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Phosphatidylinositol-3 kinase acts in parallel to the ERK MAP kinase in the FGF pathway during *Xenopus* mesoderm induction

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SUMMARY

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that can phosphorylate phosphaditylinositides leading to the cell type-specific regulation of intracellular protein kinases. PI3Ks are involved in a wide variety of cellular events including mitogenic signalling, regulation of growth and survival, vesicular trafficking, and control of the cytoskeleton. Some of these enzymes also act downstream of receptor tyrosine kinases or G-protein-coupled receptors.

Using two strategies to inhibit PI3K signalling in embryos, we have analysed the role of PI3Ks during early *Xenopus* development. We find that a class 1A PI3K catalytic activity is required for the definition of trunk mesoderm during the blastula stages, but is less important for endoderm and prechordal plate mesoderm induction or for organiser formation. It is required in the FGF signalling pathway downstream of Ras and in parallel to the extracellular signal-regulated kinase (ERK) MAP kinases. In addition, our results show that ERKs and PI3Ks can synergise to convert ectoderm into mesoderm. These data provide the first evidence that class 1 PI3Ks are required for a specific set of patterning events in vertebrate embryos. Furthermore, they bring new insight into the FGF signalling cascade in *Xenopus*.

Key words: PI3K, Mesoderm, FGF, MAP kinase, MEK, Ras, *Xenopus*, Embryo, *Brachyury*

INTRODUCTION

Phosphoinositide 3-kinases are lipid kinases that phosphorylate the 3′-OH position of phosphatidylinositols and some phosphoinositides to give rise to phospholipids such as PtdIns(3)P, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 (reviewed by Vanhaesebroeck et al., 1997; Wymann and Pirola, 1998; Leevers et al., 1999). These phospholipids in turn act as second messengers that regulate a variety of processes in cells. PI3Ks have been implicated in cultured cells in the regulation of cell proliferation, survival, cytoskeletal reorganisation and vesicular trafficking.

Three classes of PI3K catalytic subunits have been defined on the basis of their structures, *in vivo* activity and mode of regulation (reviewed by Vanhaesebroeck et al., 1997; Wymann and Pirola, 1998). Class 3 PI3Ks are homologues of the yeast vesicular protein-sorting protein Vps34p involved in the control of vesicular trafficking in the cell. They are thought to be constitutively active. Class 2 PI3Ks are large molecules whose role is poorly understood. Their presence in activated epidermal growth factor (EGF)- and platelet-derived growth factor (PDGF)-receptor complexes suggests that they may contribute to the signalling properties of these receptors (Arcaro et al., 2000). Finally, Class 1 PI3Ks are composed of a catalytic subunit coupled to an adaptor protein that links the catalytic subunit to upstream signalling components. This class of PI3Ks can be separated into two subclasses. Vertebrate Class 1A catalytic subunits, such as p110α, associate with p85 adaptors that harbour two SH2 domains and a SH3 domain, and associate with phosphorylated tyrosines within a pTyr-X-X-Met motif. This class of enzymes has been implicated in the transduction by receptor tyrosine kinases (RTKs) that bind extracellular ligands such as EGF, PDGF, or insulin. The catalytic subunits of class 1B PI3Ks associate with a different type of adaptors, such as p101, which do not contain known protein motifs. This class of enzymes is stimulated by G-protein-coupled receptors. In addition to interacting with their cognate adaptor, all class 1 enzymes interact with active Ras.

The phospholipids generated by PI3Ks will in turn regulate directly or indirectly, in a cell type-dependent manner, the activity of target proteins such as the transducing molecules Akt/PKB, PDK1, GSK3 and PKC serine/threonine kinases, guanine nucleotide exchange factors (GEFs), or phospholipase Cγ, as well as factors involved in vesicular trafficking such as human EEA1 or yeast Vps27p (Leevers et al., 1999).

The effects of loss- or gain-of-function of PI3K subunits in whole metazoa have recently been described (Wymann and

Pirola, 1998; Weinkove et al., 1999). In *Drosophila*, the class 1 PI3K catalytic subunit p110 and its adaptor protein p60 are involved in the regulation of growth and size of imaginal disks. In *Caenorhabditis elegans*, a reduced activity of the sole class 1A PI3K, AGE-1, leads to a prolonged life span. Finally, inactivation of mouse p110α or β or of p85α results in embryonic death, probably due to a proliferation defect. In view of the importance of RTK signalling during early development, it is surprising that a reduced zygotic function of Class 1A PI3K activity does not lead to patterning defects in the early animal embryos in which it has been achieved. This finding may be due to the maternal expression of PI3K genes or to functional redundancy within the family.

Early *Xenopus* embryos are patterned in the absence of cell growth and therefore provide a simple system in which to study the role of a signal transduction pathway in cell fate decisions. Important cell fate decisions taken between fertilisation and the onset of gastrulation include the specification of the three germ layers along the animal-vegetal axis, as well as the definition of the dorsoventral axis of the embryo. In this study, we have used specific synthetic inhibitors of PI3K as well as dominant negative and constitutively active forms of regulatory or catalytic PI3K subunits to study the involvement of these enzymes during early *Xenopus* embryogenesis. We find that class1A PI3Ks are required for the early steps of trunk mesoderm formation and act in the FGF signalling pathway downstream of Ras and in parallel to the ERK mitogen-activated protein (MAP) kinase.

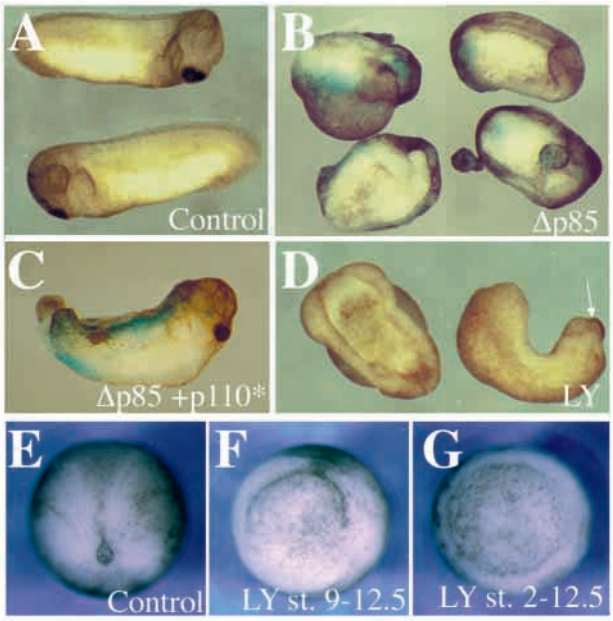

Fig. 1. Inhibition of PI3K signalling prevents gastrulation and axial development. (A,B,D) External appearance at the equivalent of the tailbud stage of WT embryos (A); embryos radially co-injected at the four-cell stage with mRNA for Δp85 (1 ng/blastomere) and NLS-β-Gal (B); or embryos treated with 80-100μM LY2941002 (D, right embryo, side view; left embryo, vegetal view). The majority of treated embryos have no apparent axial or head structures (severe phenotype, left embryos), while a minority of embryos showed reduced axial structures and a cement gland (arrow) but no eye anlage or hatching gland (mild phenotype, right embryos). (C) Rescue of embryos radially injected with Δp85 mRNA (1 ng/blastomere) by co-injection with activated p110α (p110caax, 1 ng/blastomere). In C, trunk and head but not tail development is rescued. (E-G) Vegetal views at the late gastrula stage (st. 12.5) of a control embryo (E), an embryo incubated in 80μM LY294002 between stages 9 and 12.5 (F) and an embryo incubated in 30 μM LY294002 between the two-cell stage and stage 12.5 (G). Gastrulation in embryos F and G has been severely impaired. This phenotype was fully penetrant.

