A role for Syndecan-4 in neural induction involving ERK- and PKC-dependent pathways

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INTRODUCTION

The ‘default model’ of neural induction proposes that neural development occurs as a result of the inhibition of BMP signalling in the embryonic ectoderm, and that in the absence of cell-cell signalling, ectodermal cells will adopt a neural fate (Munoz-Sanjuan and Brivanlou, 2002; Weinstein and Hemmati-Brivanlou, 1997). There is compelling evidence that BMP signalling and its modulation by endogenous inhibitors are involved in the specification of neural and non-neural domains in Xenopus (Iemura et al., 1998; Piccolo et al., 1996; Sasai et al., 1994; Smith et al., 1993). However, several challenges to the default model have originated from studies in chick and ascidians, as well as from more recent experiments in Xenopus (for a review, see Stern, 2005). There is now convincing evidence that in addition to BMP inhibition, other signals are required for neural induction. One of these is FGF, which is involved in the induction of neural tissue in chick and Xenopus, as well as in Ciona (Hongo et al., 1999; Launay et al., 1996; Streit et al., 1997; Tannahill et al., 1992; Wilson et al., 2000; Bertrand et al., 2003). It has been proposed that FGF regulates neural induction in animal caps and in Xenopus embryos by activation of MAPK, which in turn phosphorylates the BMP target Smad1, contributing to the inhibition of BMP signalling (Fuentelba et al., 2007; Graves et al., 1994; Grunz and Tacke, 1989; Hartley et al., 1994; Kuroda et al., 2005; Pera et al., 2003; Sato and Sargent, 1989). However, it is not known how the activity of FGF is regulated in the embryo to account for its role during neural induction. As it is well established that several proteoglycans (PGs) can regulate the activity of FGF, in some cases working as co-receptors, we decided to study the role of PGs as potential modulators of FGF during neural induction.

PGs are extracellular glycoproteins that contain sulphated glycosaminoglycan (GAG) chains. Biochemical and cell culture assays have implicated PGs as co-regulators of many growth factors, including FGF, HGF, Wnt, TGFβ and BMP (Bernfield et al., 1999; Iozzo, 1998). The GAG chains can be of heparan, chondroitin or dermatan sulphate (Bernfield et al., 1999; Iozzo, 1998). Syndecan-4 (Syn4) is a heparan sulphate PG reported to modulate FGF signalling in vitro (Iwabuchi and Goetinck, 2006; Tkachenko et al., 2004; Tkachenko and Simons, 2002). In addition, Syn4 interacts with chemokines (Brule et al., 2006; Charnaux et al., 2005) and with the planar cell polarity (PCP) pathway (Matthews et al., 2008; Muñoz et al., 2006). As Syn4 also interacts with fibronectin and integrins and is required for the formation of focal adhesions (Woods and Couchman, 2001), its main role has been thought to be in cell migration. However, Syn4 is also able to modulate PKC- and small GTPase-dependent intracellular signalling (Bass et al., 2007; Horowitz et al., 1999; Horowitz and Simons, 1998; Keun et al., 2004; Matthews et al., 2008).

Here, we investigate the role of Syn4 in neural induction in Xenopus. We report that Syn4 is expressed in ectoderm and becomes restricted to the neural plate. Loss-of-function experiments show that Syn4 is required for neural induction, whereas misexpression of Syn4 can induce the expression of neural markers in animal caps or ventral ectoderm. We also report that Syn4 activates two parallel pathways: the FGF/ERK pathway, previously implicated in neural induction, and the PKC/ Rac/JNK pathway.

MATERIALS AND METHODS

Xenopus embryos, animal cap assay and microinjection

Xenopus embryos were obtained as described (Newport and Kirschner, 1982). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). For normal development, embryos were incubated in 0.1× Marc’s Modified Ringer’s Solution (MMR) until they reached the appropriate stage. Animal caps were dissected at stage 9 and analysed at stage 14. Injected mRNA was synthesised using the mMessage mMachine Kit (Ambion) following the manufacturer’s instructions. For the Rac1N17 experiments, we added a poly(A) sequence that was not included in the original clone (Tahinci and Symes, 2003). Grafting of neuroectoderm has been described (Linker and Stern, 2004). For 32-cell stage injection, the cell lineage was as described (Moody, 1987).

