopus S6 kinase (Jones et al., 1988) and rat ERK-1 kinase (Patriotis et al., 1994) never induced ectopic cement gland formation. Most embryos injected with 0.5-2 ng of these RNAs developed normally and in some cases kinked anteroposterior axes and incomplete blastopore closure were observed, strongly arguing for the specificity of the GSK3β effects. Furthermore, mRNA encoding a recently cloned Xenopus GSK3β (XGSK3β) (Dominguez et al., 1995) caused the same embryonic phenotypes as overexpression of the rat GSK3β (Fig. 1 and see below).

Taken together, our observations suggest that overexpressed GSK3β specifically modulates early determination of ectodermal cell fates.

**Developmental effects of GSK3β correlate with its enzymatic activity**

Wild-type and several mutated forms of GSK3β were genetically engineered to include a myc epitope ‘tag’ at their N termini (see Materials and Methods) in order to allow immunoprecipitation of the overexpressed proteins from embryonic lysates. RNAs synthesized in vitro from these myc-tagged constructs were injected into animal ventral blastomeres of the 8-cell-stage embryos to confirm that the myc epitope does not interfere with the developmental effects of GSK3β. Whereas myc-GSK3β and myc-Y216F overexpression gave rise to embryos with ectopic cement glands, embryos injected with myc-tagged ΔE and ΔH mRNAs developed normally (not shown). The same mRNAs were injected into the animal pole region of both blastomeres of the 2-cell embryos to analyze expression levels of GSK3β in the injected embryos and to measure GSK enzymatic activity. Embryos were lysed at the late blastula stage and the lysates were analyzed by Western blotting or by immunoprecipitation with anti-myc antibodies followed by in vitro kinase assays using myelin basic protein as substrate. Consistent with the functional overexpression studies, lysates from embryos injected with myc-tagged GSK3β and the Y216F RNAs revealed kinase activity, whereas no kinase activity was immunoprecipitated from Xenopus embryos overexpressing GSK3β deletion mutants (Fig. 3A). We observed no significant decrease of kinase activity in embryos overexpressing the Y216F variant of GSK3β when compared to the wild type, indicating that phosphorylation of Y216 is not absolutely required for GSK3β enzymatic activity under our experimental conditions. Finally, Western blotting with anti-myc antibodies showed that all proteins were expressed at comparable levels (Fig. 3B). These results suggest that the effect of GSK3β on ectodermal cell fates in the early embryos requires enzymatically active GSK3β protein.

**Induction of ectopic neural tissue by GSK3β**

Embryos with ectodermal abnormalities caused by GSK3β overexpression were examined histologically (Fig. 4). Consis-
tent with our morphological observations, embryos injected with GSK3β or Y216F mRNA contained characteristic columnar cement gland cells that were located inappropriately. Serial sections of 31 out of 38 embryos that were injected with GSK3β mRNA revealed ectopic neural tissue located between the neural tube and an ectopic cement gland, or expansion of the neural tube in the area adjacent to an ectopic cement gland (e.g., Fig. 4D). Additional neural tissue was usually positioned more anterior to or at the same anteroposterior level with an ectopic cement gland.

The neural retina did not form or was underdeveloped in sections from embryos injected with GSK3β into an animal dorsal blastomere. Moreover, brain structures adjacent to the deficient eyes were also malformed, suggesting that GSK3β overexpression affects formation of the brain which is necessary for development of the normal eye. In most cases, ectodermal pattern was disturbed only on one side with the other side unaffected, e.g. the second remaining eye was always complete (as in Fig. 4A). This result suggests that the effects of GSK3β are only evident in cells that contain the injected message.

To characterize developmental effects of GSK3β at the molecular level, embryos injected with GSK3β or XGSK3β mRNA were subjected to whole-mount in situ hybridization using an anterior brain marker Xotx2 (Pannese et al., 1995), pan-neural marker NCAM (Kintner and Melton, 1987) and muscle-specific actin (Dworkin-Rastl et al., 1986) probes. Ectopic tissues adjacent to the cement gland expressed both Xotx2 (Fig. 5B, D) and NCAM (Fig. 5E). In contrast, somitic tissues expressing muscle-specific actin were not affected in the injected embryos (Fig. 5C). These findings demonstrate that GSK3β affects ectodermal, but not mesodermal, cell fates and indicate that ectopic structures in GSK3β-injected embryos (Fig. 4) represent anterior neuroectodermal derivatives.

