Development 128, 2451-2460 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2724

Evidence for a role of protein kinase C in FGF signal transduction in the developing chick limb bud

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Accepted 17 April 2001

SUMMARY

In developing limbs, numerous signaling molecules have been identified but less is know about the mechanisms by which such signals direct patterning. We have explored signal transduction pathways in the chicken limb bud. A cDNA encoding RACK1, a protein that binds and stabilizes activated protein kinase C (PKC), was isolated in a screen for genes induced by retinoic acid (RA) in the chick wing bud. Fibroblast growth factor (FGF) also induced *RACK1* and such induction of *RACK1* expression was accompanied by a significant augmentation in the number of active PKC molecules and an elevation of PKC enzymatic activity. This suggests that PKCs mediate signal transduction in the limb bud. Application of chelerythrine, a potent PKC inhibitor, to the presumptive wing region resulted in buds that did not express *sonic hedgehog* (*Shh*) and developed into wings that were severely truncated. This observation suggests that the expression of *Shh* depends on PKCs. Providing ectopic SHH protein, RA or ZPA grafts overcome the effects of blocking PKC with chelerythrine and resulted in a rescue of the wing morphology. Taken together, these findings suggest that the responsiveness of *Shh* to FGF is mediated, at least in part, by PKCs.

Key words: FGF, FGFR, Protein kinase C, RACK1, Retinoids, Sonic hedgehog, Chick

INTRODUCTION

Numerous signaling molecules that regulate vertebrate limb development have been identified including sonic hedgehog (SHH), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and retinoic acid (RA; reviewed by Johnson and Tabin, 1997; Martin, 1998). The wealth of information on the signaling molecules themselves contrasts with a more limited knowledge of the underlying signaling mechanisms. RA is required for early development (Helms et al., 1996; Lu et al., 1997; Niederreither et al., 1999) and when applied to the limb bud induces an ectopic zone of polarizing activity (ZPA; Noji et al., 1991; Wanek et al., 1991), presumably as a result of activating *Shh* (Riddle et al., 1993), *Bmp2* (Francis et al., 1994) and *Fgf4* (Laufer et al., 1994; Niswander et al., 1994). It is likely that in such RA-exposed tissue, genes and proteins are activated to mediate the feedback loops involved in ZPA formation and limb growth. Using a PCR-based subtractive screening technique, we searched for genes that are activated upon ectopic RA exposure. We found *RACK1* that encodes a receptor for activated C-kinase, a protein that specifically binds and stabilizes activated protein kinase C (PKC; Ron et al., 1994).

PKCs are phospholipid-dependent serine threonine kinases that regulate cell growth and differentiation (Clemens et al., 1992; reviewed by Jaken, 1996; Newton, 1996; Murray et al., 1997). They consist of a regulatory domain and a catalytic domain that contains a substrate- and a RACK1-binding site. These sites are blocked in inactive PKC by autoinhibitory sequences located in the regulatory domain (Mochly-Rosen et al., 1995). Binding of PKC activators induces a conformational change that enables the binding of RACK1 to activated PKC conformeres, thereby exposing the substrate-binding site. The formation of a PKC/RACK1 complex thus stabilizes the enzymatically active conformation of PKC and thereby enhances PKC activity.

Several experiments indicate that PKCs can transduce FGF signals. In cultured cells binding of FGFs to their receptors (FGFRs) activates phospholipase C γ (PLCγ) whose reaction products in turn trigger activation of PKC (Burgess et al., 1990). FGFR1 mutant mice, in which a tyrosine critical for the interaction between FGFR and PLCγ is mutated, have defects in patterning of the anteroposterior axis (Partanen et al., 1998). These data provide direct evidence for a role of the FGF/PLCγ/PKC signal transduction pathway in development. An alternate pathway of FGF signaling not involving PKCs occurs through Ras/mitogen-activated protein kinase (Brunet and Pouyssegur, 1997; Kouhara et al., 1997).

FGFs play an important role in limb outgrowth and patterning (reviewed by Martin, 1998). Local application of

various FGFs to the interlimb flank induces an ectopic limb bud (Cohn et al., 1995; Ohuchi et al., 1995). *Fgf10* mutant mice lack an apical ectodermal ridge (AER), do not express *Fgf8* and fail to form limbs (Min et al., 1998; Sekine et al., 1999). Limb buds of mice that lack a functional FGFR2 do not express *Fgf8* and *Shh*, express a reduced level of *Fgf10*, and fail to develop limbs (Deng et al., 1997; Partanen et al., 1998; Xu et al., 1998). Based on these data, Xu et al. (Xu et al., 1998) have proposed that FGF10 produced in the limb bud mesenchyme activates FGFR2-IIIb expressed in the AER. The ligand-bound FGFR2-IIIb then turns on FGF8 that is then secreted by the AER and binds to FGFR2-IIIc in limb mesenchyme. The resultant activation of FGFR2-IIIc initiates the expression of *Shh* and also maintains expression of *Fgf10*.

An important finding of this study is that we were able to causally link FGF signaling with PKC activation in the limb bud. Specifically, we found that FGF applied with the interlimb lateral plate locally induced RACK1 expression and, furthermore, increased PKC activity (as revealed by PKC enzymatic assays and immunohistochemistry with an antibody specific for activated PKC). When PKC activity was blocked by chelerythrine chloride, a potent inhibitor of PKCs, the expression of *Shh* was prevented and truncated wings formed. This loss of limb structures could be reversed when RA, SHH protein or a ZPA were ectopically provided to inhibitor-treated buds. Based on these observations, we propose that in the limb bud PKCs play a role in the regulation of *Shh* expression.

MATERIALS AND METHODS

RA treatment

All-*trans*-RA (10 μg/ml in DMSO) was applied to stage 20 (Hamburger and Hamilton, 1951) wing buds as described (Eichele and Thaller, 1987). After 12 hours of treatment, the AER and mesenchymal tissue adjacent to the bead was cut out in ice-cold phosphate-buffered saline (PBS) and RNA was isolated with RNAzol (Tel-Test, Friendswood, TX). Tissue used is composed of cells in which *Shh* will be expressed as a result of RA treatment (Helms et al., 1996).