Morpholino oligonucleotide and whole-mount in situ hybridisation

The Syn4 morpholino oligo (MO) was the same as that described previously (Muñoz et al., 2006; Matthews et al., 2008). For rescue experiments, we used point-mutated Syn4 as described (Matthews et al., 2008).
For in situ hybridisation, we followed the procedures described by Harland (Harland, 1991), with the modifications described by Kuriyama et al. (Kuriyama et al., 2006).

Western blot
SDS-PAGE and blotting were performed using NuPAGE Novex Bis-Tris Gels (Invitrogen) following the manufacturer’s instructions, and PVDF membrane (Amersham) was used for transfer blotting. Samples were taken from animal caps at the appropriate stages, and homogenised with buffer containing anti-phosphorylation reagent (Sigma) and protease inhibitor cocktail (Roche). Antibodies for p42/44 MAPK and phosphorylated p42/44 MAPK were used at 1/1000 (Cell Signaling) in 4% BSA in TBST, and anti c-Fos antibody (Santa Cruz) was used at 1/400 in 10% horse serum in TBST. After three washes, anti-rabbit IgG (H+L) horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch) was applied as secondary antibody at 1/25,000. Signal was visualised with luminescent HRP substrate and exposed to film (Fuji).

Confocal microscopy
The mRNA for fluorescent fusion proteins (PKCδ-EGFP or PKCα-EGFP) was injected at the 2-cell stage in both blastomeres. The membrane was visualised by co-injection of mRNA for membrane monomeric Cherry (mCherry) protein. In Fig. 5, the animal caps were dissected at stage 5, treated with 2 μM phorbol ester (Sivak et al., 2005) or 10 ng/ml FGF2 (R&D), and fixed in MEMFA for 20 minutes. In Fig. 6, mCherry mRNA with MO was injected into 16-cell stage embryos after injection of PKCα-EGFP mRNA at the 2-cell stage. Images were taken with a Leica SP2 confocal microscope.

Clones and constructs
Full-length cDNA clones (NIBB) were used for the analysis of Syn4 expression; these give a stronger signal than the probe previously published by Muñoz et al. (Muñoz et al., 2006). The National Institute for Basic Biology (Japan) reference number of Syn4.1 is XL201e11, and Syn4.2 is XL457P08aex. The cDNA of the c-Fos gene was isolated from X. laevis neurula cDNA, and initially cDNA containing the 3’ UTR was amplified by RT-PCR with the following primers (sequences according to EST clone MGC80305): Xbal-Xi-c-Fos Fw, 5’-CCGTCTAGAACAGAGCAGGAT-3’ and Xl-c-Fos Rv, 5’-ACAGAATTCACAACAA-3’. Xenopus laevis c-Fos shares 63-65% identity with c-Fos from other species (data not shown). The Xenopus laevis c-Fos ORF was amplified using the following primers: ClaI-c-Fos Fw, 5’-ATATCGTATACAGGCAAGGATTTGCAATTATA-3’ and Xi-c-Fos Rv, 5’-ACAGAATTCACAACAA-3’. Xenopus laevis c-Fos ORF was subcloned into the pCS2+-GR vector. After checking that there were no mutations in the sequence, the ORF was subcloned into the pCS2+-GR vector.

Rac activation assay
Rac activity was analysed using the Rac1 Activation Assay Biochem Kit (Cytoskeleton). Animal caps were dissected at stage 9 and cultured until stage 10.5 (data not shown) or 11.5. The total amount of protein used to bind the PAK-RBD beads was adjusted in a pilot experiment using Bradford analysis (BioRad). Around 100 animal caps were dissected for each condition, cell lysates were centrifuged to remove the yolk fraction, and the supernatants were used for the GDP/GTP-binding reaction according to the manufacturer’s instructions. Positive (GTP-bound) and negative (GDP-bound) controls were performed as described by the manufacturer. For pulldown of active Rac, 10 μg of PAK-RBD beads was applied to each sample, boiled with Laemmli sample buffer (Invitrogen) and loaded onto the gel.