**Fig. 5.** Injection of GSK3β causes ectopic expression of anterior ectodermal and neural markers. Albino embryos injected with mRNAs from rat (A) or *Xenopus* GSK3β (B) were analyzed by whole-mount in situ hybridization using XAG1 (A), Xotx2 (B). The bottom right embryo in A is a normal embryo for comparison with the other embryos that have ectopic cement gland. The arrowhead in B indicates ectopic Xotx2 expression, which is absent in the top control sibling embryo. Localization of muscle actin (C), Xotx2 (D) and NCAM (E) transcripts in wild-type embryos injected with *Xenopus* GSK3β mRNA is shown in sections prepared after whole-mount in situ hybridization. After whole-mount analysis, embryos at stage 24 (C and E) and at stage 33 (D) were sectioned. Ectopic neural tissue (indicated by arrows) expresses Xotx2 (D) and NCAM (E). Abbreviations are as in Fig. 4, except n, notochord; s, somite. Scale bar is 150 µm.
Together, these observations are consistent with the possibility that GSK3β plays a role in neural tissue development and in ectodermal patterning along the anteroposterior axis.

The effects of GSK3β overexpression on ectodermal development are cell autonomous

To further address the question of whether the ectodermal cell fates are altered only in the GSK3β-injected cells, GSK3β mRNA was co-injected with mRNA encoding β-galactosidase (lacZ RNA). Both ectopic cement gland cells and areas with eye deficiencies stained positively for β-gal activity (Fig. 6). Control embryos injected with lacZ RNA alone into an animal ventral blastomere contained lineage tracer in ventroposterior epidermis, whereas embryos that received animal dorsal injections were stained in the head region (Fig. 6). Eyes at the un.injected side of one-eye-deficient embryos were complete and were not stained with β-gal in contrast to those at the injected side, consistent with histological sections (data not shown and Fig. 4A). These results indicate that cell fate changes only occur in cells that were β-gal positive and, thus, overexpressed GSK3β, with no significant contribution from the cell population that did not receive GSK3β mRNA.

Whereas β-gal staining was highly condensed in the ectopic cement gland, weaker staining was detected between the neural tube and the ectopic cement gland (Fig. 6A). Serial sections of the β-gal-stained embryos revealed that the weakly stained tissue corresponds to ectopic neural structures (data not shown).

These observations suggest that GSK3β overexpression changes a cell’s response to inductive signals, but does not affect surrounding cells. Thus, the effects of GSK3β mRNA are cell autonomous.

Ectodermal explants injected with GSK3β RNA express anterior ectodermal markers

Based on the lineage tracing experiments, we thought it possible that GSK3β may directly convert ectodermal cells to cement glands. To address this issue, GSK3β mRNAs from rat and Xenopus cDNAs were injected into the animal pole region of 2-cell-stage embryos and ectodermal explants were isolated at the blastula stage. Even after culturing the explants until tadpole stage, morphological signs of cement gland formation were not apparent. The explants did not undergo morphogenetic movements, a typical element of mesodermal differentiation, and looked similar to untreated animal caps (not shown).

To assess which molecular markers were expressed in the injected animal caps, the explant RNA was extracted for northern analysis when sibling embryos reached stage 11 or stage 28. Animal cap explants injected with noggin, a neural inducing factor (Lamb et al., 1993), were also analyzed for comparison. Explants from embryos microinjected with either XGSK3β or rat GSK3β mRNA expressed XAG1, a cement gland marker (Sive et al., 1989), indicating that cement gland differentiation proceeds, at least, at the molecular level. An anterior brain marker, Xotx2 (Lamb et al., 1993; Pannese et al., 1995; Blitz and Cho, 1995) was also induced in GSK3β-injected animal caps at tailbud stage, whereas neural markers NCAM (Kintner and Melton, 1987) and XIF3 (Sharpe et al., 1989) were not detected (Fig. 7A). In contrast, noggin mRNA containing ectodermal explants expressed high levels of NCAM and XIF3. None of the mesodermal markers examined, including goosecoid (Blumberg et al., 1991), Xwnt8 (Christian et al., 1991), Xbra (Smith et al., 1991) and muscle-specific actin (Dworkin-Rastl et al., 1986) were expressed in GSK3β-injected animal caps (Fig. 7). Interestingly, Xotx2 expression was induced already at the early/midgastrula stages (Fig. 7B), possibly reflecting the Xotx2 role in neuroectodermal development (Pannese et al., 1995; Blitz and Cho, 1995). These results suggest that GSK3β can directly (in the absence of mesoderm) activate anterior neuroectodermal markers in the animal cap explants.