Poly(A)PCR and subtractive hybridization

Poly(A)PCR amplification was carried out as described in Brady and Iscove (Brady and Iscove, 1994) and subtractive hybridization procedure was modified from a method described by Wang and Brown (Wang and Brown, 1991). cDNA obtained by poly(A)PCR was digested with *Eco*RI and photobiotinylated. *Eco*RI-digested and biotinylated cDNAs were used as a driver for subsequent hybridization. 2.5 μg tracer was mixed with 50 μg driver for a long subtractive hybridization (LH). After LH, the residual tracer was mixed with 25 μg driver and subject to short hybridization and subtraction (SH). After three rounds of LH, SH and PCR amplification, the subtracted cDNAs were amplified by PCR. A final PCR amplification cycle with a 2 hour extension time at 72°C ensured that cDNAs were double stranded. The PCR products were subcloned into pBluescript vector resulting in a plasmid subtracted library. 6000 colonies were screened with ^{32}P-labeled subtracted cDNAs, and 72 positive clones were picked representing three different inserts.

Immunohistochemistry

Immunohistochemistry at the electron microscope level with indirect immunogold labeling was performed as described previously (Sierralta and Thole, 1992; Sierralta et al., 1995). Embryos were fixed with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), washed, dehydrated with graded ethanol, embedded in LR-Gold and sectioned at 70 nm. Sections were incubated with primary antibody (Ab) against RACK1 (IgM monoclonal Ab, diluted 1:100, Transduction Lab, Lexington, KY) or autophosphorylated PKCα/PKCβII (Sweatt et al., 1998, diluted 1:30), initially for 2 hours at 37°C and then overnight at 4°C. The attachment of the primary Ab was detected with a secondary Ab labeled with 10 nm gold. After a fixation with 0.25% glutaraldehyde, the sections were contrasted with uranyl acetate and lead citrate. The PKCα/PKCβII antibody was raised against a phosphopeptide that contains two autophosphorylation sites of PKCβII, Thr634 and Thr641, a sequence also present in PKCα.

The attachment of gold particles in nuclear or cytoplasmic areas of lateral plate mesenchyme from either the FGF-treated lateral plate or control lateral plate was analyzed with a Zeiss EM 902 microscope and a SIT 66-vidicon camera. To quantify gold tagging and to measure areas in randomly selected fields, the program AnalySIS version 2.1 (Soft Imaging Software GmbH, Muenster, FRG) was used. The maximal size of each individual area analyzed was 5.7 μm^2 and measurements were carried out at 20,000 × magnification. Eight different fields were analyzed per section and six sections were used from three different embryos. Gold particles separated by less than 10 nm from their neighbors were considered to belong to the same cluster.

For light microscopy, embryos were fixed with 4% PFA/PBS for 2 hours at 4°C and embedded in paraffin. To increase the antigen availability, de-paraffinated sections were immersed in 5% acetic acid/95% ethanol for 2 minutes. Sections were stained by standard procedures. Primary antibodies: RACK1 (diluted 1:500). The secondary antibodies were detected using a Tyramide Signal Amplification kit (NEN, Boston, MA).

FGF and SHH treatments

Heparin acrylic beads (H5263, Sigma), about 200 μm in diameter, were soaked in a 2 μl drop of 1 mg/ml recombinant human FGF2 (R&D Systems, Minneapolis, MN) or 0.87 mg/ml recombinant FGF4 (a gift from Dr V. Rosen, Genetics Institute, Cambridge, MA) for at least 1 hour at room temperature. FGF-soaked beads were then implanted into the interlimb flank of stage 14 embryo at somite 22/23 level, as described by Cohn et al. (Cohn et al., 1995). SHH protein was produced as previously described (Roelink et al., 1995). SHH was delivered from heparin acrylic beads that were soaked for 5 hours in 5 μl of a 5 mg/ml solution.

Protein kinase C assay

After 20 hours of FGF treatment in ovo, the interlimb lateral plate surrounding the FGF bead and the contralateral interlimb flank were dissected in ice-cold PBS. Tissue was homogenized as described (Gonzalez et al., 1993). To determine PKC activity in lateral plate homogenates, PKC-specific substrate NG(28–43) (Klann et al., 1993) was incubated with 5 μg total protein for 5 minutes at 32°C. Co-factor-dependent PKC activity is defined as activity observed in the presence of co-factors (100 μM calcium chloride, 320 μg/ml phosphatidylserine and 30 μg/ml dioctonoylglycerol) minus the autonomous PKC activity, defined as activity in the presence of 2 mM EGTA.

PKC inhibitor treatment

AG1-X2 ion exchange beads of 250 μm diameter (BioRad) were soaked overnight in 20 μl of 6.5 mM chelerythrine chloride (BIOMOL, Plymouth Meeting, PA). Stage 14 embryos were slightly stained with Neutral Red, two incisions that were made with a tungsten needle into the lateral plate at somite levels 15 and 20 and beads were pushed into these incisions thereby contacting mesenchyme.