MATERIALS AND METHODS

Embryo manipulations

Adult pigmented *Xenopus laevis* were obtained from Nasco (Wisconsin, USA) and the CNRS *Xenopus* breeding centre (Rennes, France). LY294002 (Sigma) was used at concentrations ranging from 20 μM to 100 μM and replaced every 4 hours. Wortmannin was used at concentrations from 10 to 100 nM and replaced every 4 hours. Cycloheximide (CHX, Sigma), FGF2 (Sigma) and activin β were used, respectively, at concentrations of 10 μg/ml, 50 ng/ml and 4 units/ml. The efficiency of the cycloheximide treatment was witnessed by the gradual cell division arrest observed in treated embryos, probably as a result of arrested cyclin synthesis.

In situ hybridisations and β-galactosidase staining

Whole-mount in situ hybridisation with digoxigenin-labelled RNA probes was performed as described (Gawantka et al., 1995). Whole-mount β-gal staining was as in Sanes et al. (Sanes et al., 1986).

Antibodies

Whole-mount immunostainings with the 12/101 (muscle) and MZ15 (notochord) antibodies were performed as described (Darras et al 1997). The antibodies directed against the activated form of ERK or against all forms of ERK were purchased from Sigma and used in western blot experiments (Chesnel et al., 1997) at dilutions of 1:3000 and 1:2000, respectively.

mRNA injection

pBSRN3-Δp85 was obtained by cloning the *Bam*HI/*Eco*RI insert from pGEX-Δp85 (Dhand et al., 1994) into the *Bgl*II and *Eco*RI sites of pBSRN3 (Lemaire et al., 1995). pCS2+p110caax was obtained by cloning the *Bam*HI/*Xho*I insert from pLHA110caax (pLHA110; Dhand et al., 1994) into pCS2+. pSP64-Xp42^{D324N}, pSP64T-MEK1$^{S217E/S221E}$ and pRN3NLSβ-Gal were as in Gotoh et al. (Gotoh et al., 1995), Umbhauer et al. (Umbhauer et al., 1995) and Lemaire et al. (Lemaire et al., 1995). The construct used to synthesize mRNA for activated Ras (v-Ha-ras) is described by Whitman and Melton (Whitman and Melton, 1992). mRNAs were synthesised in vitro with Ambion mMessage mMachine kits using T3 (pBSRN3 constructs) or SP6 (pCS2+, pSP64 constructs) RNA polymerase. 5 nl of mRNA solution in water was injected in each targeted blastomere. In performing the MEK and Ras overexpression experiments, we found that the concentration of mRNA injected had to be carefully chosen as injection of 200 pg or more of MEK mRNA or of 80 pg or more of Ras mRNA led to severe cytokinesis defects (not shown). Injection of 100 pg of MEK mRNA only rarely led to *Xbra* activation and we therefore chose to inject 125-150 pg of MEK mRNA. Injection of both 20 and 40 pg of Ras mRNA led to a robust LY294002-sensitive *Xbra* activation.

RT-PCR assays

RT-PCR was carried as in Darras et al. (Darras et al., 1997) with the following additional primers and cycle numbers: *gsc* (26 cycles; forward, 5′-TGTGGAGCAGTTCAAGCTCT-3′; reverse, 5′-ATCTGGTACTTGGTTTCTT-3′); *mixer* (26 cycles; forward, 5′-ACAGCCAGAACAAGCTGGAT-3′; reverse, 5′-AATTCCATGG-TAGCTGCTCC-3′); *Mix.1* (27 cycles; forward, 5′-

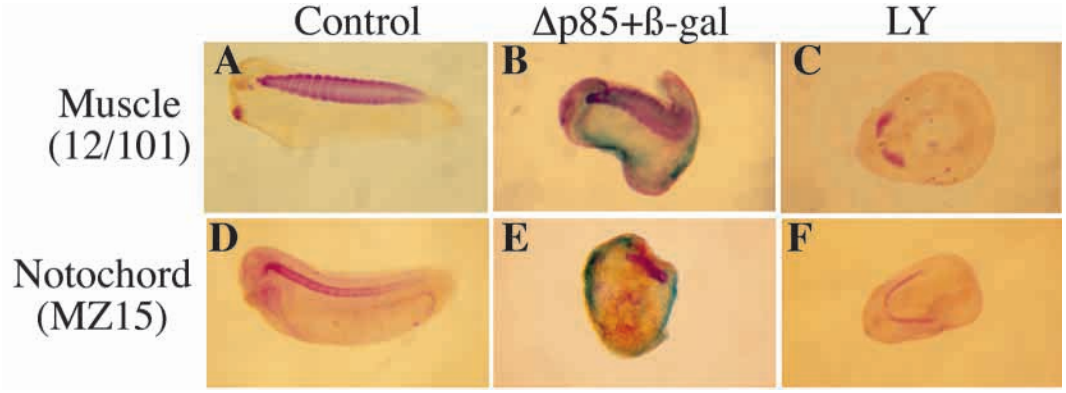

Fig. 2. Reduction of axial mesoderm differentiation in the absence of PI3K signalling. Whole-mount immunohistochemistry at the tailbud stage with monoclonal antibodies against muscle (12/101; A-C) or notochord (MZ15; D-F) on control embryos (A,D), and on embryos co-injected in the equatorial region of four-cell embryos with mRNAs for Δp85 (1ng/blastomere) and NLS-β-Gal (B, injection into one side of the embryo only; E, radial injection). In B,E, visualisation of the β-gal positive cells (blue X-gal staining) reveals that the inhibition of muscle and notochord development is restricted to the cells that have received the exogenous mRNA. (C,F) Embryos treated with 100 μM LY2941002 from stage 9 to stage 12.5.