RESULTS
Syn4 is required for neural induction
We started by examining the expression of Syn4 by whole-mount in situ hybridisation. As the mesodermal expression of Syn4 has already been described (Muñoz et al., 2006), we focused on the ectodermal expression pattern. A longer Syn4 probe (see Materials and methods), which gives stronger staining than the probe described by Muñoz et al. (Muñoz et al., 2006), allowed us to characterise the neuroectodermal expression of Syn4. During blastula stages, Syn4 is expressed transiently in a wide region of the ectoderm (Muñoz et al., 2006) and very quickly becomes enriched in the prospective neural tissues at the early and mid-gastrula stages (Fig. 1A-D). During early neurula stages, Syn4 expression was detectable only in the neural plate (Fig. 1E,G,I), resembling the expression of the neural plate marker Sox2 (Fig. 1F,H,J). To determine whether Syn4 is required for neural induction, we performed loss-of-function experiments using a mixture of two antisense morpholino oligonucleotides (Syn4 MOs), as previously reported (Muñoz et al., 2006; Matthews et al., 2008). Injection of Syn4 MO into dorsal animal blastomeres at the 8-cell stage produced a strong inhibition of the neural plate markers Sox2 and Nrp1 on the injected side, whereas no inhibition was observed when a control MO was injected into ventral animal blastomeres at the 8-cell stage (data not shown). To test the effect of Syn4 MO on the expression of Syn4, we used a synthetic RNA encoding a shorter Syn4 transcript (Syn4 XL457P08ex). The cDNA of the c-Fos ORF was amplified using the following primers: ClaI-c-Fos Fw, 5’-ATATCGTATACAGGCAAGGATTTGCAATTATA-3’ and Xi-c-Fos Rv, 5’-ACAGAATTCACAACAA-3’ and Xl-c-Fos Rv, 5’-ACAGAATTCACAACAA-3’. Xenopus laevis c-Fos shares 63-65% identity with c-Fos from other species (data not shown). The Xenopus laevis c-Fos ORF was amplified using the following primers: ClaI-c-Fos Fw, 5’-ATATCGTATACAGGCAAGGATTTGCAATTATA-3’ and Xi-c-Fos Rv, 5’-ACAGAATTCACAACAA-3’. Xenopus laevis c-Fos ORF was subcloned into the pCS2+-GR vector. After checking that there were no mutations in the sequence, the ORF was subcloned into the pCS2+-GR vector.

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Fig. 1. Dynamic expression of Syn4 in the neural plate region. Whole-mount in situ hybridisation analysis of Syn4 and Sox expression. (A) Lateral view of a stage 10.5 Xenopus embryo showing Syn4 expression in the dorsal marginal zone. Dorsal (d) to the right; ventral (v), left; animal pole to the top. (B) Fate map of a stage 10.5 embryo, shown in the same orientation as in A. NP, prospective neural plate; m, prospective mesoderm. (C) At stage 12, Syn4 expression (arrowheads) is restricted to the dorsal region of the embryo (orientation as in A). (D) Fate map of a stage 12 embryo, shown in the same orientation as in C. NP, neural plate. (E) At stage 14, Syn4 expression is seen in the neural plate, but is absent from the dorsal midline (arrowhead). Dashed line indicates the plane of the section in G. (F) Stage 14 embryo showing Sox2 expression. The expression pattern is similar to that of Syn4 in E. Dashed line indicates the plane of the section in H. Arrowhead, dorsal midline. (G) Section of a stage 14 embryo, showing Syn4 expression. No expression is observed at the midline or in mesoderm. (H) Section of a stage 14 embryo, showing Sox2 expression. (I) At stage 16, Syn4 expression is seen in the neural plate. (J) Sox2 expression at stage 16.
was injected (Fig. 2A,B,E,F). A similar inhibition of neural plate markers was observed when the injected embryos were analysed at later stages, indicating that the effect of Syn4 MO is not merely a delay in gene expression, but a true inhibition (see Fig. S1A,B in the supplementary material). As the injection at the 8-cell stage might target some mesodermal cells and affect neural induction indirectly, we used two approaches to inhibit Syn4 selectively in the prospective neural plate. First, Syn4 MO was injected at the 32-cell stage into the A1 blastomere, which is fated to contribute to the neural plate but not to the mesoderm (Moody, 1987). Sox2 expression was inhibited in descendants of these Syn4 MO-injected cells (Fig. 2C). This is specific for Syn4 because it could be rescued by co-injection of mRNA encoding a mutated Syn4 that does not bind to the MO (Fig. 2D). As an alternative approach, prospective neural plate tissue taken from an early neurula embryo injected with Syn4 MO or control MO was grafted into the early neurula of an un.injected host, creating an embryo in which Syn4 MO is present only in the neural plate. The control graft still showed normal Sox2 expression (Fig. 2G), whereas grafts of Syn4 MO-injected tissue showed loss of Sox2 expression (Fig. 2H, asterisk). These results show that Syn4 is required in the ectoderm for neural plate induction.