In situ hybridization

Synthesis of digoxigenin-tagged riboprobes from *Fgf4* (Nohno et al.,

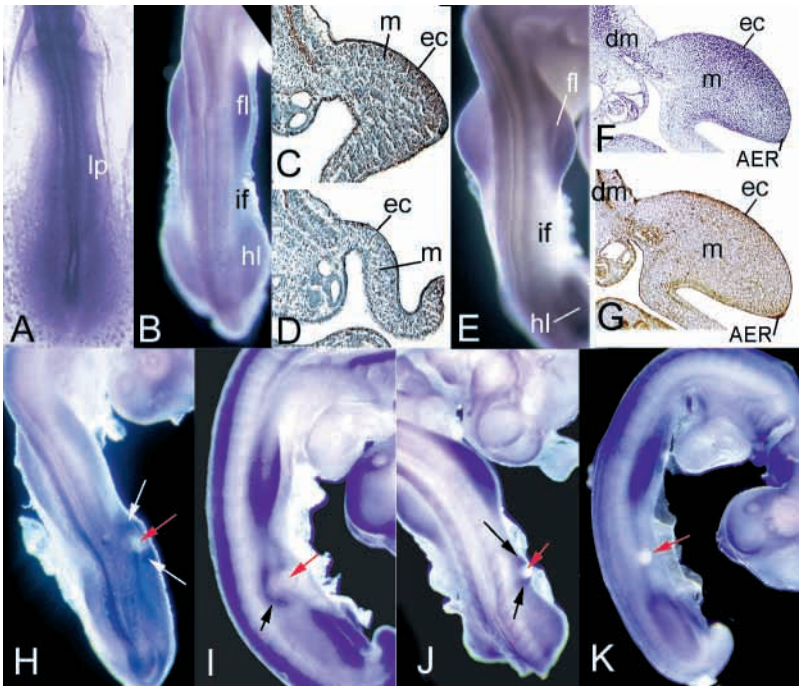

Fig. 1. Expression profile of *RACK1* during limb development and its induction by ectopic FGF. (A) At stage 13, *RACK1* was expressed uniformly in the lateral plate. (B) By stage 17 the expression of *RACK1* was augmented in the limb-forming region but was low in the interlimb flank. (C,D) By stage 17, RACK1 protein detected by immunostaining (brown deposit) was high in the wing bud (C) but was low in the interlimb region (D). (E) *RACK1* was expressed in the limb buds of a stage 18 embryo. (F,G) RACK1 mRNA (F) and protein (G) in sections through a stage 22 wing bud. (H) Ectopic expression of *RACK1* (white arrows) induced by FGF released from a bead (red arrow) was observed after 12 hours of treatment. (I,J) Two different embryos showing strong ectopic expression of *RACK1* (black arrows) upon FGF4 treatment for 24 hours. (K) A plain heparin bead (red arrow) did not induce *RACK1*. AER, apical ectodermal ridge; dm, dermomyotome; ec, ectoderm; fl, forelimb; hl, hindlimb; if, interlimb flank; lp, lateral plate; m, mesenchyme.

1997), *Fgf8* (Crossley et al., 1996), *Fgf10* (Ohuchi et al., 1997), *Msx-1* (Davidson et al., 1991), *RACK1* (cDNA encoding the entire coding region was used, generated by RT-PCR with the primers corresponding to the published chick *RACK1* sequence GenBank, A33928), *Rel/NF-κB* (Kanegae et al., 1998) and *Shh* (Riddle et al., 1993; Roelink et al., 1994) was carried out with a Stratagene RNA transcription kit. In situ hybridization was carried out as previously described (Albrecht et al., 1997).

Western blotting

Wing buds from stage 20 chick embryos were dissociated into single cells with trypsin. These cells were cultured at 37°C in 0.5 ml DMEM containing 10% fetal calf serum (Gibco-BRL) until they had attached. Thereafter, medium with or without inhibitors (BIOMOL) was added. Cells were harvested for protein isolation after 24 hours of culture. Proteins were analyzed on 12% SDS-PAGE gels and electrophoretically transferred to a nitrocellulose membrane. Blots were blocked and probed sequentially with (1) Ab against autophosphorylated PKCα/PKCβII (1:500) and (2) monoclonal antibody against PKCβ (IgG monoclonal from Transduction Lab, 1:250). Bands were visualized with horseradish peroxidase-linked secondary antibodies and developed using enhanced chemiluminescence (Pierce). The density of each band was quantified with a StudioScan desktop scanner with NIH image software.

ZPA grafts

Limb buds were dissected, incubated in 2% trypsin for 20 minutes at 4°C, rinsed in PBS containing 1% bovine serum albumin. Small pieces of ZPA were cut out using a tungsten needle and placed underneath the AER, which had been lifted by making an incision between the AER and the underlying mesenchyme.

RESULTS

Isolation of *RACK1* from RA-treated wing buds

To isolate RA-regulated genes in the developing limb bud, RA was applied to the anterior margin of Hamburger-Hamilton stage 20 chicken wing buds and after 12 hours of treatment, AER and mesenchyme adjacent of the RA-releasing bead was excised. In parallel, tissue adjacent to a control bead was collected. Using a combination of poly(A)PCR- and a PCR-based subtraction, a library was constructed enriched in genes upregulated by RA. A screen with the subtracted probe enriched for RA-induced cDNAs identified several upregulated clones, one of which encoded a 340 bp fragment of the chicken *RACK1* gene (Guillemot et al., 1989).

RACK1 expression in the developing limb

During early embryogenesis *RACK1* was broadly expressed in embryonic and extra-embryonic tissues including the lateral plate (Fig. 1A). From stage 17 onwards, *RACK1* mRNA was found in a more restricted pattern. *RACK1* mRNA was abundant in nascent limb buds with a much lower level of expression in the interlimb flank (Fig. 1B,E). Immunostaining of transverse sections using a monoclonal antibody revealed RACK1 protein throughout the limb bud mesenchyme, with cells underneath the AER, the surface ectoderm and the AER having increased immunoreactivity (Fig. 1C). Consistent with the ISH data (Fig. 1B,E), interlimb lateral plate exhibited little immunoreactivity (Fig. 1D). In limb buds of stage 22 and older embryos, *RACK1* expression was higher in distal and dorsal limb bud mesenchyme than in more proximal tissue (Fig. 1F). RACK1 protein showed a similar distribution; it was highly expressed in AER and ectoderm but was also found in distal and dorsal mesoderm (Fig. 1G).

FGFs upregulate *RACK1* expression in the interlimb flank

As RACK1 is expressed in proliferating limb mesenchyme, it may be associated with proliferation and thus be regulated by growth factors. To test this possibility, beads soaked in either FGF2 or FGF4 were placed into the lateral plate of a stage 14 embryo, opposite somite 22. At this stage *RACK1* was uniformly expressed in the lateral plate but as the embryo continued to develop towards stage 17, expression was downregulated in the interlimb lateral plate (Fig. 1E). However, after a 12 hour treatment with FGF2 (n=12) or FGF4 (n=17), *RACK1* expression surrounding the FGF bead was detected

