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The Wnt/\(\beta\)-Catenin Pathway Posteriorizes Neural Tissue in Xenopus by an Indirect Mechanism Requiring FGF Signalling

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In order to identify factors involved in posteriorization of the central nervous system, we undertook a functional screen in Xenopus animal cap explants which involved coinjecting noggin RNA together with pools of RNA from a chick somite cDNA library. In the course of this screen, we isolated a clone encoding a truncated form of \(\beta\)-catenin, which induced posterior neural and dorsal mesodermal markers when coinjected with noggin in animal caps. Similar results were obtained with Xwnt-8 and Xwnt-3a, suggesting that these effects are a consequence of activating the canonical Wnt signalling pathway. To investigate whether the activation of posterior neural markers requires mesoderm induction, we performed experiments using a chimeric inducible form of \(\beta\)-catenin. Activation of this protein during blastula stages resulted in the induction of both posterior neural and mesodermal markers, while activation during gastrula stages induced only posterior neural markers. We show that this posteriorizing activity occurs by an indirect and non-cell-autonomous mechanism requiring FGF signalling.

Key Words: Xenopus; A/P patterning; Wnt signalling; \(\beta\)-catenin; FGF signalling.

INTRODUCTION

Nieuwkoop's classical “activation/transformation” model proposes that two phases of signalling, arising from the mesoderm, are instrumental in generating anterior–posterior (A/P) pattern in the developing central nervous system. An initial “activation” signal inducing only anterior neural tissue (forebrain) is followed by a “transformation” phase, where subsequent signalling specifies more posterior neural character (midbrain, hindbrain, spinal cord). The posteriorizing signal(s) has been proposed to be distributed as a spatial or temporal gradient, with the highest concentration inducing the most posterior structures (Nieuwkoop, 1952; Slack and Tannahill, 1992).

Several candidate molecules for the initial neuralising signal have been identified. These include: noggin (Lamb et al., 1993), chordin (Piccolo et al., 1996; Sasai et al., 1995), Xnr3 (Hansen et al., 1997), cerberus (Bouwmeester et al., 1996; Piccolo et al., 1999), and follistatin (Hemmati-Brivanlou et al., 1994), all of which act as antagonists of BMP signalling (Wilson and Hemmati-Brivanlou, 1997). Furthermore, evidence from chick supports an essential role for fibroblast growth factor (FGF) signalling in combination with Wnt signalling for full neural induction (Streit et al., 2000; Wilson et al., 2000, 2001). Candidates for the transforming signal include retinoic acid (RA) (Blumberg et al., 1997; Kolm et al., 1997), FGFs (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995), and members of the Wnt family (McGrew et al., 1995, 1997). However, the precise role of each of these pathways and how they are
integrated during the establishment of A/P pattern is not well understood.

Tissue transplantation experiments have revealed that somites and paraxial mesoderm can reprogramme the expression of Hox genes and other axial markers, thus inducing a more posterior neural character (Gould et al., 1998; Grapin-Botton et al., 1997; Itasaki et al., 1996; Muhr et al., 1997, 1999; Woo and Fraser, 1997). In addition, transgenic regulatory analyses have also revealed that signals from somitic mesoderm are required in normal development to initiate the proper A-P patterns of somitic mesoderm that are required in normal development to initiate the proper A-P patterns of somitic mesoderm (N.I. et al., 1997; Woo and Fraser, 1997). In addition, transgenic regulatory analyses have also revealed that signals from somitic mesoderm are required in normal development to initiate the proper A-P patterns of somitic mesoderm are required in normal development to initiate the proper A-P patterns of somitic mesoderm (Gould et al., 1998). This demonstrates that paraxial mesoderm is an important source of posteriorizing signals that contribute to the generation of neural patterning. Therefore, to identify factors in somitic mesoderm involved in the process of posteriorization, we performed a functional screen using Xenopus animal cap explants. Pools of synthetic RNA from a chick somite cDNA library (N.I. et al., unpublished results) were coinjected with noggin RNA, which alone induces tissue of an anterior character (Lamb et al., 1993). Explants were assayed for changes in their neural A/P character, induced by factors from the somite RNA pools, using a variety of posterior markers.