To analyse the mechanism by which Syn4 MO blocks neural plate development, we asked whether it might interfere with BMP signalling. Inhibition of BMP signalling by a combination of BMP antagonists causes expansion of the early expression of Sox2 in the embryo and in animal caps analysed at stage 11 (Fig. 2LJLM) (Rogers et al., 2008). When co-injected with these BMP antagonists, Syn4 MO still blocked Sox2 expression in the embryo and in animal caps (Fig. 2K,N), suggesting that Syn4 does not function as a BMP antagonist in neural induction. Furthermore, if Syn4 is a BMP antagonist, it would be expected to dorsalise mesoderm and to induce a secondary axis, as do all BMP antagonists (Harland, 1994). Whereas chordin mRNA did induce a secondary axis, no such effect was observed after injection of Syn4 mRNA (see Fig. S2A,B in the supplementary material), consistent with the notion that Syn4 does not block BMP signalling.

**Overexpression of Syn4 neuralsises the ectoderm**

The above results suggest that Syn4 is required for neural plate formation. To test whether Syn4 can induce a neural fate, Syn4 mRNA was injected at the 32-cell stage into the A4 blastomere (which does not contribute cells to the neural plate) (Moody, 1987). Inhibition of BMP in this blastomere does not induce neural tissue (Linker and Stern, 2004). By contrast, injection of Syn4 mRNA into A4 did lead to induction of Sox2 and Sox3 in the ventral epidermis (Fig. 3A,B; see Fig. S2C,D in the supplementary material) and to inhibition of epidermal marker expression (Fig. 3C,D), without induction of mesodermal markers (Fig. 3G-J). The induction of neural markers by Syn4 is not transient, as they were still expressed at the late neurula stages (see Fig. S2H,I in the supplementary material). Interestingly, this neuralisation by Syn4 was not blocked by co-injection of a MO against chordin (see Fig. S2E-G in the supplementary material) (Oelgeschläger et al., 2003), which is consistent with the idea that neural induction by Syn4 is BMP independent. Furthermore, this induction of Sox2 is cell-autonomous to the descendants of the injected cell, as revealed by co-injection of nuclear β-galactosidase as a lineage tracer: all Sox2-positive, epidermal keratin (EpK)–negative cells exhibited X-Gal staining in the nucleus (Fig. 3E,F). Finally, overexpression of Syn4 induced neural plate markers and inhibited epidermal markers in isolated animal caps (Fig. 3K-N; see Fig. S2J,K in the supplementary material), without expression of mesodermal markers (not shown). Together, these gain- and loss-of-function experiments support a role for Syn4 in neural plate development.

**The GFG/MAPK signalling pathway is required for neural induction by Syn4**

As Syn4 is a proteoglycan that binds growth factors, including FGF, through its extracellular GAG chains, but can also modulate intercellular signalling through its intracellular domain (Couchman,
we tested a set of deletion constructs of Syn4 for neuralising ability. mRNA for each of these constructs was injected into the A4 blastomere of a 32-cell stage embryo and their ability to induce neural tissue was compared with that of full-length Syn4 mRNA.

Deletion of the GAG-binding domain (Syn4ΔGAG) caused a modest, but reproducible, loss of neural induction ability (see Fig. S3 in the supplementary material), whereas deletion of the intracellular domain (Syn4ΔCytCherry) had a stronger effect (see Fig. S3 in the supplementary material). Together, these experiments implicate both the extracellular and intracellular domains of Syn4 in neural induction (see Fig. S3 in the supplementary material).