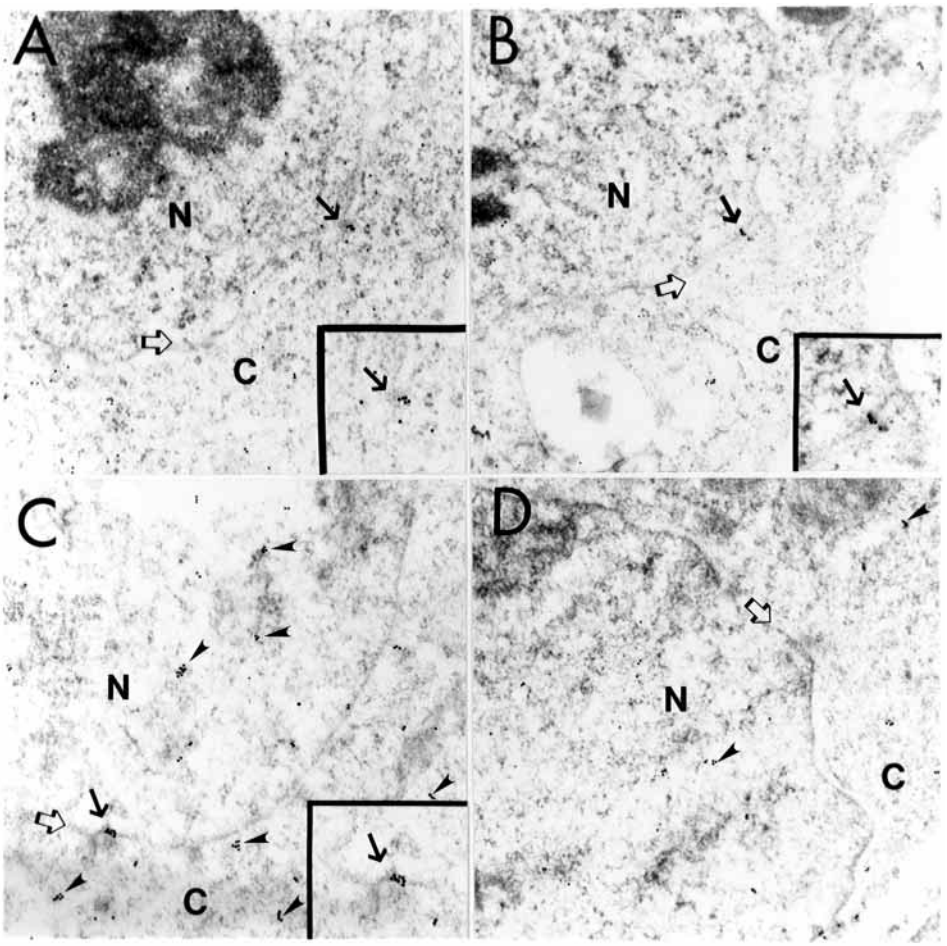

Fig. 2. Subcellular localization of RACK1 and the autophosphorylated forms of PKCα/PKCβII. (A,B) RACK1 (A) and PKCα/PKCβII (B) in wing bud mesenchymal cells of a stage 16/17 embryo. (C,D) PKCα/PKCβII in a mesenchymal cell from an ectopic nascent bud that developed from lateral plate treated with FGF4 (C) and in a cell on the contralateral side of the same embryo (D). In A-C, black arrows point at molecules at the nuclear pore; these areas are enlarged in the inserts. Arrowheads in C,D indicate clusters of gold particles. White arrows point to the nuclear envelope. C, cytoplasm; N, nucleus.

(Fig. 1H). This result was even more pronounced when FGF treatment extended over a period of 24 hours (Fig. 1I,J; *n*=8). Control beads did not induce expression of RACK1 in tissue around the implant (Fig. 1K).

RACK1 protein colocalizes with PKC

Biochemical binding data demonstrate that RACK1 binds and stabilizes activated PKC (Ron et al., 1994; Rotenberg and Sun, 1998). RACK1 has particularly high affinity for PKCα and PKCβ; therefore, we examined by immunohistochemistry whether the activated form of PKC was also expressed in RACK1-positive tissues. RACK1 was detected using a monoclonal antibody, whereas PKCα and PKCβII were visualized with a rabbit polyclonal antibody directed against a functionally critical autophosphorylation region present in autophosphorylated forms of PKCα and PKCβII (see Material and Methods; Sweatt et al., 1998). Upon activator binding PKCs will autophosphorylate (Mochly-Rosen and Koshland, 1987) and this antibody will bind to such activated form of PKCα and PKCβII.

Electron micrographs in Fig. 2A,B show mesenchymal cells of stage 16/17 wing bud tissue immunostained with the antibodies against RACK1 (Fig. 2A) and activated PKCα/PKCβII (Fig. 2B). RACK1 and activated PKCα/PKCβII are both present in the cytoplasm and in the nuclei. Gold particles attached to RACK1 or to activated PKC were associated with nuclear pores (Fig. 2A-C, inserts), raising the possibility that RACK1 and activated PKC are transported between cytoplasm and nucleus. The immunohistochemical analyses demonstrate that RACK1 and activated PKC are present in the same subcellular compartments of the limb mesenchyme cells and therefore have the potential to interact directly during limb growth.

PKC is activated by FGF

FGF treatment of interlimb lateral plate sustains *RACK1* expression that would otherwise be downregulated (Fig. 1H-J). Because RACK1 stabilizes activated PKC, there should be an augmentation of PKC enzymatic activity and an increase in the number of activated PKC molecules in FGF-treated cells. To investigate this possibility, FGF4 was applied to interlimb lateral plate and 24 hours later, PKC activity and the number of autophorsphorylated PKCα/PKCβII molecules in tissue surrounding the bead was determined by in vitro PKC activity assays and quantitative immunogold electron microscopy.

Lateral plate tissue homogenates exhibited autonomous PKC activity (i.e. activity seen in the absence of co-factors, Ca^{2+} and phospholipids) and co-factor-dependent activity (Table 1). There was no significant change in autonomous PKC activity between control and FGF-treated tissue. However, co-factor-dependent PKC activity was significantly increased in FGF treated tissue. In five independent experiments, the ratio of co-factor and autonomous activity increased by 64% on FGF treatment (paired *t*-test, $P<0.008$). It is possible that we do not see a greater increase in PKC activity because the tissue

Table 1. Phosphorylation of $NG_{(28-43)}$ peptide by lateral plate homogenates

	Autonomous PKC activity (fmole/minute/μg)	Co-factor-dependent PKC activity (fmole/minute/μg)	Ratio±s.d.
FGF treated (*n*=5)	406.906	806.658	2.253±0.283
Control (*n*=5)	435.536	534.968	1.372±0.250