One clone isolated from this screen encoded a truncated form of β-catenin, a cytoskeletal component and an intracellular mediator of Wnt signalling. Cytosolic β-catenin is degraded by the proteosome using a mechanism that requires phosphorylation by GSK-3. Stimulation of cells by Wnt proteins leads to the inhibition of GSK-3 activity and stabilization of β-catenin, which subsequently translocates into the nucleus. There, it forms a complex with members of the TCF/LEF family of transcription factors and activates the expression of target genes (Cadigan and Nusse, 1997). The β-catenin clone isolated from our screen encoded an amino-terminal truncated protein lacking the first 87 amino acids, which contain the phosphorylation sites essential for regulation by GSK3 (Lu et al., 1997; Yost et al., 1996). This suggests that the protein product of this variant might be stable and capable of maintaining high levels of Wnt signalling through the canonical pathway.

The isolation of this variant of β-catenin in a screen for posteriorization provides further support for the involvement of the Wnt signalling pathway in A/P patterning of neural tissue. Hence, in this study, we investigated the mode of action by which β-catenin induces changes in the A/P character of noggin-treated animal caps. We demonstrate that truncated β-catenin induces both posterior neural and dorsal mesodermal markers when coinjected with noggin. Similar results were obtained with Xwnt8 and Xwnt3a, suggesting that these activities may be a general property of Wnt signalling. Furthermore, we utilized an inducible form of β-catenin to precisely control the timing of events and see whether it was possible to induce posterior neural markers in the absence of mesoderm during gastrula stages. We demonstrate that posteriorization mediated by the Wnt/β-catenin pathway occurs by a noncell-autonomous mechanism, which requires cell-to-cell contact. Finally, we show that downstream of the Wnt/β-catenin pathway, FGF signalling is necessary for induction of posterior neural markers, providing another link in the coordinated activities of these pathways.

MATERIALS AND METHODS

DNA Constructs and RNA Synthesis

To prepare GR-LEFΔNβCTA, LEFΔNβCTA (Vlieminckx et al., 1999) was amplified by PCR and fused in frame to the C terminus of the ligand binding domain of the human glucocorticoid receptor with a pentaglycine bridge (details upon request). Other constructs were as published: noggin (Smith and Harland, 1992), Xwnt8 (Christian et al., 1991), XFD (Amaya et al., 1991). Plasmids were linearized and used for in vitro synthesis of capped RNA by standard methods.

Xenopus Embryos, Microinjection, and Animal Cap Explants

Xenopus embryos were obtained by in vitro fertilization (Smith and Slack, 1983), chemically dejellied using 2% cysteine hydrochloride (pH 7.8–8.1), maintained in 10% normal amphibian medium (NAM; Slack, 1984) and staged according to Nieuwkoop and Faber (1967). Xenopus embryos were injected in 75% NAM containing 4% Ficoll type 400 (Sigma). Synthetic RNA was injected in a volume of 10 nl. Care was taken with respect to both the size of the animal caps and the stage of dissection, as previous work has shown that the ability of Wnt8 to trigger mesoderm formation in presumptive ectoderm can result from large caps or caps from later stages (stage 10) (Sokol, 1993). To avoid this problem, all Xenopus animal caps were dissected at stage 8 and the territory cut comprised less than 50% of the animal hemisphere, as recommended by Silvé et al. (2000). Caps were cultured in 75% NAM. Treatment with dexamethasone (Sigma) was done at a final concentration of 1 μM in 75% NAM or 10% NAM for animal caps or whole embryos, respectively. Dissociation of animal cap cells was achieved by culturing in calcium- and magnesium-free medium (CMFM; Sargent et al., 1986) in the absence or presence of 1 μM DEX.

Histology, β-Gal Staining, and Whole-Mount in Situ Hybridization

For histological analyses, specimens were fixed, sectioned, and stained as described (Green et al., 1990). For β-gal staining, tissues were fixed in MEMFA for 1 h at room temperature, washed in PBS containing 0.01% Tween 20, and stained in PBS containing 1 mg/ml X-gal, 5 mM K,Fet(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% NP-40. After staining, samples were refixed in MEMFA for 1 h and processed for in situ hybridization as described (Jones and Smith, 1999). Probes were BF-1 (Bourguignon et al., 1998), En-2 (Hemmati-Brivanlou et al., 1991), Krox-20 (Bradley et al., 1993), and Hoxb9 (Sharpe et al., 1987).