CGCAATTAATCCCAAAGAGG-3′; reverse, 5′-GAAACATTGCCC-TGTTAGCC-3′); *Sox17α* (27 cycles; forward, 5′-GATGG-TGGTTACGCCAGCGA-3′; reverse, 5′-TGCGGGGTCTGTACTT-GTAG-3′); *Apod* (26 cycles; forward, 5′-GGAACATGCATT-CTCTGCCG-3′F; reverse, 5′-ATCGGATCCTTCATCCAGTG-3′); *Xnot* (26 cycles; forward, 5′-CAGAGCAGCTGGAGAAGCTG-3′; reverse, 5′-CAGTGTGATCTGAGCTGTCT-3′).

RESULTS

PI3K is required for gastrulation and axial mesoderm formation in *Xenopus* embryos

To test the involvement of PI3K during early *Xenopus* development, we overexpressed a truncated form of p85, a class 1A-specific regulatory subunit (Vanhaesebroeck et al., 1997). This truncated form, Δp85, is unable to bind to catalytic subunits but retains its ability to bind to its target phosphotyrosines via its SH2 domain. Its overexpression has thus been shown to block the access of wild type p85 and to uncouple class 1A catalytic subunits from the receptor (Dhand et al., 1994). Overexpression of Δp85 impaired cell movements during gastrulation (not shown), leading to an open blastopore phenotype at the tailbud stage (Fig. 1B). A strong decrease in the level of expression of differentiated markers of muscle and notochord was observed at the same stage (Fig. 2B,E), suggesting that the failure of treated embryos to gastrulate originated from a default in mesoderm formation. Co-injection of a lineage tracer revealed that Δp85 acted at short range (Fig. 2B,E).

A major function of p85 is thought to be to target the catalytic form of PI3Ks to the membrane. To confirm that the effect observed in our experiments was due to the interference with the formation of phosphatidyl-3-inositides, we tested whether the phenotype caused by overexpression of Δp85 could be rescued by overexpression of an activated, membrane-targeted, (p110caax) form of the class-1A PI3K catalytic subunit p110α (Didichenko et al., 1996; Reif et al., 1996). As expected co-injection of mRNA for Δp85 (1 ng) and p110caax (1 ng) mRNAs in the marginal zone led to a rescue of head and trunk/tail structures (Fig. 1C, Table 1) in 45% of embryos while co-injection of 1 ng of Δp85 mRNA and 0.5 ng of p110caax mRNA only led to the rescue of 6% of embryos. Our inability to rescue all injected embryos may stem from the difficulty to achieve an optimal ratio of Δp85 and p110caax proteins or from a geographically broader domain of activation of p110 than normally found in embryos.

Table 1. Defects at the tailbud stage of embryos injected with various mRNAs

Treatment	*n*	Normal (%)	Mild (%)	Severe (%)
Control	53	100	0	0
St. 9-12.5 LY294002 (100 μM)	56	0	27	73
β-gal (1 ng)	31	100	0	0
Δp85 (1 ng)	185	0	19	81
Δp85 (1 ng) + p110caax (0.5 ng)	17	6*	65	29
Δp85 (1 ng) + p110caax (1 ng)	29	45*	27.5	27.5
Δp85 + Ras (10 pg)	36	64*	8	28
Δp85 + Ras (20 pg)	59	80*	0	20

Each blastomere of two-cell embryos was injected with the indicated mRNA amount and the embryos were scored for defects at the tailbud stage. The mild phenotype corresponds to embryos similar to the right embryo of Fig. 1D, while the severe phenotype corresponds to embryos similar to the left embryo of the same panel.

*Embryos scored as normal had a normal head and trunk but could show posterior defects such as those seen in Fig. 1C.

To further confirm that PI3K is required for gastrulation movements and mesoderm differentiation, and to obtain a more homogeneous inactivation of PI3K than achieved by mRNA injection, we treated embryos with two pharmacological inhibitors of PI3K: LY294002 (Vlahos et al., 1994) and wortmannin (Arcaro and Wyman, 1993). In agreement with Umbhauer and colleagues (Umbhauer et al., 1995), treatment of whole embryos with wortmannin did not lead to specific defects in embryo patterning. This may reflect either the poor penetration of wortmannin in *Xenopus* embryos, its instability or the interference of Δp85 with wortmannin-insensitive enzymes (see Discussion). In contrast, treatment of embryos with 80-100 μM LY294002 from stage 9 to the end of gastrulation greatly interfered with gastrulation movements. At the early gastrula stage, the lip formed on the dorsal side as in control embryos (not shown), but gastrulation then stopped and, by stage 12.5, the blastopore remained widely open (Fig. 1F). A similar effect could be obtained using a lower concentration of inhibitor (25 μM) provided embryos were treated from the two-cell stage (Fig. 1G). This requirement for a longer treatment when lower concentrations are used may reflect the slow penetration of the drug in the embryo. By the

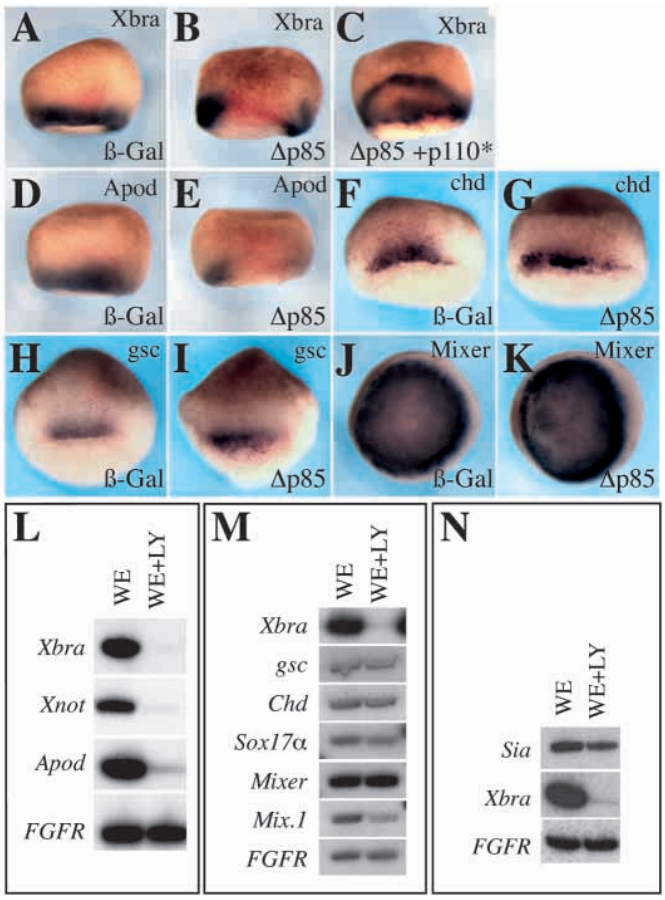

Fig. 3. Effect of the inhibition of PI3K signalling on the *in vivo* expression of early patterning genes. (A-K) Embryos were injected equatorially at the four-cell stage in one or two blastomeres with mRNA for NLS-β-Gal alone (A,D,F,H,J), with Δp85 (0.5 ng/blastomere; B,E,G,I,K), or with Δp85 (0.5 ng/blastomere) and p110caax (0.5 ng/blastomere, C), and cultured until stage 10.5-11 before fixing and whole-mount *in situ* hybridisation with the indicated probe (top right of each part). (L) RT-PCR analysis of the expression of early trunk mesodermal markers in stage 10.5 whole embryos (WE) treated between stage 8.5 and 10.5 with 100 μM of LY294002 (LY). (M) RT-PCR analysis of the expression of early mesodermal and endodermal markers in stage 10.5 whole embryos (WE) treated between stage 8.5 and 10.5 with 100 μM of LY294002 (LY). (N) RT-PCR analysis of the expression of *Xbra* and *Siamois* (*Sia*) at stage 10 in control embryos or embryos incubated in LY294002 (LY; 25 μM) between the four-cell stage and stage 10.

