CHARACTERIZATION OF THE FGF RECEPTOR SIGNALING COMPLEX IN Xenopus laevis DURING EARLY EMBRYONIC DEVELOPMENT

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Characterization of the FGF Receptor Signaling Complex in *Xenopus laevis* during Early Embryonic Development.

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A thesis submitted to the School of Graduate

Studies for the partial fulfillment of the

Requirements for the degree of

Masters of Science

Faculty of Medicine

Memorial University of Newfoundland

St. John's Newfoundland

Dedicated to my parents for their support throughout my education. They are, and always will, be my pillar of strength. Thank-you

Abstract

A stable complex is formed between the fibroblast growth factor receptor (FGFR) and SH2-containing proteins, during early development in *Xenopus laevis*. The results presented in this thesis demonstrate that phosphorylation of PLCγ1 and its association with FGFR1 occurs during mesoderm induction *in vitro*. PLCγ1 is involved in intracellular signaling pathway of FGF during the time mesoderm induction is occurring in the embryo. PLCγ1 is specifically phosphorylated during early- to mid-blastula stages, yet the expression level of PLCγ1 protein is constant throughout these stages of development. It is speculated that PLCγ1 is involved in a negative feedback loop, down-regulating the FGFR. The timing of the *in vitro* experiments (30-minute treatment of FGF) provides evidence that FGF may be a component of the vegetal inducing signal. There were seven other phosphorylated bands co-immoprecipated with PLCγ1 or FGFR during early embryo development. These substrates were identified as phosphatidylinositol 3-kinase (PI3′K), GTPase-activating protein (Gap), SHP2 phosphatase, Nck and three nck-associated proteins: NAP123, NAP

81 and NAP65. Son of Sevenless (SOS) and growth factor receptor binding protein2 (GRB2) were identified in this complex. Neither SOS nor GRB was phosphorylated. This report provides evidence for the importance of these substrates for FGFR signaling during early embryonic development.

Acknowledgements

I am deeply indebted to my supervisors Drs Laura Gillespie and Gary Paterno, who have supported my Masters program and offered valuable support through my years working with them. I am grateful to them for their guidance and years of enjoyable work experience. They are excellent teachers, who gave me the opportunity to gain vast experience and knowledge in the science industry. Thank-you.

I appreciate all of the help provided by the staff at the Microcomputer/Workstation Resource Center (MWRC), for their valuable computer assistance.

I would like to thank the students and staff of Dr.Alan Pater's Lab for allowing me to use their computer facilities during the writing of this thesis.

I am grateful to the faculty and staff of the Terry Fox Labs for providing a fabulous work environment. It was a pleasure to go to work with intelligent friends who gracefully offered their assistance.

Finally, my love and appreciation go to my husband, Cory, for his support and understanding during the duration of my educational program.

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List of Abbreviations and Symbols Used

AC	(animal cap)
aFGF	(acidic FGF)
Anti-XFR	(Anti-*Xenopus* FGFR)
AP	(Ammonium persulfate)
Bek	(Bacterial expressed kinase)
bFGF	(basic FGF)
BMP-4	(bone morphogenetic proteins)
BSA	(Bovine Serum Album)
$Ca(NO_3)_2.4H_2O$	(Calciun nitrate)
CML	(Chronic myelogenous leukemia)
DAG	(diacyl glycerol)
DEAE-HPLC	(diethylaminoethyl-highperformanceliquid chromotography)
ECDGF	(Embryonal carcimoma-derived growth factor)
EDTA	(ethylenediame tetracetic acid disodium salt hydrate)
EGF	(epidermal growth factor)
EGFR	(EGF Receptor)
eFGF	(embryonic FGF)
FGF	(Fibroblast Growth Factor)
FGFR	(FGF receptor)
Flg	(FMS-like gene FGFR1)
GAP	(GTPase-activation protein)
GDP	(guanine di-phosphate)
GRB2	(Growth factor receptor-binding protein)
GTP	(Guanine tri-phosphate)
HRP	(hydrogen peroxide)
Ig-like	(Immunogolbulin-like)
IP	(immunoprecipitated)
IU	(International units)
kDa	(kilodalton)
MAPK	(Mitogen Activating protein kinase)
MBT	(mid-blastula transition)
M-CSF	(macrophage colony-stimulating factor)
MEK	(MAP kinase kinase)
$MgSO\ 4.7H_20$	(magnesium sulfate)
MIF	(Mesoderm Inducing Factors)