RNA Isolation and RT-PCR

Fifteen animal caps or two embryos were processed for RNA isolation by using the TRIzol reagent (Gibco BRL) according to the manufacturer's protocol.
Absence of mesoderm (McGrew et al., 1995, 1997). In contrast, we find that coinjection of increasing RNA concentrations of truncated β-catenin with noggin induces both posterior neural and dorsal mesoderm markers (Fig. 1). At low concentrations of β-catenin RNA (20 pg/embryo), we observe induction of En-2, Krox-20, and Hoxb9 (Fig. 1, lane 5), indicating a posteriorization of the noggin-induced neural tissue. At higher concentrations of β-catenin RNA (100 and 500 pg/embryo), we observe induction of NCAM and muscle actin, demonstrating that the posteriorization of the noggin-induced neural tissue is a direct consequence of activating the Wnt pathway. At low concentrations of β-catenin RNA (20 pg/embryo), we observe induction of En-2, Krox-20, and Hoxb9 (Fig. 1, lane 5), indicating a posteriorization of the noggin-induced neural tissue. At higher concentrations of β-catenin RNA (100 and 500 pg/embryo), we observe induction of NCAM and muscle actin, demonstrating that the posteriorization of the noggin-induced neural tissue is a direct consequence of activating the Wnt pathway. At low concentrations of β-catenin RNA (20 pg/embryo), we observe induction of En-2, Krox-20, and Hoxb9 (Fig. 1, lane 5), indicating a posteriorization of the noggin-induced neural tissue. At higher concentrations of β-catenin RNA (100 and 500 pg/embryo), we observe induction of NCAM and muscle actin, demonstrating that the posteriorization of the noggin-induced neural tissue is a direct consequence of activating the Wnt pathway.
the activation of the Wnt pathway or is secondary to the induction of mesoderm in the animal cap. To address this point, we designed experiments to test whether the two processes could be separated. We generated a dexamethasone (DEX)-inducible form of β-catenin, in which the LEF-1 DNA binding domain and the C-terminal trans-activation

**FIG. 2.** Xwnt8 induces posterior neural and dorsal mesodermal markers in animal caps when coinjected with noggin and ventral mesodermal markers when injected alone. (A) RT-PCR of animal caps assayed when sibling embryos reached stage 24. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while explants from embryos injected with noggin RNA (500 pg/embryo; lane 4) expressed NCAM and BF-1 but not posterior neural or mesodermal markers. Posterior neural markers and the dorsal mesodermal marker muscle actin were induced in explants coinjected with noggin and Xwnt8 (50 pg/embryo; lane 5). Xwnt8 RNA alone (lane 6) induced the ventral mesoderm marker α-globin and Hoxb9. (B) RT-PCR of animal caps assayed at stage 14 to examine mesodermal markers. Xwnt8 induced Xhox3 and coinjection of noggin resulted in an increase in the expression of Xbra and a reduction in Xhox3 expression. Embryo RNA was used as a positive control and −RT is embryo RNA processed without reverse transcriptase and served as a negative control. ef1α was used as loading control. (C) Morphology of animal caps fixed either at stage 18 (a–d) or stage 38 (e, f). Explants from uninjected (a), noggin-injected (b), and Xwnt8-injected (c) embryos did not elongate. Animal caps coinjected with noggin and Xwnt8 elongated (d). Animal caps from uninjected embryos (e, g) did not form vesicles, while those injected with Xwnt8 RNA (f, h) formed vesicles characteristic of ventral mesoderm.
domain of β-catenin (LEF\textsubscript{N}β\textsubscript{CTA}) (Vlaminckx et al., 1999) were fused to the ligand binding domain of the human glucocorticoid receptor, creating GR-LEF\textsubscript{N}β\textsubscript{CTA}. This should create a variant where the trans-activation domain is dependent upon the presence of DEX ligand to potentiate its activity. In agreement with this, GR-LEF\textsubscript{N}β\textsubscript{CTA} induced the formation of double axes and expression of the direct target genes siamois (Brannon et al., 1997) and Xnr3 (McKendry et al., 1997) in animal caps only after exposure to DEX (data not shown).

Animal caps lose their capacity to respond to mesoderm-inducing factors such as activin and FGF during gastrula stages (Green et al., 1990). If Wnt-mediated induction of posterior neural markers is independent of mesoderm formation, the activation of GR-LEF\textsubscript{N}β\textsubscript{CTA} during gastrula stages should lead to the induction of posterior neural markers in the absence of mesoderm. Therefore, we injected GR-LEF\textsubscript{N}β\textsubscript{CTA} RNA into the animal pole of 2-cell stage Xenopus embryos with and without noggin RNA and isolated animal caps at stage 8. We then added DEX at different stages, cultured the animal caps until late neurula stages, and examined gene expression by RT-PCR. In animal caps injected with noggin and GR-LEF\textsubscript{N}β\textsubscript{CTA}, addition of DEX at stage 9 induced the posterior neural markers En-2, Krox-20, and Hoxb9 and dorsal mesodermal markers (Figs. 3A and 3B, lane 6). Consistent with the idea of posteriorization, it also suppressed the anterior neural marker BF-1. However, when DEX was added at stage 12, while En-2 and Krox-20 were still induced, Hoxb9 and mesodermal markers were not induced and BF-1 was only partially suppressed (Figs. 3A and 3B, lane 7). No posteriorization or mesoderm induction was observed when DEX was added at stage 15 (Fig. 3A, lane 8) or when DEX was not present in the culture medium (Figs. 3A and 3B, lane 5). These results show that Wnt signalling can posteriorize neural tissue in the absence of mesoderm in a stage-specific manner.