tailbud stage, the majority of LY294002-treated embryos lacked axial structures and a well patterned head but occasionally formed anterior structures such as the cement gland (Fig. 1D, Table 1). Lack of recognisable axial structures in LY294002-treated embryos was accompanied, as in the case of Δp85-injected embryos, by a strong reduction in the expression of muscle and notochord markers (Fig. 2C,F). No rescue of the LY294002 phenotype was observed following overexpression of p110caax (not shown). This result is not surprising as LY294002 acts by interfering with the ATP-binding site of the kinase (Vlahos et al., 1994) and could thus be predicted to block the action of p110caax as well as that of the endogenous enzyme.

Although we cannot exclude that LY294002 may interfere with other pathways in addition to PI3K, the similarity of the phenotypes resulting from the overexpression of Δp85 and the treatment with LY294002 suggests that a PI3K activity is required for the formation of head structures and trunk axial and paraxial mesoderm in *Xenopus* embryos.

PI3K signalling is required during the late blastula stages for trunk mesoderm induction

The structures affected in PI3K-impaired embryos are formed in response to two classes of pregastrula events: mesoderm induction and organiser formation. To test which of these processes are affected, we analysed the expression of mesoderm and organiser markers in early gastrulae in which PI3K signalling had been disrupted by overexpression of Δp85 or treatment with LY294002.

As shown in Fig. 3, overexpression of Δp85 in the marginal zone led to a complete down regulation of the trunk mesoderm markers *Xbra* (Smith et al., 1991; Fig. 3A,B) and *Apod* (Stennard et al., 1999; Fig. 3D,E) but interfered little with the expression of the organiser markers *chordin* (Sasai et al., 1994; Fig. 3F,G) and *goosecoid* (Cho et al., 1991; Fig. 3H,I). The expression of the endodermal markers *Mixer* (Henry and Melton, 1998; Fig. 3J,K) and *Mix.1* (Rosa, 1989; not shown) were also not noticeably affected. Consistent with the ability of p110caax to rescue the phenotypes induced by overexpression of Δp85, co-injection of mRNA for Δp85 and p110caax rescued the expression of *Xbra* (Fig. 3C) in the marginal zone of a third of co-injected embryos (11/32; as a comparison, *Xbra* was lost in 22/24 embryos injected solely with Δp85). As shown on Fig. 3C, in rescued embryos the animal boundary of the *Xbra* expression domain was often shifted towards the animal pole, suggesting that during normal embryogenesis PI3K activity is restricted to the equatorial region of the embryo.

As expected, treatment of embryos with LY294002 also repressed the trunk mesodermal markers *Xbra*, *Apod* and *Xnot* (Von Dassow et al., 1993) (Fig. 3L) while *chordin*, *goosecoid*, *Sox17α* (Hudson et al., 1997), *Mix.1* and *Mixer* were not affected (Fig. 3M). Likewise, expression of the early organiser gene *Siamois* was not affected by treatment with LY294002 (Fig. 3N).

The similarity between the effects of Δp85 and LY294002 confirm that PI3K is required for embryonic patterning and establish that PI3K signalling is required before gastrulation for the formation of trunk mesoderm but appears less important for the early specification of endoderm and the organiser. It is worth noting however, that the loss of head structures in embryos in which PI3K signalling has been impaired (Fig. 1B,D) may occur independently from the loss of trunk mesoderm formation described here (see Discussion). Yet, because trunk mesoderm induction appears to be a very early patterning event requiring PI3K signalling, we focused our attention on the mode of action of PI3K in this process.

PI3K acts during mesoderm induction in the FGF signalling pathway

Mesoderm induction can be reconstituted in vitro by treating animal cap tissue with mesoderm inducers such as activin and FGF. Treatment of animal caps with activin (4 units/ml) leads to the appearance of convergent-extension movements similar to those observed during normal gastrulation (Smith et al.,