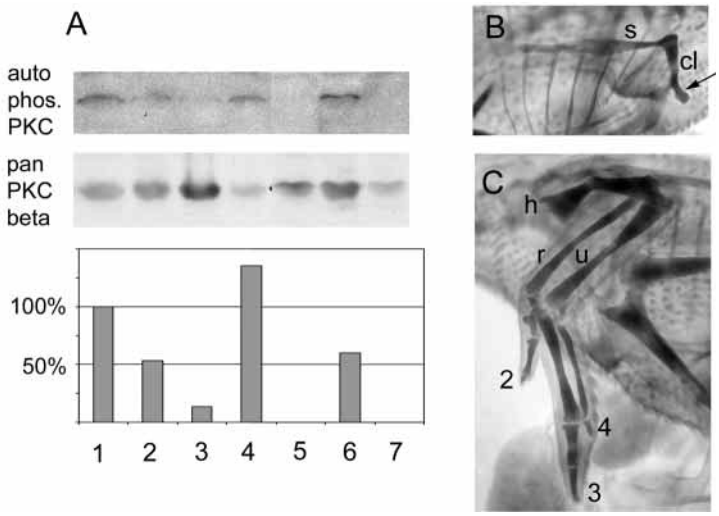

Fig. 3. The effects of PKC inhibitors on PKC activation and limb development. (A) Western blot (top panel) and quantification (lower panel) of activated PKCα/PKCβII in primary limb bud cell cultures treated with PKC inhibitors. 1, medium only; 2, 100 nM Go 6976; 3, 1 μM Go 6976; 4, 13 μM chelerythrine chloride; 5, 130 μM chelerythrine chloride; 6, 30 μM sphingosine; 7, 300 μM sphingosine. In all cases, except for the low dose treatment with chelerythrine chloride, PKC inhibitors were effective in reducing PKCα/PKCβII autophosphorylation. Quantification of PKCα/PKCβII autophosphorylation was achieved by densitometry. Percent activity was normalized to PKCβ detected by a pan PKCβ antibody. Lane 1 (no inhibitor added) was defined as 100% activity. (B) Treatment of the presumptive wing bud with chelerythrine chloride produced a completely truncated wing. The scapula (s) was shortened and the clavicle (cl) had a small protrusion (arrow). (C) Contralateral wings were always normal showing humerus (h), radius (r), ulna (u) and digits 2, 3, and 4.

analyzed is presumably larger than the domain of PKC activation around the FGF-releasing bead.

A gold-tagged secondary antibody directed against the antibody for the activated form of PKCα/PKCβII, permitted direct electron microscopic visualization and counting of activated PKC molecules. Attachment of gold particles was observed in the cytoplasm and in the nuclei of the mesenchymal cells of the ectopic wing bud that developed from FGF-treated lateral plate (Fig. 2C) and in control tissue (Fig. 2D).

In FGF-treated cells, the gold particles were often clustered, indicating the formation of oligomeric protein complexes (compare Figs 2C,D). The gold-labeled antibodies used were free of aggregates, as revealed by inspection of coated grids sprayed with these secondary antibodies only (data not shown). Counting of cluster number and cluster size in thin sections from three different embryos revealed that more and larger clusters of activated PKCα/PKCβII were seen on the FGF-treated side than on the control side (Table 2). In all cases there is a statistically significant increase (analyzed by ANOVA) of the abundance of gold particles per μm^2 in the nucleus of FGF-treated tissue, suggesting an increase in the number of activated PKCα/PKCβII molecules. Moreover, a significant increase was also seen in three out of six cases in the cytoplasmic compartment. FGF treatment increased the total number of activated PKCα/PKCβII molecules in the nucleus by 45% and by 34% in the cytoplasm (Table 3). This table also shows that there were more gold particles per cluster in the nuclei and cytoplasm of FGF-treated cells. For example, five clusters containing six gold particles were detected in the nuclei of FGF-treated cells but such large clusters were absent in the nucleus of control cell. Likewise, 17 clusters of five gold

Table 2. The abundance of activated PKCα/βII in FGF4-treated and normal lateral plate mesoderm

	Section number											
	1		2		3		4		5		6	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
Particles/μm^2 (mean±s.d.) in FGF-treated tissue	4.60±1.25	4.72±1.26	3.89±0.44	2.87±0.53	4.85±2.10	3.85±1.02	4.26±0.59	3.91±1.53	5.76±0.45	5.65±1.49	6.36±0.96	5.76±1.05
Particles/μm^2 (mean±s.d.) in control tissue	3.10±1.24	2.76±0.52	2.00±0.45	1.85±0.31	2.91±0.98	2.42±0.76	2.60±1.05	3.16±1.08	4.74±0.31	4.45±1.08	5.36±0.47	5.82±1.48
ANOVA *t*-test (FGF treated versus control)	$P<0.05$	$P<0.005$	$P<0.001$	$P<0.001$	$P<0.01$	$P<0.005$	$P<0.005$	Not significant	$P<0.001$	$P<0.1$	$P<0.05$	Not significant

In each section, eight fields were randomly selected from the FGF-treated side and eight fields from the contralateral side. Gold particles were counted in nucleus and cytoplasm. Sections 1 and 6 derive from one embryo; sections 2, 3 and 5 derive from another embryo; section 4 is from a third embryo.

Table 3. Clustering and abundance of activated PKCα/βII in normal or FGF4-treated lateral plate mesoderm

	Total number of gold particles	Number of clusters with 6 particles	Number of clusters with 5 particles	Number of clusters with 4 particles	Number of clusters with 3 particles	Number of clusters with 2 particles	Number of single gold particles	Total area analyzed (μm^2)
FGF treated (nuclear)	1175	5	17	32	48	149	490	237.99
Control (nuclear)	758	0	1	13	37	112	366	222.58
FGF treated (cytoplasm)	862	0	6	18	53	112	377	197.92
Control (cytoplasm)	621	0	3	10	26	78	332	191.95