We also investigated the properties of the inducible β-catenin variant in the absence of noggin. In animal caps injected only with GR-LEF\textsubscript{N}β\textsubscript{CTA}, DEX addition at stage 9 resulted in the induction of NCAM, BF-1, Hoxb9, and mesodermal markers with ventral character (Figs. 3A, lane 10 and 3B, lane 9). Addition of DEX at later stages did not induce any neural or mesodermal markers (Figs. 3A, lanes 11 and 12 and 3B, lane 10). To rule out the possibility that distinct activities of GR-LEF\textsubscript{N}β\textsubscript{CTA} at different stages were a consequence of degradation of the protein, we analysed the protein levels by Western blot using an anti HA antibody against a tag present in the construct (Fig. 3C). The levels of GR-LEF\textsubscript{N}β\textsubscript{CTA} remain stable until at least stage 16. This is consistent with other reports, indicating that GR fusion proteins are remarkably stable (Kolm and Sive, 1995; Tada et al., 1997).

Overall, these results show that the competence for induction of neural tissue by GR-LEF\textsubscript{N}β\textsubscript{CTA} is restricted to late blastula stages. Furthermore, activation of GR-LEF\textsubscript{N}β\textsubscript{CTA} at stage 9 has the ability to induce ventral mesoderm, which is dorsalized in the presence of noggin. This is consistent with our earlier experiments using the truncated form of β-catenin and Xwnt8 and confirms that the chimeric variant functions as an inducible modulator of Wnt signalling.

**Activation of GR-LEF\textsubscript{N}β\textsubscript{CTA} in Intact Xenopus Embryos**

To investigate the consequences of activating GR-LEF\textsubscript{N}β\textsubscript{CTA} in whole embryos, GR-LEF\textsubscript{N}β\textsubscript{CTA} and β-gal RNAs were coinjected in one animal/dorsal blastomere at the 8-cell stage. Embryos were cultured in the presence or absence of DEX and gene expression was analysed by in situ hybridization. DEX addition at stage 9 suppresses BF-1 expression (Fig. 4B) and shifts the expression domains of En-2, Krox-20, and Hoxb9 anteriorly (Figs. 4F, 4J, and 4N, respectively). Addition of DEX at stage 12 resulted in similar effects on the expression of BF-1, En-2, and Krox-20 (Figs. 4C, 4G, and 4K), but Hoxb9 was unaffected (Fig. 4O). Neural markers were unchanged by the addition of DEX at stage 15 or in the absence of DEX by comparison to the uninjected sides of the embryos (Figs. 4A, 4D, 4E, 4H, 4I, 4L, 4M, and 4P). These findings show that ectopic expression of GR-LEF\textsubscript{N}β\textsubscript{CTA} in whole embryos results in neural markers being expressed in more anterior locations, suggesting that anterior tissues acquire a more posterior character. Furthermore, the competence of animal caps for the posteriorizing action of GR-LEF\textsubscript{N}β\textsubscript{CTA} is similar to the response in intact embryos.

**Induction of the Posterior Neural Markers by GR-LEF\textsubscript{N}β\textsubscript{CTA} Occurs in a Noncell-Autonomous Manner**

Activation of the Wnt/β-catenin signalling pathway can induce posterior neural markers and suppress anterior character in both noggin-injected animal caps and in whole embryos. However, the molecular mechanism by which the pathway causes these effects is not clear. It has been suggested that En-2 is directly regulated by the Wnt pathway, in a mechanism that is dependent on TCF sites present in the En-2 promoter (McGrew et al., 1999). The induction of other posterior neural markers may occur directly or indirectly. To address this point, we investigated whether the induction of En-2 and Krox-20 by β-catenin occurs in a cell-autonomous manner. First, noggin and GR-LEF\textsubscript{N}β\textsubscript{CTA} were coinjected in one animal blastomere of 16-cell stage embryos with β-gal RNA as a lineage tracer. Animal caps were dissected at stage 8, cultured in the presence or absence of DEX, fixed at stage 20, and processed for β-gal staining followed by in situ hybridization with En-2 or Krox-20 probes. In the absence of DEX, no induction of En-2 or Krox-20 occurs (Figs. 5A and 5B). Addition of DEX at stage 9 (Figs. 5C and 5D) or stage 12 (Figs. 5E and 5F) resulted in the induction of En-2 and Krox-20. In all cases, induction of these genes occurred outside of the β-gal...
stained area. This suggests that a non-cell-autonomous
mechanism may be operating in the induction of these
genes by GR-LEF\textsubscript{DN}bCTA.