DISCUSSION

In the present study, we report that overexpression of GSK3β leads to specific alterations in neuroectodermal cell fates. Embryos injected with GSK3β mRNA develop ectopic cement glands and contain frequent eye and brain abnormalities. In our experiments, these embryonic phenotypes never arose spontaneously or after overexpression of other serine/threonine kinases, ERK-1 and S6 kinase (data not shown). The ability of GSK3β to affect ectodermal cell fates is dose-dependent and appears to require GSK3β kinase activity, since all tested enzy-
matically inactive forms of GSK3β RNA with short in-frame deletions in the coding region did not have any effect on embryonic development (Fig. 3). Furthermore, properties of a Xenopus homologue of GSK3β were indistinguishable from those of GSK3β, also causing eye deficiencies and ectopic cement gland formation (Figs 5, 7).

Interestingly, we found that the variant of GSK3β with a Y216F substitution, which was immunoprecipitated from injected embryos, retained kinase activity (Fig. 3). The same mutation has been reported to decrease the efficiency of c-Jun phosphorylation by the enzyme isolated from the baculovirus-infected cells (Hughes et al., 1993). The observed differences could be due to differences in substrate-specificity of GSK3β variants (myelin basic protein versus c-Jun). Alternatively, the kinase activity may depend on the differential phosphorylation of GSK3β when it is produced in different cell types (Xenopus embryos versus SF9 cells used by Hughes et al., 1993). Thus, whereas it is possible that our experimental conditions do not permit to distinguish small changes in the enzyme’s activity, it seems that phosphorylation of Y216 is not essential for GSK3β function.

Our data indicate that GSK3β can activate ectopic Xotx2 transcription in a cell autonomous manner (Figs 5–7). Furthermore, GSK3β mRNA is sufficient to trigger Xotx2 expression in animal caps as early as at stage 10.5. It has been recently reported that overexpression of Xotx2 on its own leads to formation of ectopic cement glands (Pannese et al., 1995; Blitz and Cho, 1995). Xotx2 is normally expressed in presumptive neuroectoderm at early gastrula stage (Pannese et al., 1995; Blitz and Cho, 1995) and is thought to be involved in neural patterning. Together, these observations suggest that GSK3β may exert its effects on neuroectodermal fates through activation of Xotx2.

Embryos injected with GSK3β RNA into an animal dorsal blastomere frequently lack one eye, suggesting that GSK3β interferes with eye development. Vertebrate eye formation is initiated by evagination of the optic vesicle from the presumptive diencephalon (a posterior part of the forebrain). The optic vesicle shapes into the optic cup (composed of neural and pigmented retina) and induces the lens in apposing ectoderm (Grainger, 1992). Our histological analysis indicates that cells similar to neural retina, but not pigmented retina, form in the brains of injected embryos, but that the optic vesicle fails to evaginate from the neural tube (data not shown, Fig. 4A). These observations suggest that GSK3β may interfere with proper signaling controlling morphogenetic movements required for optic vesicle formation. Taken together with the observed induction of Xotx2 in animal caps and in injected embryos, one possible explanation of our findings is that overexpression of GSK3β shifts positional information about neuroectodermal cell fates anteriorly along the AP axis.