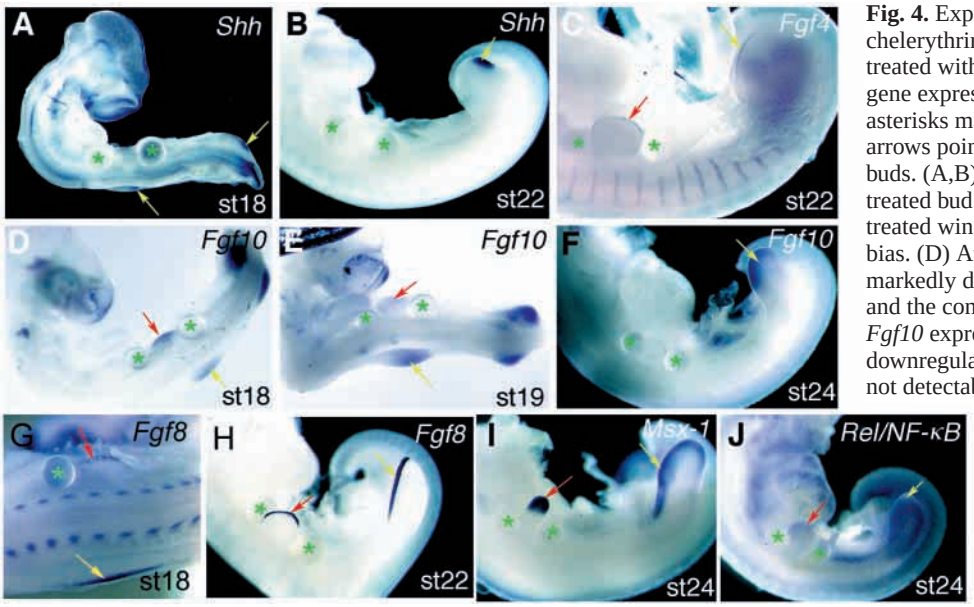

Fig. 4. Expression of various limb patterning genes in chelerythrine chloride-treated wing buds. Embryos treated with chelerythrine chloride were analyzed for gene expression at the stage indicated. Green asterisks mark the slow release beads and yellow arrows point to the normal expression in untreated buds. (A,B) *Shh* transcripts were never detected in the treated bud. (C) *Fgf4* expression in the AER of the treated wing bud (red arrow) has lost its posterior bias. (D) At stage 18, *Fgf10* expression was not markedly different between the treated (red arrow) and the contralateral wing bud. (E) At stage 19, *Fgf10* expression in the treated wing (red arrow) was downregulated. (F) By stage 24, *Fgf10* mRNA was not detectable in the treated bud. In the embryo shown in G, the level of *Fgf8* expression in the AER of the treated wing bud is reduced. By contrast in H, *Fgf8* expression in the AER was not affected. (I) *Msx-1* expression (red arrow). (J) The size of *Rel/NF-κB* expression domain (red arrow) was reduced in the treated wing bud.

particles were seen in the nuclei of FGF treated cells, whereas there was only one such case in control cells. Taken together, our experiments show that FGF increased RACK1 expression, augmented total PKC activity, increased the number of activated PKCα/PKCβII molecules as well as the size of oligomeric complexes.

Blocking PKC signaling in wing tissue results in a truncated wing

RACK1 induction concomitant with PKC activation resulting from FGF treatment of interlimb lateral plate raises the possibility that PKC mediates FGF signaling in the limb bud. To test this hypothesis, we disrupted PKC signaling in the presumptive wing region using PKC inhibitors. As such inhibitor experiments had previously not been carried out in embryos, we first tested the efficacy of the competitive inhibitors chelerythrine chloride (Herbert et al., 1990), Go6976 (Martiny-Baron et al., 1993) and sphingosine in primary limb bud mesenchyme cultures. Chelerythrine chloride and Go6976 are both specific inhibitors for PKC, while sphingosine also inhibits other kinases (Herbert et al., 1990; Martiny-Baron et al., 1993). To assess inhibitor efficacy, we measured the relative quantity of activated PKC protein in cells after inhibitor treatment. Activated PKCα/βII was detected and quantified using the antibody against the autophosphorylated PKCα/PKCβII. A Western blot of protein extracts from limb bud cells cultured for 24 hours with or without inhibitor present is depicted in Fig. 3A. Compared with untreated tissue, the amount of activated PKCα/βII was greatly reduced upon treatment with 1 μM Go6976 (lane 3), 130 μM chelerythrine chloride (lane 5) or 300 μM sphingosine (lane 7). By contrast, the amount of phosphorylated PKCα/βII was reduced by about 50% with a tenfold lower concentration of Go6976 (lane 2) and sphingosine (lane 6). At the lower concentration chelerythrine chloride (lane 4) was not effective. We conclude that at the higher concentration, all three inhibitors effectively block PKC signaling in limb bud mesenchyme cultures.

Next, all three inhibitors were absorbed onto AG1-X2 beads and released into 1 ml PBS. The amount of compound released was monitored by HPLC as a function of time. Only chelerythrine chloride was absorbed and subsequently released from AG1-X2 at significant amounts over a time span of approximately 36 hours (data not shown). Therefore, such beads presoaked in either 3.25 or 6.5 mM inhibitor were implanted into stage 14 embryos at the anterior and posterior boundaries of the wing field. The resulting PKC inhibition led to buds that were significantly smaller (see Fig. 4) and wings were severely truncated in a majority of cases (Table 4, Fig. 3B). In all cases, the contralateral wings of treated embryos were normal (Fig. 3C), which indicated that the effect of the inhibitor at was local.

PKC inhibitor affects the expression of a subset of limb patterning genes

In an attempt to define the pathways for which PKC signaling is required, we examined a series of molecular markers implicated in limb development. The expression of *Shh* was not detectable in treated wing buds at any stage examined (n=11; stage 18-24; Fig. 4A,B). *Fgf10* expression was initially not affected (n=8, Fig. 4D) but after stage 19 it was markedly down-regulated (n=11, Fig. 4E) and transcripts were not detectable by stage 24 (n=3, Fig. 4F). In a normal chick wing bud *Fgf4* expression is elevated posteriorly. In chelerythrine chloride-exposed buds this gene was still expressed but the posterior bias of expression was lost (Fig. 4C, n=10) suggesting that the anteroposterior limb axis was affected. In the case of *Fgf8* (n=6) some embryos showed overall reduced expression in the AER (Fig. 4G), while others showed no significant decrease in the intensity of expression in the AER (Fig. 4H). The expression domains of *Msx-1* and *Rel/NF-κB* encompass the progress zone (Ros et al., 1992; Bushdid et al., 1998; Kanegae et al., 1998) and were similar in intensity in non-treated and treated limbs at stage 20-22 (data not shown). By stage 24, the expression of *Msx-1* (Fig. 4I, n=11) and *Rel/NF-κB* (Fig. 4J, n=6) could still be detected although the expression domains themselves were much reduced in size, reflecting the reduced size of the treated buds. Taken together, these experiments demonstrate that blocking PKC signaling has specific effects on gene expression, leaving the expression