Syn4 is known to modulate FGF activity (Tkachenko et al., 2004) and FGF is involved in neural induction (Fuentealba et al., 2007; Kuroda et al., 2005; Streit et al., 2000; Wilson et al., 2000). This raises the possibility that the effects of Syn4 gain- and loss-of-function are due to interference with FGF signalling. Neural induction by Syn4 in animal caps (Fig. 4A,B) was inhibited by co-injection of a dominant-negative FGF receptor (XFD-1) (Fig. 4C), as well as by the presence of the FGF receptor inhibitor SU5402 (Fig. 4D) or the MEK inhibitor U0126 (Fig. 4E), but not by the inactive analogue U0124 (Fig. 4F). Moreover, Syn4 induced phosphorylation of MAPK in animal caps cultured to stage 12.5 (the stage at which neural induction was analysed) (Fig. 4G, lanes 1, 2). Note that although FGF promotes MAPK phosphorylation at early gastrula stages (Sivak et al., 2005), this effect is not maintained when...
the animal caps are cultured until stage 12.5 (Fig. 4G, compare lanes 1 and 3). However, a high level of MAPK phosphorylation was observed at these stages in animal caps treated with FGF and Syn4 (Fig. 4G, lane 4). Together, these results indicate that Syn4 cooperates with FGF to activate the FGF/MAPK signalling pathway and that this activation is required for neural induction.

**Syn4 inhibits the PLC-PKC pathway**

The above results implicate the extracellular GAG-binding domain of Syn4 in neural induction. However, other experiments presented above revealed that deletion of the intracellular domain of Syn4 has an even stronger effect on neural induction.

The PLC/PKC pathway is involved in both FGF and Syn4 signalling (Simons and Horowitz, 2001; Sivak et al., 2005). Moreover, PKC has been implicated in neural induction (Otte et al., 1988; Otte et al., 1989; Otte et al., 1990; Otte and Moon, 1992), although this has never been clearly connected with FGF or BMP signalling. Activation of the PLC/PKC pathway by FGF leads to translocation of PKCδ to the membrane (Kinoshita et al., 2003; Sivak et al., 2005). We tested whether this change in localisation is Syn4 dependent. PKCδ-GFP distribution was diffuse in untreated animal caps (Fig. 5A-C), but on addition of the PKC activator, phorbol ester (PMA), or of FGF2, the fusion construct translocated to the cell membrane and colocalised with membrane Cherry (Fig. 5G-L). Strikingly, overexpression of Syn4 not only failed to promote PKCδ-GFP membrane translocation (Fig. 5D-F), but also inhibited translocation triggered by FGF2 (Fig. 5M-O). These results suggest that Syn4 modulates FGF signalling by inhibiting PKCδ activity.

**PKCδ and PKCα as downstream effectors of Syn4 during neural induction**

As Syn4 inhibits PKCδ activity and induces neural tissue, we asked whether direct inhibition of PKCδ is sufficient to neuralise ventral ectoderm. Injection of a dominant-negative PKCδ RNA (DN-PKCδ) (Kinoshita et al., 2003) into the A4 blastomere induced Sox2 (Fig. 6A), whereas injection of wild-type PKCδ mRNA into the endogenous neural plate region led to inhibition of neural plate marker expression (see Fig. S1C,E in the supplementary material). Furthermore, neural induction by Syn4 mRNA was inhibited by co-injection of PKCδ mRNA (Fig. 6B). These results support the conclusion that Syn4 inhibits PKCδ expression and that this inhibition is required for the neuralising activity of Syn4.

To understand more about the mechanism of neural induction by Syn4, we analysed some candidate downstream effectors of this PKCδ inhibition. It has been shown in many systems that PKCδ and PKCα activities repress each other (Kinoshita et al., 2003; Choi and Han, 2002) and that PKCα is implicated in neural induction (Otte et al., 1988). Consistent with these findings, we found that injection of PKCα mRNA into the A4 blastomere induces Sox2 (Fig. 6C) and inhibits EpK (see Fig. S1H in the supplementary material), whereas co-injection of PKCδ mRNA (Fig. 6D) blocks this process.