Although ectopic cement glands and neural tissue could be readily identified morphologically and molecularly in embryos overexpressing GSK3β (Figs 4, 5), these structures failed to form in ectodermal explants containing GSK3β. Furthermore, activation of XAG1 and Xotx2 transcription in ectodermal explants was not accompanied by NCAM and XIF3 expression. These findings are in a sharp contrast to concomitant activation of neural and cement gland markers in animal caps overexpressing noggin (Fig. 7). These observations indicate that formation of neural tissue and cement gland in the embryo overexpressing GSK3β depends on additional cooperating factors. We would like to hypothesize that GSK3β triggers only the initial step of neural induction involving activation of Xotx2 as well as cement gland markers. The second step, activation of NCAM expression and neural tissue patterning, would require further signaling from other regions of the embryo (see also Sive et al., 1989; Itoh and Kubota, 1991; Drysdale and Elginson, 1993).

Drosophila Shaggy/zw3 was proposed to function down-stream of Notch and upstream of achaete-scute gene products in a lateral inhibition pathway during Drosophila neurogenesis (Ruel et al., 1993). Interestingly, a Xenopus homologue of achaete-scute, XASH-3 (Turner and Weintraub, 1994; Ferreiro et al., 1994) was shown to cause expansion of the neural plate in embryos injected with XASH-3 mRNA, consistent with the idea that the function of achaete-scute genes is conserved in evolution. Since the neuralizing activity of XASH-3 was evident in the whole embryos, but not in animal caps, it was hypothesized that neural induction is required to reveal this XASH-3 activity (Turner and Weintraub, 1994; Ferreiro et al., 1994). Overexpression of a truncated form of Xotch (Xenopus homologue of Notch) that lacked most of the extracellular region led to neural and mesodermal hypertrophy and to inhibition of cement gland differentiation (Coifman et al., 1993).

**Fig. 7.** Overexpression of GSK3β mRNA activates transcription of anterior ectodermal markers in animal caps. Animal pole region of both blastomeres of the 2-cell embryos was injected with 2 ng of XGSK3β RNA (lane 1), with 0.4 ng of rat GSK3β RNA (lane 2), or with 0.05 ng of Noggin RNA (lane 3). Animal caps were excised from blastulae at stage 8 and cultured until the equivalent of stage 28 (A) or stage 11 (B), then, total explant RNA was extracted for northern analysis. Lane 4, control animal caps from uninjected embryos; lane 5, control sibling embryos. Cytoplasmic actin RNA cross-reacting with a muscle-specific actin probe (two upper bands) in A and fibronectin RNA in B indicate equal loading. RNA from 10 animal caps or from 2 embryos was loaded in each lane.
Although the developmental effects of Notch, Xotch, XASH-3 and GSK3β are not the same, the ability of GSK3β to modulate neuroectodermal cell fates is consistent with the idea that components of the Notch signaling pathway also operate in vertebrates. Further studies of Notch signal transduction should lead to better understanding of the molecular mechanisms involved.

In addition to its role in neurogenesis, Shaggy functions in the wingless signaling pathway operating during segmentation and during imaginal disc development in Drosophila (Perrimon, 1994). Interestingly, dorsalventral axis determination in Xenopus appears to involve vertebrate homologues of Drosophila segment polarity genes mediating wingless signaling. Wnt genes, Xenopus dishevelled and β-catenin are implicated in dorsal axis determination in Xenopus (Sokol et al., 1991; Smith and Harland, 1991; Sokol et al., 1995; Heasman et al., 1994). Recent studies have shown that overexpression of a presumed dominant negative form of GSK3β caused a secondary body axis formation in Xenopus embryos (Pierce and Kimelman, 1995; He et al., 1995; Dominguez et al., 1995). These results strongly support the view that the Wnt signaling pathway is conserved in both Drosophila and in vertebrate embryos. It remains to be investigated if Wnt signaling is also required for anteroposterior patterning of neuroectoderm.

Recently, it has been proposed that Notch and Wnt signaling are closely connected in Drosophila (Cousso and Martinez-Arias, 1994). Our results support the idea that both GSK3β and Wnts are involved in neural tissue determination. They are consistent with neuralizing properties of Xenopus dishevelled homologue (Sokol et al., 1995) as well as with the finding that Wnt1 is essential for mid/hindbrain development in mice (McMahon and Bradley, 1990). Future studies should evaluate how GSK3β interacts with its upstream regulators and downstream targets in order to define molecular mechanisms by which GSK3β modulates anterior ectodermal development.

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