Concomitantly, we tested whether cell communication
within the animal cap is required for the induction of neural
markers. Animal caps from embryos injected with
\textit{noggin} RNA were cultured in the presence or
absence of DEX in calcium- and magnesium-free medium
(CMFM), which causes dissociation of cell-to-cell contact.
As a positive control, intact animal caps expressed posterior
markers (Fig. 6A, lanes 6 and 7) when DEX was added to the
culture medium. Dissociation of animal cap cells com-
pletely blocked the induction of the posterior neural mark-
ers and muscle actin and the suppression of BF-1 (Fig. 6A,
lanes 9 and 10). Induction of the known direct targets of the
Wnt pathway, siamois and \textit{Xnr3}, was not affected by the
dissociation treatment (Fig. 6B, lanes 5, 6, and 7, 8). These
results demonstrate that the induction of posterior neural
markers by GR-LEF\textsubscript{DN}bCTA occurs in a non-cell-
autonomous manner and requires cell-to-cell contact.

\section*{FGF Signalling Is Required for the Induction of
Posterior Neural Markers by GR-LEF\textsubscript{DN}bCTA}

The indirect nature of the posteriorisation by \(\beta\)-catenin
raises a question as to the identity of the signals that
mediate these inductive events. Possible candidates include
members of the FGF family of secreted proteins, which
induce posterior neural markers in \textit{noggin}-injected animal
caps and posteriorize neural plate explants (Cox and

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{A: An inducible form of \(\beta\)-catenin, GR-LEF\textsubscript{DN}bCTA, results in expression of both posterior neural and mesodermal markers when
activated at stage 9, but only posterior neural markers when activated at stage 12 in coinjections with \textit{noggin}. (A) RT-PCR of animal caps
analysed at stage 22. Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while explants from
embryos injected with \textit{noggin} RNA (500 pg/embryo; lane 4) expressed NCAM and BF-1 but not posterior neural or mesodermal markers.
Posterior neural and mesodermal markers were detected in tissues from embryos coinjected with \textit{noggin} RNA and GR-LEF\textsubscript{DN}bCTA RNA (50 pg/embryo; lanes 5–8) after the addition of DEX at stage 9 (lane 6). Posteriorization occurred in the absence of mesoderm induction when
DEX was applied at stage 12 (lane 7). When DEX was added at stage 15 (lane 8), neither posteriorization nor mesoderm induction occurred.
In the absence of \textit{noggin}, activation of GR-LEF\textsubscript{DN}bCTA at stage 9 but not at later stages induced the expression of NCAM, BF-1, and Hoxb9
(lane 10). (B) RT-PCR of animal caps assayed for mesodermal markers at stage 14. Mesodermal markers were detected in caps expressing
GR-LEF\textsubscript{DN}bCTA when DEX was added at stage 9 (lanes 6 and 9) but not at stage 12 (lanes 7 and 10). \textit{Noggin} dorsalized the mesoderm
induced by GR-LEF\textsubscript{DN}bCTA, as the expression of \textit{Xbra} increased and \textit{Xhox3} diminished. –RT is a negative control, embryo is a positive
control, and \textit{eF1\textalpha} served as loading control. (C) GR-LEF\textsubscript{DN}bCTA protein levels are similar between blastula and neurula stages. Embryos
were injected with 50 pg/embryo of GR-LEF\textsubscript{DN}bCTA RNA, ectodermal explants were dissected at stage 8 and collected at the indicated
stages. Two cap equivalents of protein were analysed by Western blotting using the mouse anti-HA monoclonal antibody 12CA5 after
SDS-PAGE. The band corresponding to GR-LEF\textsubscript{DN}bCTA is indicated by an arrowhead and the positions of molecular mass markers (kDa)
are indicated on the left of the panel.}
\end{figure}
Recent evidence has also shown that Wnt and FGF signalling work in concert to regulate early events in neural induction (Wilson et al., 2001). Furthermore, loss-of-function experiments have shown that FGF signalling is required for posterior neural development (Holowacz and Sokol, 1999; Ribisi et al., 2000).