mRNA	(messenger RiboNucleic Acid)
MZ	(marginal zone)
NAK	(Nck-Associated kinase)
NAP	(Nck-Associated protein)
NAM	(Normal amphibian medium)
NF200	(anti-neurofilament)
NGFR	(nerve growth factor receptor)
PDGF	(platelet-derived growth factor)
PECAM-1	(platelet endothelial cell adhesion molecule)
PI	(Phosphotidylinositol)
PI3'K	(phosphatidylinositol 3'-kinase)
PI-4,5-P2	(phosphatidylinositol-4-phosphate)
PIP2	(phosphatidylinositol 4,5-bisphosphate)
PKC	(protein kinase C)
PLCγ1	(Phospholipase Cγ1)
PMSF	(phenylmethylsulfony fluoride)
PTPases	(phosphotyrosine phosphatases)
PY	(anti-phosphotyrosine)
RGB	(Running gel buffer)
Rpm	(revolutions per minute)
RTK	(receptor tyrosine kinase)
SDS	(Sodium dodecyl sulfate)
SDS-PAGE	(SDS-polyacrylimide gel electrophorisis)
SH2	(Src homology$_2$ domain)
SOS	(Son of sevenless)
SSB	(SDS sample buffer)
TEMED	(N,N,N,N'-Tetra-Methyletylenediamine)
TGFβ	(transforming growth factor beta)
TLCK	(1-chloro-3-tosylamido-7-amino-2 heptone hydrochloride)
Y	(Tyrosine)
UV	(ultra-violet)
VZ	(vegetal zone)
WASP	(Wiskott-Aldrich syndrome protein)
WB	(Western blots)
Wnt	(Wingless)
Xbra	(Xenopus brachyury)
XTC	(Xenopus tissue culture)
Xwnt-8	(Xenopus Wingless 8)

Chapter 1

1.1 Introduction

Cell-cell interactions are essential for proliferation and differentiation of cells during embryogenesis. Receptor-ligand combinations that activate signal transduction pathways are known mediators of cell-cell interactions. The expression pattern of these signaling molecules and receptors determines which cells can exchange particular types of information. Once the target cell has received the signal there is an alteration of biochemical pathways that control gene expression, the cell cycle, or metabolism within the target cell.

Fibroblast growth factors (FGFs) are responsible for a mechanism important for cell-cell interaction. The FGF family consists of about twenty family members (Klint and Claesson-Welsh, 1999). FGF-1 and FGF-2, also called acidic FGF (aFGF) and basic FGF (bFGF), exhibit the widest expression among cells (McKeehan, 1998). FGFs act through the FGF receptor (FGFR) where activation leads to tyrosine autophosphorylation of the receptor (Ullrich and Schlessinger, 1990). Tyrosine phosphorylation sites serve as high affinity binding sites for SRC homology (SH2) domain-

containing signal transduction molecules (reviewed in Isaacs, 1997). These molecules initiate a cascade of events, which eventually result in biological responses, often involving changes in gene transcription.

FGFs are regulatory peptides which function in tumorigenesis, repair of tissue injury, and embryonic development (Godspodarowicz, 1991). bFGF is important in the field of vasculogenesis and angiogenesis where it was the first identified growth factor for vascular endothelial cells and serves as an autocrine growth factor, controlling the proliferation, migration, and differentiation of that cell type (reviewed in Godspodarowicz, 1990). FGFs have various physiological effects depending on the specific cell type. They can induce mitogenesis, support cell survival and either induce or inhibit cellular differentiation (Godspodarowicz, 1990). *In vivo,* FGFs have been shown to participate in mesoderm induction and to be important for normal gastrulation movements in early *Xenopus laevis* embryo development (Slack *et al.*, 1987, Reviewed in Slack, 1994). Inappropriate FGF expression or altered FGF signaling may contribute to a number of pathologies, such as cancer, diabetic retinopathy, rheumatoid arthritis, and arteriosclerosis (Chabrier, 1996, Coffin *et al.,* 1995). Mutations in FGFR lead to skeletal disorders such as Achondroplasia (dwarfism) or Crouzon syndrome. However, the exact

mechanisms that give rise to all of these diseases remain largely unknown. A fuller understanding of the biology of the various FGFs and the FGFR mechanism may enable the development of therapeutic approaches that can alter receptor function.