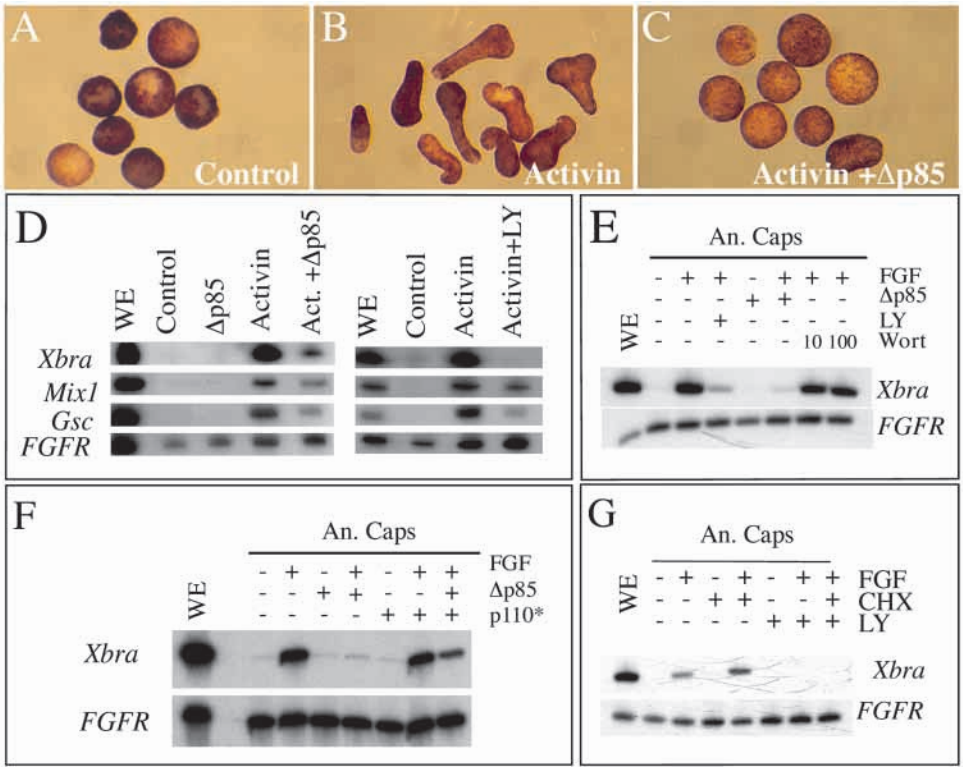

Fig. 4. PI3K signalling is required for activin- and FGF-mediated mesoderm induction in animal caps. (A-C) Animal caps from uninjected embryos (A,B) or embryos injected at the two-cell stage with Δp85 mRNA (1 ng/blastomere, C) were explanted around the mid-blastula stage and cultured in the absence (A) or presence of 4 units/ml of activin (B,C) until the equivalent of stage 16. Δp85 prevents the activin-induced elongation of the caps. (D) Animal caps from embryos uninjected or injected at the two-cell stage with mRNA for Δp85 (1ng/blastomere, animal injection) were excised at stage 8.5, treated with activin (4 units/ml) alone or in combination with LY294002 (LY, 100 μM), frozen at stage 10 and analysed by RT-PCR for the expression of *Xbra*, *Mix.1* and *goosecoid* (*gsc*). (E) Animal caps from uninjected embryos or embryos injected, when indicated, at the two-cell stage with mRNA for Δp85 (1 ng/blastomere, animal injection) were excised at stage 8, treated when indicated with FGF2 (50 ng/ml) alone or in combination with LY294002 (100 μM) or wortmannin (10 and 100 nM), frozen at stage 10 and analysed by RT-PCR for the expression of *Xbra*. (F) Animal caps from control embryos or from embryos injected with mRNA for Δp85 (250 pg/blastomere), p110caax (125 pg-blastomere), or a combination of both, were explanted during the blastula stages and cultured when indicated in 50 ng/ml FGF2 until the early gastrula stage and processed for RT-PCR with *Xbra* primers. (G) Animal caps were excised at stage 8, treated with FGF2 (50 ng/ml) alone or in combination with LY294002 (100 μM) or cycloheximide (CHX, 10 μg/ml) as indicated, and frozen at stage 10 for RT-PCR analysis of *Xbra* expression (top panels). In D-G, the ubiquitously expressed FGF receptor 1 gene (FGFR) is used as a loading control.

1995; Fig. 4B). These movements were inhibited in caps in which PI3K signalling had been interfered with by overexpressing Δp85 (Fig. 4C) or by treatment with LY294002 (not shown). However, incubation of animal caps in even high concentrations (100 μM) of LY294002 or overexpression of Δp85 was not sufficient to completely block the early transcriptional response to activin signaling. At the early gastrula stage, *Xbra* was strongly downregulated, but activation of *gsc* or *Mix.1* was more resistant to the inhibitor (Fig. 4D).

Blocking the FGF pathway prevents activation of *Xbra* by activin, while the activation of *gsc* and *Mix.1* at the early gastrula stage is less affected (Amaya et al., 1993; Cornell et al., 1994). This parallel between the effect of inhibiting FGF and PI3K signalling, combined to the previous implication of class-1A PI3K in signalling by tyrosine kinase receptors suggested that PI3K signalling might act in the FGF signalling pathway during mesoderm formation. Consistent with this proposition, Ryan et al. have reported the association of p85 with the FGF receptor in *Xenopus* embryos (Ryan et al., 1998). This study, however, provided no indication for a functional requirement for PI3K in the FGF pathway.

Three genes, *Xbra*, *Apod* and *Xnot*, have been characterised as FGF target genes during early *Xenopus* embryogenesis and could be used to test a role for PI3K in the FGF pathway in animal cap cells. As described above, all three genes are downregulated by LY294002 treatment in whole embryos. FGF signalling is necessary for the normal mesodermal expression of *Xbra* in vivo (Amaya et al., 1993), and is sufficient to induce ectopic expression of this gene in animal caps (Smith et al., 1991). FGF signalling has also been shown to be sufficient for *Xnot* and *Apod* induction at the mid-gastrula stage (von Dassow et al., 1993; Horb and Thomsen, 1997). Stennard and colleagues, however, reported that FGF could activate *Xbra* but not *Apod* at the early gastrula stage, suggesting that this latter gene may be a late FGF-response gene (Stennard et al., 1999). The same may apply to *Xnot*. We find that, in contrast to *Xbra*, both *Apod* and *Xnot* are very poorly induced by FGF in animal caps at the early gastrula stage (not shown), suggesting that their regulation by FGF may not be direct. We therefore chose *Xbra* as a readout for FGF signalling in our experiments. Treatment of animal caps with LY294002 (20 μM) or overexpression of Δp85 both blocked the induction of *Xbra* and of differentiated ventral mesoderm by FGF2 (50 ng/ml) (Fig. 4E and not shown). Inhibition of *Xbra* activation by Δp85 was specific as it could be rescued by co-injection of p110 caax (Fig. 4F). Interestingly, this latter experiment also indicates that activation of PI3K is by itself not sufficient to activate *Xbra* expression. Consistent with the absence of effect of wortmannin on whole embryos, treatment of animal caps with this inhibitor failed to block *Xbra* expression (Fig. 4E). In whole embryos, FGF is involved both in the direct, i.e. protein synthesis-independent, activation of *Xbra* as well as in the maintenance of this gene via the establishment of a regulatory loop (Isaacs et al., 1994). To test whether PI3K was required for the direct activation of *Xbra*