To ask whether FGF signalling is required for the induc-

FIG. 4. Activation of GR-LEFI\textsubscript{N}\beta CTA in intact Xenopus embryos results in posteriorization of the neural tube. One animal/dorsal blastomere of 8-cell stage embryos was injected with 50 pg of GR-LEFI\textsubscript{N}\beta CTA RNA and 200 pg of \beta-gal RNA. Embryos were fixed at stages 18–22, processed for \beta-gal staining to reveal the injected side (light blue staining) and for whole-mount in situ hybridization with probes specific for BF-1 (A–D), En-2 (E–H), Krox-20 (I–L), and Hoxb9 (M–P) (purple staining). With no addition of DEX, the markers show an identical pattern of expression on the injected and uninjected sides (A, 24/25; E, 20/20; I, 20/20; M, 24/24). When DEX was added at stage 9, an anterior shift of the markers resulted (B, 25/30; F, 26/30; J, 25/28; N, 20/32). Addition of DEX at stage 12 affected BF-1 (C, 20/32), En-2 (G, 22/28), and Krox-20 (K, 17/25) but not Hoxb9 (O, 3/29). Addition of DEX at stage 15 did not affect any of the markers (D, 3/28; H, 2/27; L, 2/28; P, 1/25). The position of the markers is indicated with a white arrowhead on the injected side and a black arrowhead on the control side. Panels A–D are frontal views (dorsal is up) all other panels are dorsal views (anterior is up).
We coinjected embryos with noggin, GR-LEF\(^\Delta N\)\(\beta\)CTA RNA, and XFD, a truncated FGF receptor 1, which blocks FGF signalling (Amaya et al., 1991). Animal caps were isolated at stage 8 and cultured in the presence or absence of DEX. In caps coinjected with noggin and GR-LEF\(^\Delta N\)\(\beta\)CTA, addition of DEX at stage 9 led to the induction of the posterior neural markers and muscle actin (Fig. 7A, lane 6). Coinjection of 100 pg/embryo of XFD RNA suppressed both the expression of muscle actin and posteriorization of the explants. The expression of En-2, Krox-20, and Hoxb9 was reduced, while expression of BF-1 recovered (Fig. 7A, lane 7). Increasing the amount of XFD RNA to 500 pg/embryo resulted in a stronger suppression of the posterior neural markers (Fig. 7A, lane 8). In explants cultured in DEX from stage 12, the presence of XFD led to a complete suppression of En-2 and Krox20 (Fig. 7A, lanes 10 and 11). In summary, inhibiting FGF signalling with XFD suppressed the posteriorizing effect of GR-LEF\(^\Delta N\)\(\beta\)CTA.

XFD was introduced as RNA, which is translated and becomes active soon after injection. So, it was possible that the suppression of the posterior neural markers was due to an early effect on the competence of the animal cap to respond to the activation of GR-LEF\(^\Delta N\)\(\beta\)CTA. To address this question, we used the specific inhibitor of the tyrosine kinase activity of the FGF receptor, SU5402 (Calbiochem) (Mohammadi et al., 1997), which can be applied simultaneously with DEX. We injected embryos with noggin and GR-LEF\(^\Delta N\)\(\beta\)CTA RNA, dissected animal caps at stage 8, and cultured them in the presence of DEX and SU5402. Increasing amounts of SU5402 suppressed the induction of posterior neural markers either when added at stage 9 (Fig. 7B, lanes 7 and 8) or stage 12 (Fig. 7C, lanes 10 and 11), providing further evidence for the requirement of FGF signalling in the induction of posterior neural markers by GR-LEF\(^\Delta N\)\(\beta\)CTA.

These results suggest that Wnts might work in part through activation of FGFs. To investigate whether FGFs themselves are induced in animal caps following activation of GR-LEF\(^\Delta N\)\(\beta\)CTA, animal caps explanted from embryos injected with noggin and GR-LEF\(^\Delta N\)\(\beta\)CTA RNA were analysed by RT-PCR. Activation of GR-LEF\(^\Delta N\)\(\beta\)CTA at stages 9 and 12 induced FGF3 and FGF8 (Fig. 7C, lanes 6 and 7), while eFGF was induced only when DEX was added at stage 9. In the absence of noggin, FGFs are only induced when GR-LEF\(^\Delta N\)\(\beta\)CTA is activated at stage 9 (Fig. 7C, lane 9). These results show that FGFs are induced in response to Wnt/\(\beta\)-catenin signalling. This suggests that FGFs are a critical aspect of the noncell-autonomous inductive events following activation of Wnt/\(\beta\)-catenin pathway.