In conclusion, our data support the hypothesis that activation of PKCα and inhibition of PKCδ promote neural induction, and that these two kinases antagonise each other. The inhibition of PKCδ expression by Syn4 mRNA and the inhibition of PKCδ expression by PKCα mRNA prompted us to analyse the relationship between Syn4 and PKCα in neural induction. We found that the induction of Sox2 by PKCα mRNA (Fig. 6E) is inhibited by co-injection of Syn4 MO (Fig. 6F), whereas dominant-negative PKCα RNA blocks neural induction by Syn4 (Fig. 6G). Observations in cultured cells indicate that Syn4 recruits phosphatidylinositol 4,5-bisphosphate (PIP2) and translocates PKCα to the membrane (Keum et al., 2004).

We analysed the localisation of a PKCα-EGFP fusion protein that retains its neuralising activity when injected into ventral ectoderm (Fig. 6H). PKCα-EGFP expressed in animal caps showed a spontaneous membrane localisation that was not affected by co-injection with control MO (Fig. 6I-K). However, mosaic expression of Syn4 MO (cells labelled with an asterisk in Fig. 6L-N) led to a complete absence of PKCα-EGFP from the membrane, indicating that Syn4 is required for the activation of PKCα. We therefore propose that neural induction by Syn4 is mediated by activation of PKCα and that this activation requires Syn4.

**Syn4 induces neural tissue in a MAPK- and PKCα-dependent manner.** What is the link between the MAPK and PKCα activities? PKCα is known to activate MAPKs, including p38-MAPK, ERK and JNK (Mauro et al., 2002; Rucci et al., 2005; Seo et al., 2004; Skeletzorowski et al., 2005; Wensheng, 2006). However, we found no evidence that neural induction by PKCα depends on MAPK. First, induction of Sox2 in animal caps by PKCα (Fig. 7A, lane 3) was not inhibited by the MEK inhibitor U0126 (Fig. 7A, lane 4), in spite of the strong inhibition of phosphorylated MAPK (p-MAPK in Fig. 7A, lane 4). Second, PKCα did not affect the phosphorylation of MAPK, as analysed by western blot (Fig. 7A,B). Therefore, our results do not support a direct link between MAPK activity and PKCα during neural induction, a discovery that prompted us to look for downstream effectors of PKCα in neural induction.

**Rac/AP-1 as downstream effectors of PKCα during neural induction**

We have recently shown that Syn4 is a repressor of the small GTPase Rac during neural crest migration in vivo (Matthews et al., 2008), whereas the Syn4/PKC/Rac/RhoA signalling complex...

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**Fig. 5. Membrane translocation of PKCδ by Syn4.** *Xenopus* animal caps analysed by confocal microscopy after injection/treatment as indicated. (A-C) Control animal cap shows cytoplasmic localisation of PKCδ. (D-F) Animal caps injected with Syn4 mRNA. PKCδ shows cytoplasmic distribution. (G-L) Phorbol ester (PMA; G-I) or FGF2 (J-L) triggers the translocation of PKCδ into the membrane. (M-O) Syn4 inhibits the translocation of PKCδ activated by FGF2.
appears to be a key regulator of cell migration in vitro (Couchman, 2003). Could a similar pathway be involved in neural induction? Our results showed that the normal levels of Rac activity found in a control animal cap (Fig. 7C, AC lane 2) are strongly inhibited by expression of Syn4 (Fig. 7C, Syn4 lane 4). Furthermore, our data suggest that the inhibition of Rac activity by PKCa is a requirement for neural induction. Neural induction by PKCa misexpression (Fig. 7D) was inhibited by co-injection of a constitutively active form of Rac (Fig. 7E,F). In addition, expression of active Rac in the neural plate led to inhibition of the endogenous neural plate (see Fig. S1D,F in the supplementary material). By contrast, injection of a dominant-negative form of Rac into the A4 blastomere strongly induced Sox2 (Fig. 7G), supporting the hypothesis that inhibition of Rac activity by Syn4/PKCa can induce neural tissue.