1.2 *Xenopus laevis*: a Scientific Model

Xenopus laevis, the African clawed frog, was utilized for studying signaling mechanisms of the FGFR. The advantages of this system are:

1) Eggs develop outside the female and are large enough (1-2mm) that microinjections and dissections are possible.

2) *Xenopus* care and maintenance is minimal in the laboratory setting. The oocytes can be obtained by a simple injection of chorionic gonadotropin and large numbers of relatively hardy eggs can be obtained.

3) *Xenopus* development is well characterized and documented thus standardizing the classification of the development process.

4) The embryos of *Xenopus* are extremely hardy and are highly resistant to infection after microsurgery.

5) *In vitro* fertilization of the embryos allows the population of eggs to be at the same stage and gives accessibility to all stages.

6) Every cell in the embryo contains yolk that serves as a nutrient supply. This nutrient supply enables fragments of the embryo to be incubated in minimal salt medium during experimental conditions.

7) The overall structure of frog embryos and their development is very similar to that of higher vertebrates.

1.3 Early Embryonic Development

Before fertilization, *Xenopus* eggs have an animal-vegetal polarity, which is visible as a pigmented animal half and an unpigmented vegetal half. The animal hemisphere contains small and medium sized yolk platelets, whereas the vegetal hemisphere contains large yolk platelets. The unfertilized egg is enclosed in a protective vitelline membrane, which is embedded in a gelatinous coat (Wolpert, 1998).

After fertilization, the vitelline membrane lifts off the egg surface and within fifteen minutes the egg rotates within it and the pigmented animal region points upwards. Shortly after fertilization the egg cortex rotates 30% relative to the inner cytoplasm. Cortical rotation determines the future dorsal side of the *Xenopus* embryo, which develops opposite the site of sperm entry and is called the gray crescent (Gerhart *et al.*, 1986; Vincent *et al.*, 1986). The Nieuwkoop center is below the gray crescent on the dorsal side. This region is able to exert a special influence on surrounding tissue and can determine how the cells will develop. The sperm provides a spatial cue that orients the rotation of the cytoplasm, which is essential for dorsal development. If subcortical rotation is blocked, for example by ultra-violet (UV) irradiation of the embryo, then proper axis formation is inhibited (Vincent and Gerhart, 1987). These 'rotationally challenged' embryos are rescued when embedded in agar to mimic the rotation (Vincent and Gerhart, 1987). The direction of cytoplasmic movement determines the future dorsal and ventral side of the embryo. This is the first step in pattern formation during embryonic development.

First cleavage occurs about 90 minutes after fertilization at room temperature and divides the cell through the animal and vegetal hemispheres,

separating the egg into right and left halves. The second cleavage is at right angles to the first cleavage, and separates dorsal from ventral side. The third cleavage is along the equator separating the animal from the vegetal half. Subsequent cleavages occur at approximately 30-minute intervals, at room temperature, until blastula stage (Nieuwkoop and Faber, 1975).

As division continues, a cavity or blastocoel forms in the center of the embryo and is surrounded by a thin layer of ectodermal cells and a deeper layer of endodermal cells. At blastula, the embryo exists as a hollow sphere (six hours of development, 5000 cells) (Nieuwkoop and Faber, 1975).

Maternal contribution of proteins and messenger RNA (mRNA) accumulated during oogenesis are required for normal development of the embryo to the middle of blastula stage. Transcription of the embryonic genome begins during blastula stage at a point known as the mid blastula transition (MBT)(Newport and Kirschner, 1982). At stage 10, nine hours after fertilization, gastrulation begins. During gastrulation there is an involution of presumptive endoderm and presumptive mesoderm to the correct positions for further development of these body structures (Wolpert, 1998) (See figure 1.1).