**DISCUSSION**

In this paper, we show that members of the Wnt pathway cooperate with noggin to induce posterior neural and dorsal mesodermal markers in animal caps. The use of an inducible form of \(\beta\)-catenin enabled us to demonstrate that during gastrula stages Wnt/\(\beta\)-catenin signalling can induce posterior neural markers in the absence of mesoderm. Furthermore, our results show that these activities occur by an indirect and noncell-autonomous mechanism requiring cell-to-cell contact. FGF signalling appears to be part of this
mechanism as we have shown that FGFs are upregulated by an inducible form of β-catenin and the induction of posterior neural markers by Wnt/β-catenin pathway requires FGF signalling. These findings raise a number of interesting issues with respect to Wnt signalling and A/P neural patterning in vertebrate embryos.

Wnt/β-Catenin Signalling in Neural Patterning and Mesoderm Induction

In this study, Xwnt8, β-catenin, and truncated β-catenin functioned in a similar manner when coexpressed with noggin in animal caps. Posterior markers such as En-2, Krox-20, and Hoxb9 were induced together with the dorsal mesoderm marker muscle actin. Members of the Wnt pathway have previously been shown to induce posterior neural markers in the absence of mesoderm (McGrew et al., 1995, 1997). The basis of these different results is not clear, but could be due to the amount of RNA injected. We observe the induction of posterior markers in the absence of mesoderm with low concentrations of β-catenin RNA. Wnt signalling has been reported to induce either dorsal (Sokol, 1993) or ventral mesoderm (Chakrabarti et al., 1992; Christian and Moon, 1993) in animal caps. These differences have been related to the size of the animal cap and the time at which the explant is isolated (Sokol, 1993). In our assays, activation of the Wnt/β-catenin signalling pathway in animal caps induced ventral mesoderm and coinjection of noggin led to the dorsalisation of this mesoderm, as reported previously (Lamb et al., 1993; Smith and Harland, 1992). Our results in explants confirm recent observations in whole embryos, where activation of the Wnt/β-catenin signalling pathway at late blastula stages, by the use of an heat-shock inducible system, led to the ectopic expression of ventral mesodermal markers (Hamilton et al., 2001).

These findings prompted us to ask whether or not the induction of the posterior neural markers by members of the Wnt pathway is dependent on the induction of mesoderm. Our results with the inducible GR-LEFΔNβCTA construct clearly show that, at stage 12, posteriorization of neural tissue can occur independently of mesoderm formation. Furthermore, the competence of the animal cap to respond to the posteriorizing effects of GR-LEFΔNβCTA ends by midneurula stages (Fig. 3A). The animal cap anal-

FIG. 6. Induction of posterior neural markers by GR-LEFΔNβCTA requires cell-to-cell contact. (A) RT-PCR analysis showing that intact explants from embryos coinjected with noggin (500 pg/embryo) and GR-LEFΔNβCTA (50 pg/embryo) RNA expressed posterior neural and mesodermal markers when DEX was added at stage 9 (lane 6) and only posterior neural markers when DEX was added at stage 12 (lane 7). Dissociation of the explants blocked the induction of the posterior neural and mesodermal markers (lanes 9 and 10). As a negative control, DEX was not added to the culture medium (lanes 5 and 8). (B) Dissociation of animal cap cells did not affect the induction of direct targets of TCF/β-catenin. Activation of GR-LEFΔNβCTA at stage 9 induced Siamois and Xnr3 in both intact (lane 6) and dissociated (lane 8) explants. As a negative control, DEX was not added to the culture medium (lanes 5 and 7). Animal caps were dissected at stage 8 and cultured intact (in 75% NAM) or dissociated in calcium- and magnesium-free medium (CMFM) until stage 11 (B) or stage 22 (A). Animal caps from uninjected embryos (lane 3) did not express neural or mesodermal markers, while noggin-injected explants expressed only anterior neural markers. eF1α served as loading control, –RT as a negative control, and embryo RNA as a positive control.
FIG. 7. FGF signalling is required for induction of posterior neural and mesodermal markers by GR-LEF\(\beta\)CTA. (A) XFD suppressed the induction of the posterior neural and mesodermal markers by GR-LEF\(\beta\)CTA. RT-PCR detected the induction of posterior and mesodermal markers in explants cojected with noggin (500 pg/embryo) and GR-LEF\(\beta\)CTA (50 pg/embryo) when DEX was added at stage 9 (lane 6). Coinjection of XFD RNA (100 pg/embryo) caused a reduction in the expression of En-2, Krox-20, and Hoxb9, suppressed muscle actin, and reestablished BF-1 expression (lane 7). Coinjection of 500 pg/embryo XFD RNA resulted in the suppression of all posterior neural markers (lane 8). When DEX was added at stage 12, XFD blocked the induction of En-2 and Krox-20 (lanes 10 and 11). In the absence of DEX, 500 pg/embryo XFD RNA caused a reduction in the expression of NCAM and BF1 (lane 13) (B) The FGF signalling inhibitor SU5402 blocked the induction of posterior neural markers when added simultaneously with DEX at stage 9 or stage 12. Both 10 \(\mu\)M (lanes 7 and 10) and 50 \(\mu\)M (lanes 8 and 11) of SU5402 suppressed the induction of posterior neural and mesodermal markers. Addition of 50 \(\mu\)M of SU5402 at stage 9 in the absence of DEX did not affect the expression of NCAM or BF1 (lane 12). (C) FGFRs are induced by GR-LEF\(\beta\)CTA. RT-PCR of animal caps analysed when sibling embryos reached stage 14 detected the expression of eFGF, FGF3, and FGF8 when DEX was added at stage 9 (lanes 6 and 9). FGF3 and FGF8 expression was also detected when DEX was added at stage 12 (lane 7). –RT is a negative control and embryo is a positive control. eF1\(\alpha\) served as loading control.