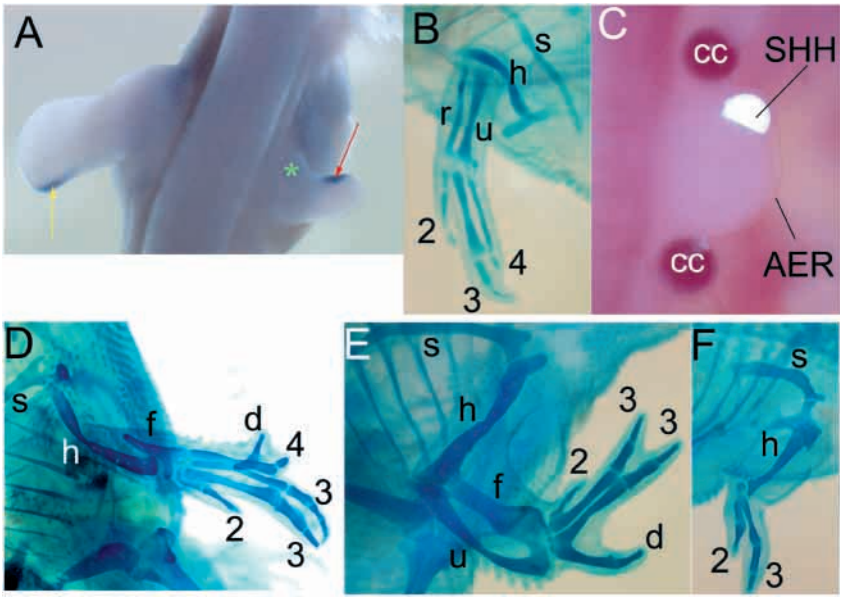

Fig. 5. Rescue of chelerythrine chloride-treated limb buds by RA, a ZPA or SHH (A) Ectopic *Shh* expression in chelerythrine chloride treated wing buds. A yellow arrow indicates normal posterior *Shh* expression in the left wing bud. A red arrow indicates ectopic *Shh* expression induced by a now buried RA bead implant indicated by a green asterisk. (B) An example of the typical skeletal pattern of 10-day-old RA-rescued wing. A complete hand plate is produced, but reversed in polarity, owing to the placement of the RA bead at the anterior side of the wing bud. (C) Placement of chelerythrine chloride beads (cc) and a SHH bead in the wing bud. (D) Skeletal pattern of 10-day-old ZPA-rescued wing. Polarity is reversed due to placement of ZPA at anterior of wing bud. (E,F) Skeletal patterns of 10-day-old SHH-rescued wings. In E, polarity is reversed, owing to placement of SHH bead at anterior side of the wing bud. 2, digit 2; 3, digit 3; 4, digit 4; d, digit; f, forearm element; h, humerus; r, radius; s, scapula; u, ulna.

of some genes largely unaffected, while others are either downregulated or are not expressed at all.

Application of RA, ZPA grafts and SHH overcome PKC inhibition

The most striking and earliest effect of PKC inhibition we detected was the complete absence of *Shh* expression. This raises the possibility that PKC may control *Shh* expression. If this is the case, it should be possible to counteract PKC inhibition by providing ectopic SHH. This was achieved in three ways: RA application (to induce *Shh*), grafting a ZPA and application of SHH protein. In a first set of experiments, a RA-releasing bead was implanted at the anterior margin of stage 20 wing buds that had received (at stage 14) beads soaked in 6.5 mM chelerythrine chloride. Because the RA bead was implanted at the anterior margin, rescue should give rise to a wing with a reversed polarity, which can be clearly identified as patterned from an anteriorly located signaling center. We found that RA applied to chelerythrine-treated wing bud resulted in the induction of *Shh*. Of 13 RA-treated embryos, eight embryos showed a well-formed right wing bud with a prominent domain of ectopic *Shh* expression at the anterior margin (Fig. 5A). Moreover, a morphological analysis of embryos that were sacrificed at day 10 showed a remarkable rescue. Although buds not treated with RA invariably resulted in truncations (Table 4), two thirds of the 30 RA-treated buds produced a wing with a forearm and a hand plate. Of the 20 cases, 15 developed wings that had digits 3 and 4 reversed in polarity, with 4 being anterior to 3 (Fig. 5B).

In a second series of experiments, a ZPA graft was implanted under the AER at the anterior margin of stage 20 wing buds that had been treated with chelerythrine chloride at stage 14. Embryos that were sacrificed at day 10 showed a result similar to that of RA treatment. The ZPA implant rescued bud outgrowth and enabled the formation of distal wing structures. Specifically, four of the six wings treated this way showed a reversal in polarity of the digit pattern (Fig. 5D). In a third series of rescue experiment, SHH protein was applied to stage 20 wing buds that had been treated with chelerythrine chloride at stage 14 (Fig. 5C). In several instances, this treatment resulted in the formation of distal structures (Fig. 5E), although we never recovered a digit 4. Moreover, six out of 13 wings displayed a hand plate with reversed polarity, in which digit 3 was anterior to digit 2 (Fig. 5F).

Collectively, these rescue experiments demonstrate that providing exogenous SHH to wing buds whose PKC signaling had previously been blocked, can in a significant number of cases restore limb development. Because exogenous SHH can counteract PKC inhibition, we conclude that *Shh* is one of the main targets of PKC signaling.

DISCUSSION

When RA is applied locally to the chick limb bud it induces a ZPA and this process involves the activation of various signaling molecules including *Shh*, *Fgf4* and *Bmp2*. It is likely that RA treatment also induces genes encoding signal transducers. We applied a PCR-based differential screening technique to RA-treated chick wing buds and isolated RACK1,

Table 4. Patterns resulting from chelerythine chloride treatment

Chelerythrine chloride concentration	Number of cases	Truncation	Humerus only	Humerus + one or two forarm elements	Humerus + one or two forarm elements + d or 2	Humerus + one or two forarm elements + d or 2 + 3	Normal
6.5 mM	73	20	15	22	8	5	3
3.25 mM	19	3	1	8	2	1	4

a receptor for activated PKC that is highly conserved from *Hydra* (Hornberger and Hassel, 1997) to human (Guillemot et al., 1989). Binding of RACK1 to activated C kinases enhances the ability of PKCs to phosphorylate their substrates (reviewed in Mochly-Rosen, 1995).