1.4 Mesoderm Induction

The blastula embryo can be subdivided into different regions on the basis of the cell type each region will develop (fate map). The animal cap (AC) or presumptive ectoderm comprises of the upper half of the blastocoel roof. The region around the equator, or marginal zone (MZ) is the presumptive mesoderm and the bottom of the embryo consists of the yolky vegetal zone (VZ) or presumptive endoderm (See Figure1.2). At blastula stage, the equatorial cells, which contact both the animal and vegetal cells in the equator, differentiate into mesoderm. If the stage-8 embryo is divided into explants of animal, marginal, and vegetal cells and cultured in salt solution, the animal explants will develop into epidermis; marginal zone explants primarily into mesoderm tissues; and vegetal explants into endodermal cells. However, animal caps will also form mesoderm if they are combined with vegetal pieces or treated with FGF (Nieuwkoop, 1975)(See Figure 1.2B). These results suggest that the marginal zone cells develop into mesoderm because of their proximity to the vegetal cells that secrete an inducer. In the normal embryo, the cells in the animal hemisphere are thought to be too far from the source of the inducer and differentiate into ectoderm instead of mesoderm. The suggested inducer probably involves a member of the transforming growths factor beta (TGFβ)

Figure 1.1 Time Course for the Developing *Xenopus* Embryo

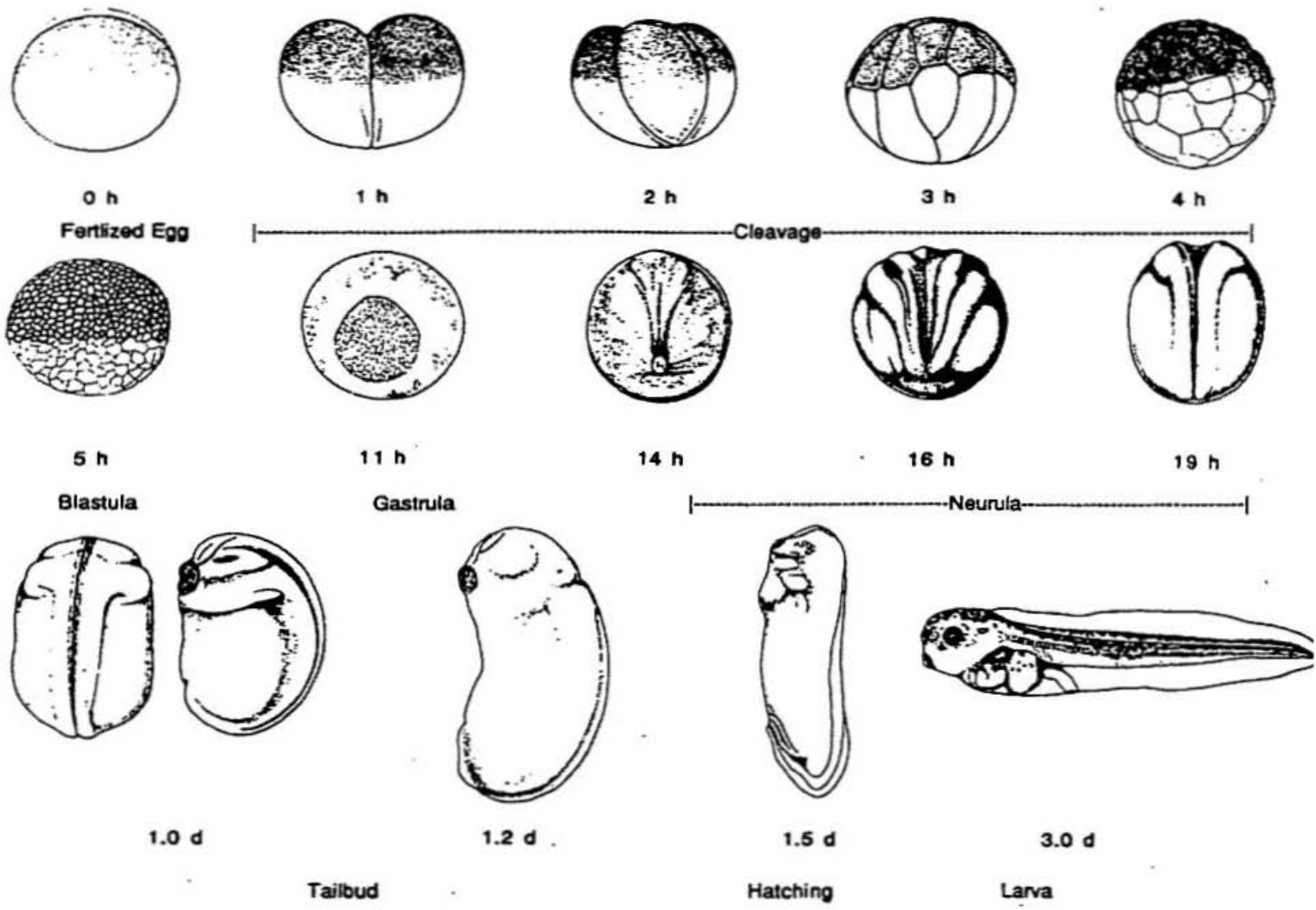

Figure 1.1 This figure represents stages of the developing *Xenopus* embryo showing cleavage furrows and developmental processes. The time after fertilization that is required for embryos to reach each stage is represented in days (d) or hours (h) (Nieuwkoop and Faber, 1975).

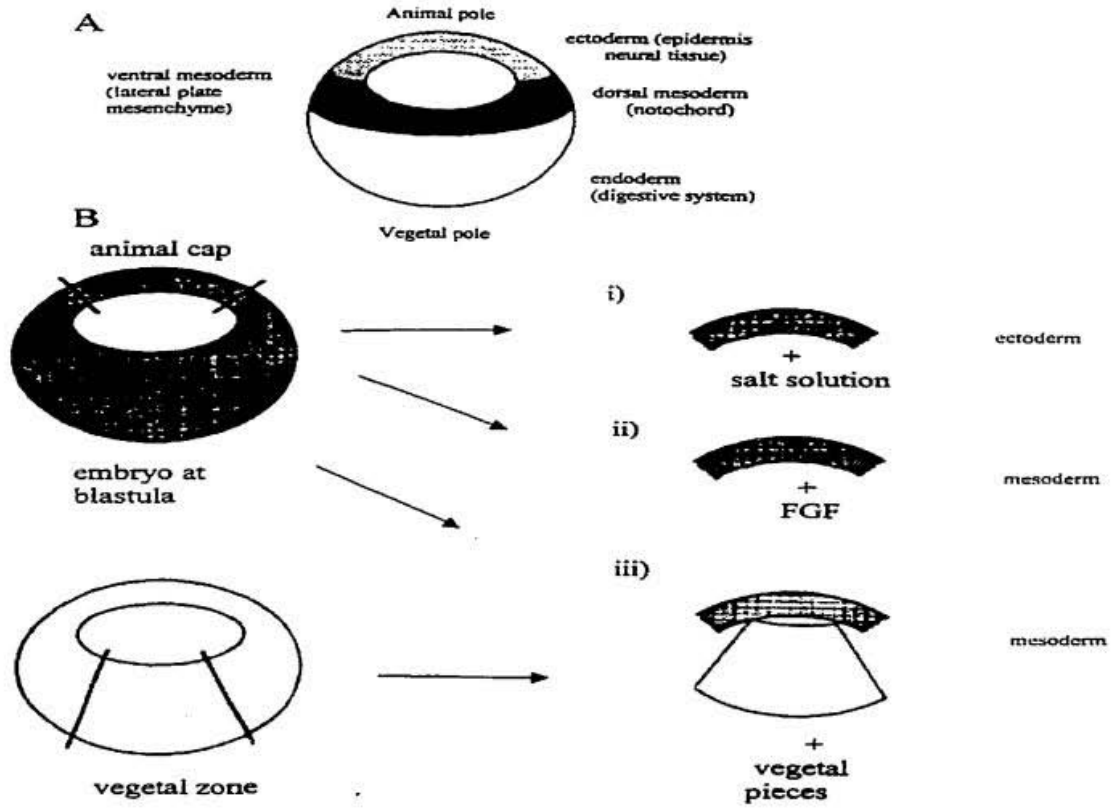

Figure 1.2 Fate Map of a *Xenopus* embryo A) Shows a blastula embryo subdivided into three regions. 1) presumptive ectoderm of the embryo (light stippling) 2) presumptive mesoderm (black area) is located around the equator, which will develop into mesodermal tissue as indicated and 3) presumptive endoderm in vegetal hemisphere (white area).