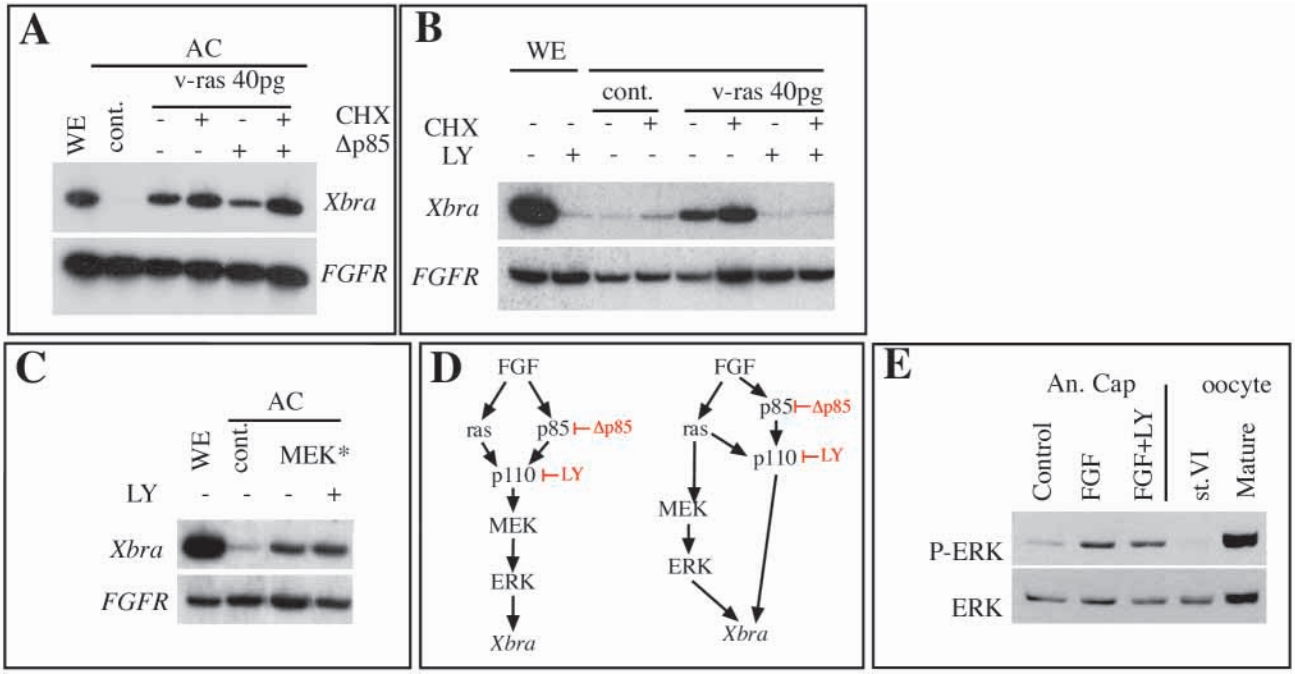

Fig. 5. Position of PI3K in the FGF signalling cascade. (A) Embryos uninjected or injected at the two cell-stage animally with the indicated combination of 40 pg/blastomere of Ras (v-ras) mRNA and/or 1 ng of Δp85 mRNA were incubated with cycloheximide (CHX, 10 μg/ml) from stage 7 onwards where stated. Animal caps were excised at stage 8 and frozen at stage 10 for RT-PCR analysis of *Xbra* expression. (B) Embryos uninjected or injected animally at the two-cell-stage with 40 pg/blastomere of Ras mRNA were incubated when indicated with LY294002 (25 μM) from the four-cell stage onwards and/or with cycloheximide (CHX, 10μg/ml) from stage 7 onwards. Analysis of *Xbra* expression at stage 10 was performed as in A. (C) Effect of LY294002 treatment on the expression of *Xbra* at stage 10 in animal caps injected at the two-cell stage with $MEK1^{S217E/S221E}$ mRNA (125 pg/blastomere) and treated with LY294002 in the same way as in panel B. (D) Two models of action of PI3K in the FGF pathway. The identity of the molecules blocked by Δp85 and LY294002 (red) is shown. (E) Western blot analysis of the effect of LY294002 on the activation by phosphorylation of ERK in response to FGF signalling. Animal caps from control embryos or embryos preincubated in 100 μM LY294002 for 2 hours were explanted at stage 9 and cultured for 20 minutes in Modified Barth Saline (MBS) or MBS + 100μM LY294002. ERK is present in equal amounts in fully grown stage VI and progesterone-matured oocytes, but is only activated in the latter. The stage VI and mature oocytes lanes demonstrate the specificity of the anti-activated ERK antibody used. The low level of activation of ERK in control animal caps may be due to the wounding of the cells during the explantation (LaBonne and Whitman, 1997). P-ERK, activated ERK; ERK, total ERK.

by FGF, we prevented de novo protein synthesis with cycloheximide (CHX, 10 μg/ml), and compared the ability of FGF to directly activate *Xbra* in the presence or absence of 20 μM LY294002. As shown in Fig. 4G, the PI3K inhibitor efficiently blocked the direct activation of *Xbra* by FGF, demonstrating that PI3K acts in the FGF signalling pathway upstream of direct transcriptional targets.

Position of PI3K in the FGF signalling cascade

Induction of ventral mesoderm by FGF is thought to be mediated by the Ras-MAPK signalling cascade (LaBonne et al., 1995; Gotoh et al., 1995; Umbhauer et al., 1995) and we next wanted to position PI3K with respect to three key components of this cascade: Ras, MEK and the ERK MAP kinase.

Overexpression of constitutively active Ras leads to the direct, CHX-insensitive activation of *Xbra* (Fig. 5A and Whitman and Melton 1992). Co-overexpression of Δp85 had no effect on the direct induction of *Xbra* by activated Ras (Fig. 5A). This suggests that p85 acts upstream or in parallel to Ras in this assay. A similar epsitatic relationship was also observed in the embryo: overexpression of activated Ras mRNA (20 pg/bl) was sufficient to rescue axial development in 80% of embryos injected radially with Δ*p85* mRNA (Table 1). Previous reports have established that activation of PI3K can be achieved either by binding of PI3K via their p85 subunit to the receptor complex or by direct binding of activated Ras to the catalytic subunit of PI3K (Vanhaesebroeck et al., 1997; Wymann and Pirola, 1998). This suggests that in our experiments, the relatively high level of expression of Ras is sufficient to directly activate the catalytic subunit of PI3K, thereby bypassing the requirement for a functional p85. Confirming the necessity for a catalytic PI3K activity downstream of Ras, treatment of Ras-injected caps with 25 μM LY294002 during the cleavage and blastula stages prevented the cycloheximide-insensitive activation of *Xbra* (Fig. 5B). Based on these experiments, the relative position of Ras, p85 and PI3K catalytic subunits is indicated on Fig. 5D.

Similar epistatic experiments were performed with $MEK^{S217E/S221E}$, an activated form of the MAP kinase kinase, MEK1 (Umbhauer et al., 1995). In this case however, we found that LY294002 did not prevent the activation of *Xbra* by MEK1 during the blastula stages (Fig. 5C). Taken together, the data presented here indicate that LY294002-sensitive PI3Ks act downstream of Ras but not of MEK.