Mechanism of Induction of the Posterior Neural Markers by the Wnt/\(\beta\)-Catenin Pathway

Together, our data show that Wnt signalling posteriorizes neural tissue by an indirect mechanism. The noncell-autonomous induction of En-2 and Krox-20 expression by GR-LEF\(\beta\)CTA in animal caps is consistent with the localized expression of GR-LEF\(\beta\)CTA in whole embryos resulting in an anterior displacement of posterior neural markers outside of the injected cells (Fig. 4). Dissociation of animal caps blocked the induction of posterior neural markers but did not block the induction of the known direct targets of the Wnt/\(\beta\)-catenin pathway, siamois and Xnr3.

It has been suggested that En-2 is directly regulated by the Wnt/\(\beta\)-catenin pathway, via LEF/TCF sites present in the En-2 promoter (McGrew et al., 1999). These sites are required for the activation of a reporter by noggin and Xwnt3a in animal caps, and may be essential for the Wnt1-dependent expression of En-2 during development. However, in mice, Wnt3 was shown to be required for the maintenance of En-2 expression but not for its initiation (Danielian and McMahon, 1996; McMahon et al., 1992). It is possible, however, that other Wnt family members may play a direct role in the initiation of En-2 expression. The activation of the posterior neural markers by an indirect mechanism as revealed by our results indicates that activation of En-2 expression by Wnt/\(\beta\)-catenin signalling may also occur independent of these Lef/TCF sites.
dorsal mesoderm, which would then act on the ectodermal/ neural tissue.

**FGF Signalling Is Required for Wnt/β-Catenin Posteriorization**

We have shown that FGF signalling is required for the induction of posterior neural markers by the Wnt/β-catenin pathw.

FGF3 and FGF8 are induced in animal caps as a consequence of the activation of GR-LEF1/βCTA. In a previous report (M.Grew et al., 1997), FGF was shown to block the repression of the anterior gene Otx-2, but not the induction of En-2 and Krox-20, when co-injected with noggin and GR-LEF1/βCTA. In our experiments, increasing amounts of FGF RNA completely blocked the induction of all posterior markers, including En-2 and Krox-20, when GR-LEF1/βCTA was activated at stage 9 or stage 12. These results were confirmed by the ability of SU5402 to suppress the induction of the posterior neural markers when added simultaneously with DEX, suggesting that FGF signalling is required after the activation of GR-LEF1/βCTA.

Finally, we show that FGF3 and FGF8 are induced in animal caps as a consequence of the activation of GR-LEF1/βCTA. During late gastrula and early neurula stages, FGF3 (Tannahill et al., 1992) and FGF8 (Christen and Slack, 1997) are expressed in the posterior ectoderm and mesoderm, which is consistent with a role for these proteins as posteriorising factors. Xwnt8 is also expressed in the posterior/lateral mesoderm and has been suggested to induce expression of the posterior neural marker Pax3 (Bang et al., 1999). The requirement for FGF signalling and the induction of FGFs by Wnts suggests a model in which regulation of FGFs by Xwnt8 is essential for the promotion of posterior fates during embryonic development. In summary, our results have provided insight into some of the underlying mechanisms associated with the roles of Wnt signalling in A-P patterning and suggest that FGF signalling is an integral aspect of the posteriorizing action of the Wnt/β-catenin pathway.

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