The role of RACK1/PKC in the developing limb bud

RACK1 is also inducible by FGF applied to the interlimb flank, suggesting a link between FGF signaling and RACK1 function. Furthermore, our biochemical and immunohistochemical studies of FGF-treated interlimb flank showed that FGF not only induced RACK1, but also resulted in an increase in PKC activity. It has previously been shown that PKCs are activated by Ca^{2+}, phosphatidylserine (PS) and diacylglycerol (DAG), which are generated in response to FGFR-mediated activation of PLCγ (Burgess et al., 1990). Activated PKC molecules are stabilized by binding to RACK1 (Mochly-Rosen et al., 1991; Ron and Mochly-Rosen, 1994; Ron et al., 1994; Ron and Mochly-Rosen, 1995; Csukai et al., 1997). It thus appears that FGFs have two effects in the limb bud: they activate PKCs via the FGFR/PLCγ pathway and at the same time they increase the concentration of RACK1. Such an increased concentration of RACK1 has the benefit of stabilizing active PKC conformers and this enables a more efficient phosphorylation of PKC target proteins.

Although we show that ectopic FGF increased PKC activity, this does not prove that PKCs are required for normal limb development. Evidence for this comes primarily from our inhibitor studies showing that preventing PKC activation resulted in wing buds that failed to express *Shh* and did not maintain *Fgf10* expression. Moreover, such PKC inhibitor-treated buds were markedly smaller and produced highly dysmorphic wings. Importantly, supplying exogenous SHH reversed this outcome, suggesting that the chief consequence of PKC inhibition is preventing *Shh* expression. The absence of *Shh* provides strong evidence for the absence of a ZPA. Hence, PKC signaling seems to be required for the formation of a ZPA.

It has long been proposed that FGFs released from the ectoderm regulate *Shh* expression. Specifically, it is thought that FGF8 secreted by the presumptive AER binds to FGFR2-IIIc in the mesenchyme and as a result of this, the *Shh* gene is activated (Grieshammer et al., 1996; Crossley et al., 1996; Xu et al., 1998; reviewed by Martin, 1998). We posit that ligand-bound FGFR2-IIIc transmits its signal through a RACK1/PKC complex. Although *Fgf8* is still expressed in chelerythrine chloride-treated buds, the ligand-bound receptor does not transmit the signal to the *Shh* gene. Note, however, that this explanation cannot fully account for the wing defects we observe. Recent work shows that mice lacking *Fgf8* in their limb buds exhibit delayed expression of *Shh* and have dysmorphic limbs (Lewandoski et al., 2000; Moon and Capecchi, 2000). Defects are, however, not as severe as in wings that form from chelerythrine-treated buds. Hence, it is likely that PKC not only mediates FGF8/ FGFR2-IIIc signaling but is also required for additional pathways. The nature of these pathways remains elusive but most likely can be studied by identifying PKC substrates.

A PKC substrate of relevance in limb development may be IκB, whose phosphorylation results in an inactivation and degradation of IκB in the IκB/NF-κB complex (Steffan et al., 1995 and references therein). IκB degradation results in a release of NF-κB, which then translocates to the nucleus where it regulates target gene expression. *Rel/NF-κB* is expressed in the progress zone, and inhibition of NF-κB by expression of transdominant-negative IκB protein repressed *Shh* expression and resulted in truncated limbs (Bushdid et al., 1998; Kanegae et al., 1998). It is tempting to speculate that blocking of PKC with chelerythrine results in a failure to degrade IκB and consequently NF-κB cannot enter the nucleus to exert its regulatory function. In addition to IκB, there are presumably additional PKC substrates in the limb region that are phosphorylated as a result of FGF signaling.

The failure to maintain *Fgf10* expression in buds exposed to chelerythrine could be due to the absence of SHH. Alternatively, maintaining *Fgf10* expression could also directly require PKCs. Once limb buds are formed, a reciprocal interaction between AER and ZPA sustain limb outgrowth (Zwilling, 1955; Rubin and Saunders, 1972). FGFs released from the AER and SHH present in the ZPA are key molecular players that maintain bud outgrowth via a feedback mechanism (Moon et al., 2000; Sun et al., 2000). As is the case in early limb development, AER-derived FGFs may act through FGFR2-IIIc and it is possible that RACK1/PKC also transduce the signal from this receptor at later stages of limb development. This possibility is supported by the fact that RACK1 and activated PCK (H.-C. L. and G. E., unpublished) are both expressed in limb buds of stage 19 and older.

Although an inhibition of PKC signal transduction by chelerythrine results in striking and selective changes in gene expression, the cellular mechanisms by which this is achieved remains to be investigated. One possibility is that the inhibitor induces apoptosis. Note, however, expression of several genes characteristic for the progress zone and the AER still occurs arguing against global cell death. A more likely possibility is that inhibition of PKCs reduces the rate of cell proliferation. This would explain the smaller size of the limb buds, and is consistent with the general view that PKCs regulate cell proliferation. Reduction in size could, however, also result from the absence of a ZPA that has long been known to provide proliferative signals to remainder of the bud (Cooke and Summerbell, 1980). Another issue is that chelerythrine treatment predominantly affects gene expression in mesenchyme and not in the AER. Recall that the chelerythrine-delivering beads were implanted into flank mesenchyme and hence had limited contact with the overlying ectoderm and the AER. This point is illustrated in Fig. 5C.

In summary, the results presented here point to the importance of PKCs in mediating limb development. A major unresolved issue concerns the nature of PKCs substrates in the limb bud. Finding such substrates will help understanding the mechanism by which PKCs signal not only during limb development, but also in the numerous other developmental processes in which FGFs are involved.

We thank Alexander Prokscha, Ingrid Boenig and Marianne Michael for expert technical assistance, Drs Juan Carlos Izpisúa-Belmonte and Sumihare Noji for cDNA probes, and Drs Kerby C. Oberg, Kimberley Sweeney for comments on the manuscript. Dr J. David Sweatt and his colleagues provided the rabbit polyclonal antibody against autophosporylated PKCα and PKCβII and the advice for PKC activities assay. Genetics Institute, Cambridge, MA, provided us with recombinant FGF4 protein. This work was supported by a Grant from the Max-Planck Society and the National Institutes of Health to G. E. (HD 20209).

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