Two models for the FGF signalling cascade could account for these results. PI3K could act in between Ras and MEK

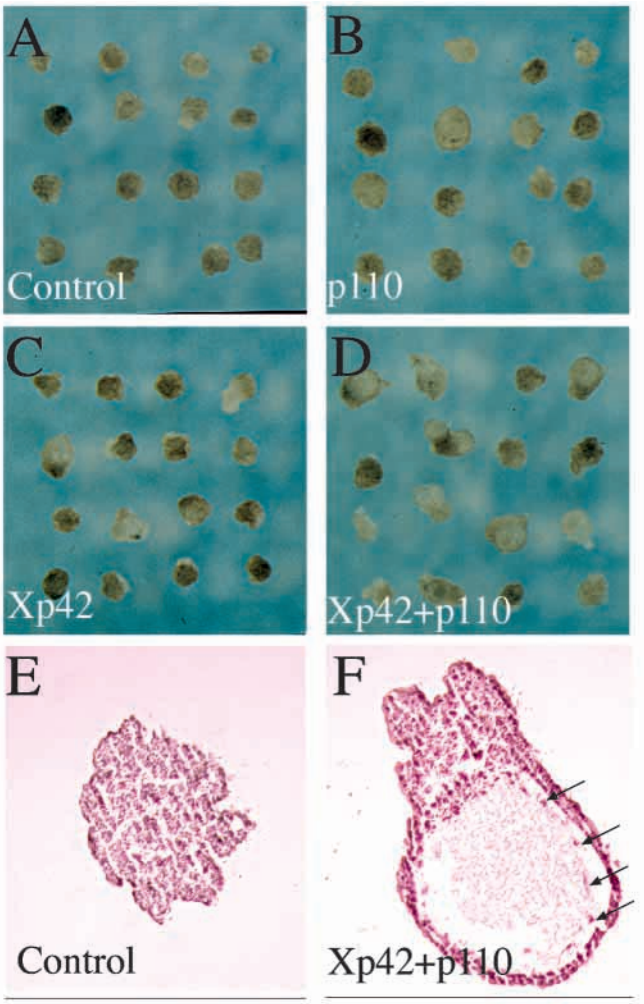

Fig. 6. Activated ERK and p110 synergise during mesoderm induction in animal caps. (A-D) Whole-mount photography of stage 37/38 animal caps explanted at stage 9 from control embryos (A), embryos injected with 250 pg of mRNA for p110caax, a membrane-targeted form of the p110α class 1A PI3K (B), embryos injected with 200 pg of mRNA for Xp42^{D324}, an activated form of ERK (C) or embryos co-injected with both mRNAs (D). (E,F) Histological staining of sectioned animal caps at stage 37/38. (E is a control cap; the cap in F expresses activated ERK and p110α). The presence of a large fluid-filled cavity lined with mesothelial cells (arrows) is characteristic of ventral mesoderm differentiation (Smith, 1993). This analysis did not reveal significant difference between the mesodermal types induced by the different mRNA injections.

(Fig. 5D, left). Alternatively, Ras may activate two separate cascades, a PI3K-dependent pathway and the classical MAP kinase pathway (Fig. 5D, right). The rapid activation of ERK in response to FGF (Hartley et al., 1994) should be PI3K dependent in the first model but not in the second. As shown in Fig. 5E, treatment of the animal caps with a high concentration (100 μM) of LY294002 had no effect on the FGF-mediated activation of the ERK, thus favouring the second model.

We conclude that a PI3K-dependent signalling pathway is required downstream of ectopic Ras and in parallel to the MEK/ERK pathway.

Cooperation between ERK and p110

We finally tested whether ectopic expression of the constitutively active Class1A PI3K catalytic subunit p110caax was sufficient to convert ectoderm into mesoderm and whether PI3K and ERK signalling could synergise during mesoderm induction. Fig. 6 summarises the results of one out of three independent experiments that gave similar outcomes.

By Stage 40, overexpression in animal cap cells of 500pg of *p110caax* mRNA induced histologically recognisable ventral mesoderm (Fig. 6F) in 7.0% of analysed caps (*n*=57) (Fig. 6B); By comparison, only 1.6% of control uninjected animal caps (*n*=62) developed mesodermal characteristics. This weak mesoderm induction activity of p110caax was insufficient to cause enhanced expression of *Xbra* in *p110caax*-expressing caps (Fig. 4F). Injection of higher amounts of *p110caax* mRNA did not enhanced the percentage of mesodermalised caps (not shown).

To assay for a synergy between MAPK and p110caax, we injected animal caps with low amounts (200 pg) of mRNA for *Xp42^{D324N}*, an active form of ERK (Umbhauer et al., 1995), alone or with 250 pg of mRNA for *p110caax*. While injection of *Xp42^{D324N}* mRNA caused formation of ventral mesoderm in 11.1% of animal caps (*n*=54; Fig. 6C), co-injection of both mRNAs led to ventral mesoderm formation in 39.7% of the injected caps (*n*=58; Fig. 6D). Hence, PI3K and ERK cooperate to trigger mesodermal differentiation in animal cells, though this cooperation was not sufficient to trigger activation of *Xbra* in injected caps as assayed by RT-PCR (not shown). This suggests that additional factors act in parallel to the ERK and PI3K pathways in mesoderm induction. The observed cooperation provides further evidence for the functional importance of the activation by FGF of synergistic parallel ERK and PI3K pathways.

DISCUSSION

The data reported here show that a PI3K activity is required for the proper patterning of the *Xenopus* embryos. More precisely, our results indicate that the impairment of FGF-signalling downstream of Ras and in parallel to ERK is a likely cause for the loss of trunk mesoderm observed as result of the inhibition of PI3K signalling. This study thus provides the first evidence that PI3Ks, in addition to their previously documented roles (Wymann and Pirola, 1998), are major players in vertebrate embryonic patterning.

Identity of the PI3K involved in mesoderm formation

Vertebrate catalytic subunits of PI3Ks have been sorted into three classes. Only Class 1A subunits have been shown to require hetero-dimerisation with p85 adaptors. Our demonstration that Δp85 blocks mesoderm formation both in vivo and in FGF-treated caps, and that this effect can be rescued in vivo by overexpressing a Class 1A PI3K, suggests the involvement of a Class 1A PI3K in early embryonic patterning. Consistent with the involvement of a PI3K, LY294002, used at a concentration (20 μM) at which this compound is thought to be specific to PI3Ks (Vanhaesebroeck and Waterfield, 1999), blocks mesoderm formation in vivo and in FGF-treated caps in a similar way to Δp85.

The fact that wortmannin, a structurally unrelated inhibitor

of PI3Ks has no effect on embryonic patterning raises the possibility that the enzyme targeted by LY294002 and Δp85 might be wortmannin independent, or that wortmannin is not efficient in embryos or explants. Several arguments make us favour the latter possibility. First, all known catalytic subunits have been shown to be sensitive to both wortmannin and LY294002 (Wymann and Pirola, 1998), with the exception of the class II PI3K C2γ subunit, which is relatively resistant to wortmannin (IC_{50}=420 nM) but is not known to associate with p85. Secondly, previous data suggest that PDGF signalling is required for proper cell migration and gastrulation (Ataliotis et al., 1995) and can be blocked by wortmannin in isolated mesoderm cells (Symes and Mercola, 1996). Should wortmannin be active in an embryo context, it should elicit at least a phenotype similar to the inhibition of PDGF signalling. In our hands, however, wortmannin had no effect on embryos. We therefore conclude that wortmannin is not an efficient inhibitor of PI3K signalling in embryos. This may stem from the instability of this compound in aqueous solutions, its interaction with extracellular proteins (Vanhaesebroeck and Waterfield, 1999) or its inability to penetrate compact embryonic tissue.