Hitherto, the Syn4/PKCa/RhoA/Rac pathway has only been implicated in cell migration. Our data suggest that it also has an important role in cell specification. What could be the downstream target of Rac that is required for neural plate development? Rac is known to activate the c-Jun NH2 kinase (JNK), which promotes dimerisation of c-Jun and downregulates formation of the AP-1 complex (c-Jun/c-Fos) (Boyle et al., 1991). Previous reports suggest that the AP-1 complex is required for neural induction and that it binds directly to the promoter of the neural plate gene Zic3 during this inductive process (Leclerc et al., 1999; Lee et al., 2004). One hypothesis is that the PKCa/Rac pathway facilitates formation of the heterodimeric AP-1 complex (c-Fos/c-Jun) during neural induction. To test this hypothesis, we constructed a hormone-inducible derivative of *Xenopus* c-Fos that cannot homodimerise (Halazonetis et al., 1988; Nakabeppu et al., 1988); it will only form the heterodimer c-Fos/c-Jun when c-Fos is overexpressed, and is therefore expected to increase formation of the AP-1 complex. A similar approach has been described using c-Fos-ER to activate AP-1 in fibroblasts (Reichmann et al., 1992). We made a construct containing the *Xenopus* c-Fos gene fused to the human glucocorticoid receptor (GR; see Materials and methods). Ectopic expression of c-Fos-GR was achieved by injecting mRNA into the A4 blastomere and adding dexamethasone at stage 10.5, which induced Sox2 (Fig. 7H), whereas embryos that were not treated with dexamethasone did not upregulate Sox2 (Fig. 7I). In addition, western blot analysis revealed that PKCa increases c-Fos protein levels in animal caps (Fig. 7B).

Overexpression of c-Fos-GR was also used to rescue neural induction inhibited by activation of Rac. Animal caps injected with chordin mRNA expressed Sox2 (Fig. 7J,K), and, as expected, this induction was inhibited by expression of activated Rac (Fig. 7L). However, this inhibition of neural induction could be reversed by activation of the c-Fos-GR construct with dexamethasone (Fig. 7M). It should be noted that activation of c-Fos-GR is sufficient to neuralise the animal caps (Fig. 7N,O). In conclusion, our data are consistent with the idea that PKCa promotes the formation of AP-1 complexes that are required for neural induction through the inhibition of Rac.

### DISCUSSION

Here we demonstrate that Syn4 plays an important role in neural induction and identify the signalling pathways required for neural induction by Syn4. Inhibition of Syn4 in the ectoderm of whole embryos or in animal caps leads to strong inhibition of neural plate markers. Overexpression of Syn4 in ventral epidermis or animal caps is sufficient to induce neural tissue. At least two parallel signalling pathways are involved in this neural induction: FGF/MAPK and PKCa/Rac/AP-1. We propose that the localised expression of Syn4 in the neural plate is required to modulate these two pathways.

The role of Syn4 during *Xenopus* development has recently been analysed, revealing its key role as a new element of the PCP pathways during convergent extension and neural crest migration (Muñoz et al., 2006; Matthews et al., 2008). The apparent lack of
any effect on neural plate or neural crest induction in these previous reports is likely to be due to the targeting of different regions of the embryo. In order to see the effect of Syn4 MO on neural induction, the injection has to be targeted to the prospective neuroectoderm, whereas injections into prospective mesoderm, as published by Muñoz et al. (Muñoz et al., 2006), lead to convergent extension defects. In addition, Syn4 is expressed in neural crest cells just before their migration starts, once they are already specified (Matthews et al., 2008) (and this work), which explains why the MO does not affect neural crest induction. Taken together, these previous publications and the data presented here indicate that the same signalling molecule can be involved in induction and cell migration at different times during development.

Although early findings implicating FGF in neural induction (Lamb and Harland, 1995; Alvarez et al., 1998; Hongo et al., 1999) were controversial, the evidence is now strong that FGF is indeed involved in the induction of neural tissue (Susai et al., 1994; Smith et al., 1993; Launay et al., 1996; Linker and Stern, 2004; Pera et al., 2003; Streit et al., 1998; Streit et al., 2000; Wilson et al., 2000). Moreover, FGF contributes to the inhibition of BMP signalling, at least in part by phosphorylation of Smad1 during neural induction (Fuentealba et al., 2007; Kuroda et al., 2005).

Syn4 modulates FGF signalling through its extracellular domain (containing the GAG-binding region, which will present heparin sulphates to which FGF is expected to bind) and by an effect on the transduction of intracellular signals (Hou et al., 2007; Iwabuchi and Goetinck, 2006; Horowitz et al., 2002). Our data support the idea that FGF is required for neural induction and that Syn4 is a likely modulator, by showing that the inhibition of FGF receptor and of MAPK activity impair neural induction by Syn4. Syn4 could act as a co-receptor of the FGF receptor (Hou et al., 2007) or as a presenter of the FGF ligand, through binding of FGF to the GAG side-chains, to facilitate the activation of FGF receptor (Fig. 8A).