B) **Mesoderm Induction Assay.** Tissue from the animal pole (presumptive ectoderm) is surgically removed incubated (i) in salt solution or (ii) salt solution and FGF or (iii) in contact with a vegetal piece. The model derived from this experiment is that a signal is emitted from the vegetal zone during mesoderm induction that may involve FGF.

family, but FGF has to be present in the marginal and animal cap cells to make them responsive to the inducer (Dale, 1997).

At about the 32-cell stage (stage 6) the dorsal marginal zone cells begin to become dorsal mesoderm (Gimlich, 1986). Dale and Slack (1987) found that in isolation, dorsal mesoderm forms mainly notochord, whereas ventral mesoderm gives rise to blood but no muscle is formed. When they are incubated together, however, the ventral mesoderm also forms large amounts of muscle. They suggested a three-signal model for mesoderm induction to explain their results (See Figure 1.3). The first signal originates in the dorsal side of the embryo, which induces notochord, and a small amount of muscle. The second signal, from the ventral side of the embryo induces blood. A third signal comes from the newly induced dorsal mesoderm or 'Spemann organizer'. This signal causes the ventral mesoderm adjacent to dorsal mesoderm to form muscle instead of blood. Spemann and Mangold (1924) identified the dorsal lip region of an embryo as the organizer region. This region in the developing embryo involutes during gastrulation. The Spemann organizer has to be induced itself first through the Nieuwkoop center (located opposite the site of sperm entry below the

gray crescent on the dorsal side) (Wolpert *et al.*, 1998). The 'organizer' was able to induce a whole set of dorsal and lateral structures when transplanted into the ventral side of an early gastrula embryo.

The prospective mesoderm differentiates into cardiac and skeletal muscle, notochord, bone and cartilage, blood, kidney and mesenchyme. Recent evidence suggests that factors released by the Spemann organizer inhibit the inducer (Reviewed in Heasman, 1997). In the region closest to the Spemann organizer, inhibition of ventral mesoderm is the greatest, as a result there is more dorsal mesoderm induced. The further the distance from the organizer the concentration gradient of these factors is decreased causing less inhibition. This gives rise to more ventral tissues induced by the vegetal factor. Factors such as noggin and chordin, dorsalize the embryo or factors such as bone morphogenetic protein (BMP-4) and *Xenopus wingless 8 (Xwnt-8)* ventralize the embryo (Wolpert *et al.*, 1998).