Taken together, the fact that two inhibitors, LY294002 and Δp85, acting via very different mechanisms, both lead to similar phenotypes strongly suggests that Class 1A PI3Ks are required during early embryonic patterning.

Tissues and pathways affected by a loss of function of PI3K

Work by Mercola and colleagues (Ataliotis et al., 1995; Symes and Mercola, 1996) has previously demonstrated that PDGF, which is known to act via PI3K in a variety of systems, is required for normal gastrulation. Inhibition of PDGF signalling following injection of mRNA for dominant-negative receptors causes a failure of blastopore closure and axial defects (Ataliotis et al., 1995). In these embryos, the notochord is disorganised and dorso-anterior muscle reduced. These defects are likely to be caused by an aberrant migration of dorso-anterior cells during gastrulation, rather than by an early failure of mesoderm formation as activation of *Xbra* at the mid-gastrula stage is normal (Ataliotis et al., 1995).

Although the gastrulation defects seen in Δp85-injected or LY294002-treated embryos are superficially similar to those caused by interference with PDGF signalling, our analysis shows that the former defects are much more severe. Reduction of notochord and muscle development is more pronounced and, most importantly, the early activation of trunk mesodermal genes such as *Xbra*, *Xnot* or *Apod*, is completely blocked in embryos in which PI3K has been impaired (Fig. 3). This indicates that, in addition to their potential role in PDGF signalling during gastrulation, PI3Ks are required in an earlier pathway for trunk mesoderm induction.

Two pathways have been implicated in mesoderm induction: the transforming growth factor β (TGFβ) pathway, involving molecules of the Nodal, Derrière and Activin families, and the fibroblast growth factor (FGF) pathway, proposed to act as a relay or competence factor for the action of TGFβ (Isaacs, 1997). We show that PI3K is required downstream of both Activin and FGFs for mesoderm induction. FGF has been proposed to act downstream of Activin/Nodal-like factors in the mesoderm only and its inhibition, like that of PI3K, has little effect on the regulation of vegetally expressed genes such as *Mix.1* or *gsc*, which are direct targets of TGFβ signalling (Rosa, 1989; Cho et al., 1991).

Two pieces of evidence suggest that impairment of FGF signalling by PI3K inhibitors is a major cause for the observed early loss of trunk mesoderm. First, PI3K is required for the direct activation of *Xbra* by FGF in animal cells and for the activation of the FGF targets *Xbra*, *Apod* and *Xnot* in embryos. Second, inhibition of PI3K by overexpression of Δp85 can be rescued in whole embryos by overexpression of activated Ras, a known component of the FGF signal transduction pathway.

As mentioned above, however, FGF is not the sole pathway requiring PI3K during early embryogenesis as treatment of embryos with PI3K inhibitors leads to a partial loss of head structures, a phenotype that is not observed in embryos injected with a dominant negative FGF receptor (Amaya et al., 1993). The head phenotype we observe is not due to an interference with organiser formation, since we observed very little effect on the expression of *gsc* and *chordin* in embryos overexpressing Δp85 or treated with LY294002. It has been previously shown that the PDGF pathway is involved in the migration of anterior mesendodermal cells and in head formation (Ataliotis et al., 1995). The head defects observed in our experiments may therefore, in part, be due to interference with PDGF signalling.

Towards a revised model for FGF signalling during mesoderm formation

While PI3K has been shown to be required for signal transduction in response to RTK ligands such as PDGF, insulin or EGF, its role in FGF signalling is much less clear cut. On the one hand, several molecules able to bind PI3K subunits such as dof (Vincent et al., 1998) and p85 (Ryan et al., 1998) act downstream of FGF or are found associated with the FGF receptor. Also, treatment of cultured cell lines with basic FGF can lead to a modest increase in PI3K activity (van Weering et al., 1998; Cross et al., 2000). On the other hand, inhibition of PI3K signalling seldom has a demonstrated direct effect on the response to FGF and in the few cases where this appears to be the case, the role of PI3K is limited to the reorganisation of the cytoskeleton (van Weering et al., 1998) or the regulation of exocytosis (Janecki et al., 2000). There is to our knowledge no case in which the direct activation by FGF of a target gene has been shown to be PI3K dependent. In contrast to the controversial role of PI3K in FGF signalling, activation of the MAP kinase pathway has been shown to play a crucial role in FGF signalling. On the basis of the overexpression of activated MAP kinase, it was suggested that the Ras-dependent activation of this kinase was sufficient to account for the FGF-mediated induction of mesoderm induction (Gotoh et al., 1995; Umbhauer et al., 1995) and for the direct activation of *Xbra* (Smith et al., 1991).

Using two different strategies to interfere with PI3K signalling, our study provides the first demonstration that PI3K signalling is crucial for the direct activation by FGF of *Xbra*. PI3K signalling is not involved in the activation of ERK by FGF but rather acts in parallel to the MAP kinase pathway. In contrast to what has been previously proposed, our results thus indicate that, during mesoderm induction, the FGF signalling pathway splits upstream of ERK into at least two cooperating branches. Several questions remain to be addressed. First, the

weak mesoderm induction obtained when both the ERK and PI3K pathways are activated suggests the existence of additional parallel effector pathways downstream of Ras. Several effector pathways, including Ral, Rac/Rho and phospholipase D, have been shown to act downstream of Ras and probably in parallel to ERK and PI3K (Shields et al., 2000). It will be important to test the role of these pathways in *Xenopus* mesoderm formation. It will also be important to position PI3K with respect to laloo, a recently described src-family tyrosine kinase acting in the FGF pathway (Weinstein et al., 1998). Second, while we have shown that PI3K is required for FGF signalling, we have not addressed whether this PI3K activity is modulated by FGF signalling. This could be the case, since in other systems FGF can stimulate, albeit weakly, PI3K activity (van Weering et al., 1998; Cross et al., 2000). In addition, p85 is associated to the FGF receptor in *Xenopus* embryos during gastrulation (Ryan et al., 1998). Third, we need to identify the components acting downstream of PI3K in mesoderm induction. Several downstream effectors of PI3K have been characterised in cultured cells including GSK3, PKB/Akt, $p70^{S6k}$ and the GTPases Rac and Rho (reviewed by Vanhaesebroeck et al., 1997). Our results do not support a role for GSK3 downstream of PI3K in early embryos, as expression of *Siamois*, a direct target of the β-catenin/GSK3 pathway (Brannon et al., 1997; Fan et al., 1998), is not affected by treatment with LY294002. It will be interesting to test a potential role for PKB/Akt and Rac/Rho in mesoderm induction downstream of PI3K. The availability of constitutively active or dominant negative forms of proteins acting in the Ras and PI3K pathways in other systems, coupled to the convenience of the *Xenopus* system, will help shed light on these issues.

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