However, Syn4 also plays a separate role in neural induction involving PKC (Fig. 8B). We propose that this involves inhibition of PKCδ and activation of PKCα, and that PKCα is an inhibitor of
that overexpression of PKCα inhibits the formation of the c-Jun/c-Fos dimers that form part of the AP-1 transcriptional regulator complex. Thus, the inhibition of Rac by PKCα inhibits Rac activity. Rac activates JNK, which phosphorylates c-Jun and inhibits JNK.

Studies of the preneural gene Zic3 revealed that AP-1 binds directly to the Zic3 promoter rather than to the c-Jun homodimer (Lee et al., 2004). Taken together, these data suggest that during neural induction, Syn4/PKCα might inhibit Rac to minimise JNK activity, facilitating formation of the c-Fos/c-Jun (AP-1) complex.

A role for PKCα in neural induction was first suggested almost 20 years ago (Otto et al., 1988; Otto et al., 1989; Otto et al., 1990; Otto et al., 1991; Otte and Moon, 1992) but had never been connected with the signalling pathways now known to be involved in neural induction. It was originally shown that PKCα is activated and translocated to the membrane during neural induction, and it was suggested that this is required to confer neural competence on the ectoderm (Otto et al., 1988; Otto et al., 1989; Otto et al., 1990; Otto et al., 1991; Otte and Moon, 1992). We have confirmed and extended these observations by showing that expression of PKCα in ventral ectoderm or in animal caps can act as a neuralising signal and that PKCα activity is regulated by interactions with Syn4 and PKCδ. PKCδ appears to work as a repressor of PKCα, whereas Syn4 appears to be required for PKCα activity; however, we also show that PKCα is required for the neuralising activity of Syn4. Thus, our finding allows us to propose a link between the PKC and FGF pathways, both of which have been identified previously as being involved in neural induction.

These observations have parallels in studies of migrating cells. Syn4 interacts with PIP2, and this stabilises the oligomeric structure of Syn4 and promotes the association of PKCα and Syn4 (Oh et al., 1997a; Oh et al., 1997b; Horowitz and Simons, 1998; Lim et al., 2003); the catalytic domain of PKCα binds to the cytoplasmic domain of Syn4, and PKCα is ‘superactivated’ (Lim et al., 2003; Murakami et al., 2002). This interaction between PKCα and Syn4 provides a satisfactory explanation for our observation that neural induction by Syn4 requires PKCα and vice versa. In addition, during cell migration, PKCδ phosphorylates Syn4, decreases its affinity for PIP2 and abolishes its capacity to activate PKCα (Couchman et al., 2002; Murakami et al., 2002). We have found a similar negative regulation between PKCα and PKCδ during early neural plate development.

Despite several previous reports demonstrating direct phosphorylation of MAPK by PKCα (Mauro et al., 2002; Seo et al., 2004), we found no evidence that the PKC and MAPK pathways interact during neural induction other than indirectly, through Syn4. Neuronalisation by PKCα is evidently MAPK-independent and PKCα does not affect MAPK activity. Another possibility is that Rac can affect MAPK signalling via PAK-MEK interactions, the amino acids T292 and S298 of MEK1 being essential for PAK-dependent ERK activity (Ehlen et al., 2002). However, T292 is not conserved in Xenopus MEK1 (not shown), which could explain the absence of this regulatory pathway.

During cell migration, targets of the PKC pathway include small GTPases that control cytoskeletal organisation and adhesion to the extracellular matrix (Ridley et al., 2003). Our results suggest that Syn4/PKCα inhibits Rac activity during neural induction, as it does in migrating cells (Bass et al., 2007; Matthews et al., 2008). Expression of a dominant-negative Rac neuralises ventral ectoderm strongly, whereas activation of Rac inhibits neural induction by PKCα. However, activation of Rac in ventral ectoderm has no effect on neural plate markers, but induces neural crest markers (not shown), supporting recent reports of induction of neural crest by Rac/Rho activities (Broders-Brondon et al., 2007; Guemar et al., 2007).

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/4/575/DC1

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