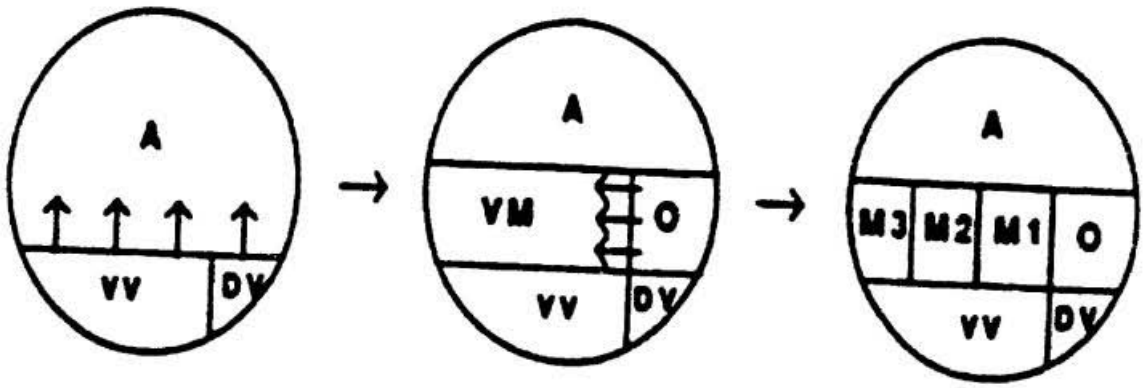

Figure 1.3 The Three Signal Model. Two mesoderm induction signals are assumed to derive from the vegetal region of the early blastula into the animal cap region (A). The dorsal-vegetal (DV) signal induces dorsal mesoderm, or 'organizer' tissue (O), while the ventral vegetal signal (VV) induces general ventral mesoderm (VM). The organizer produces a third signal probably during gastrulation that induces additional muscle. Only the most ventral cells form blood-forming mesoderm (M3). There is more dorsal mesoderm (M1) formed next to the organizer region (Dale and Slack, 1987).

1.5 Mesoderm Inducing Factors

The components of the mesoderm induction signal are unknown. There have been a number of studies to identify these components. If animal caps are cultured in XTC (*Xenopus* tissue culture) media, the animal cap will differentiate into mesoderm (Smith, 1987). The XTC cell line secretes Mesoderm Inducing Factors (MIF). It has been shown that these *in vitro* active factors are actually known cytokines or growth factors (Slack, 1987). MIFs fall into two categories: those belonging to the TGFβ superfamily and those belonging to the FGF family.

The members of the TGFβ superfamily exist as dimeric proteins linked by disulfide bridges and are initially synthesized as longer precursors and later processed (Massague, 1987). Members of the superfamily with MIF activity are TGFβ 2 and 3, activin (Smith *et al.*, 1990) and certain bone morphogenetic proteins (BMP-4) (Koster *et al.*, 1991;Jones *et al.*, 1992). A homologue of activin A was identified as the active component in mesoderm inducing XTC medium (Smith *et al.*, 1990). Studies inhibiting activin activity with follistatin did not prevent mesoderm formation in early embryos (Schulte-Merke *et al.*,

1994). Because of these findings, activin was not believed to play a role in mesoderm formation *in vivo.*

Recent evidence has now implicated activin, or a close relative that has yet to be described, to be important in development (Dyson and Gurdon, 1997). Two members of the TGFβ family shown to be expressed maternally and to have mesoderm-inducing activity are Vg1 and BMPs (Hyatt *et al.*, 1996). Vg1 is a vegetally localized maternal mRNA that becomes distributed in the vegetal half of the embryo after fertilization. It is speculated that Vg1 is important in dorsal axis formation, left-right axis determination and endoderm specification. Dyson and Gurdon designed a new dominant-negative receptor lacking the transmembrane domain selectively blocking activin function but not Vg1 function. Embryos injected with this construct lacked head structures, suggesting a deficiency in anterior differentiation. Analysis of these embryos showed that blocking activin signaling reduces the initial induction of *Xenopus brachyury (Xbra)* to 20% of the wild-type level. *Xbra* is an immediate-early (independent of protein synthesis) pan-mesodermal gene marker (Smith *et al.*, 1991) and a reduction in *Xbra* suggests a role for activin in initial mesoderm induction.

FGFs are heparin binding growth factors that have also been found to be important in early embryo development (Slack *et al.*, 1987). The name FGF was derived from the assay used to identify their growth promoting activity, stimulation of DNA synthesis in fibroblasts (Slack, 1994). Subsequently, it became apparent that these growth factors were able to promote the growth of mesodermal and neuroectodermal cells both during embryogenesis and in the adult (Fernig and Gallagher, 1994).

Three types of FGF have so far been cloned from *Xenopus* that are direct homologues of each mammalian type: FGF-2, FGF-3 and embryonic FGF (eFGF) (Reviewed in Slack, 1994). All three types of Xenopus FGF are expressed in early development. FGF-2 and eFGF are present in the oocyte and fertilized egg and are therefore available at the time of mesoderm induction. FGF has been found to be responsible for the maintenance of *Xbra* and *Xbra* activates expression of FGF (Schulte-Merker and Smith, 1995). As mentioned earlier, *Xbra* is an immediate-early pan-mesodermal gene marker whose expression decreases with the inhibition of activin. Overexpression of *Xbra* in presumptive ectoderm after injection of its mRNA causes formation of ventral mesoderm (Schulte-Merker and Smith, 1995). When eFGF is inhibited

by the dominant negative FGFR, in *Xenopus*, there is a change of dorsal mesoderm such that it moves around the blastopore lip instead of elongating in an antero-posterior direction (Isaacs *et al.*, 1994). In these embryos there is a reduction in *Xbra* expression during gastrulation. eFGF and *Xbra* are able to activate the expression of each other, suggesting that they are components of an autocatalytic regulatory loop (Isaacs *et al.*, 1994). If the activity of the FGF family is inhibited by overexpression of a dominant-negative FGFR, there is a reduction in mesoderm formation, abnormalities arising from and inhibition of normal gastrulation movements and defects in the formation of the posterior parts (Isaacs *et al.*, 1996). The authors speculate that the mesoderm formation and cell movement defects are attributable to loss of *Xbra* expression, and the posterior defects were due to a lack of posterior gene activity. Overexpression of the eFGF gives rise to a posteriorized phenotype, in which posterior genes are expressed in a more anterior position (Isaacs *et al.*, 1996)

It is not clear exactly how these growth factors work together in mesoderm induction and axial patterning. There are various reports to identify the exact components of the signal in the three-signal model explained earlier. From these experiments, there have been modifications of the original model.

UV-irradiated embryos have provided an insight into the mechanism of dorsal patterning of mesoderm. UV-irradiated embryos form mesoderm that does not contain muscle tissue or notochord, but contains mesenchyme, mesothelial tissue and blood tissue (Smith and Slack, 1983). The embryos lack dorsal/ventral and anterior/posterior axes. UV irradiation is effective at blocking this dorsal pathway when it is applied to the vegetal pole of fertilized eggs shortly after sperm entry, or to the vegetal pole of full-grown oocytes (Holwill *et al.*, 1987). This suggests that a component of the dorsal pathway must be established during oogenesis and localized to the cortex of the vegetal pole before maturation. In embryos irradiated after fertilization, Gerhart (1989) found that the UV disrupts cortical cytoplasmic movements by disorganizing microtublues and preventing the normal displacement of a dorsalizing activity to the dorsal side of the embryo. The exact nature of this dorsalizing activity is not fully understood. One approach used to identify molecules important in this mechanism is to determine which molecule will rescue these UV-ventralized embryos (Slack, 1994). Another experimental approach to identify molecules important in pattern formation uses the

depletion of mRNA from the oocyte and studies the effect that depletion has on development (Heasman *et al.*, 1994). This method uses antisense oligodeoxynucleotides to target-specific mRNAs, which are then cleaved by endogenous RNase H. Combinations of these two experimental approaches have led to advancement in the understanding of the dorsal/ ventral pathway.

Dorsal axis formation in *Xenopus* appears to depend upon cytoplasmic components homologous to those of a signaling pathway in *Drosophila,* which is initiated by wingless (Wnt), a secreted protein. This *wingless*-initiated pathway has been shown to be of critical importance in the dorso-ventral patterning of *Drosophila.* Several *Xenopus* members of the *Wnt* family (*Xwnt* genes) are able to rescue the ventralized phenotype of UV-irradiated *Xenopus* embryos: *Xwnt-8* (Smith and Harland, 1991), *Xwnt-8b* (Cui *et al.*, 1995) and *Xwnt 11* (Ku and Melton, 1993).

BMPs have been implicated in the development of the ventral pathway. Suppression of BMP4 activity in the UV-irradiated embryo leads to a restoration of axial structures, although not head structures (Steinbeisser *et*

al., 1995). This suggests that the overactivity of the BMP pathway may be partly responsible for the phenotype that results from UV irradiation.

The patterning mechanism that has emerged in recent years consists of an interaction between dorsal and ventral cells (Smith and Harland, 1992). The time at which this interaction occurs is not yet known. In addition to signals in the previous model, a fourth signal has been speculated, establishing further pattern within the mesoderm, by interacting with the third signal. The secreted dorsalizing molecules, such as noggin and chordin are products of genes transcribed in the Spemann organizer. They convert ventral mesodermal tissue to dorsal mesodermal types when they are overexpressed on the ventral side of the embryo (Smith and Harland, 1992) (See Figure 1-4).

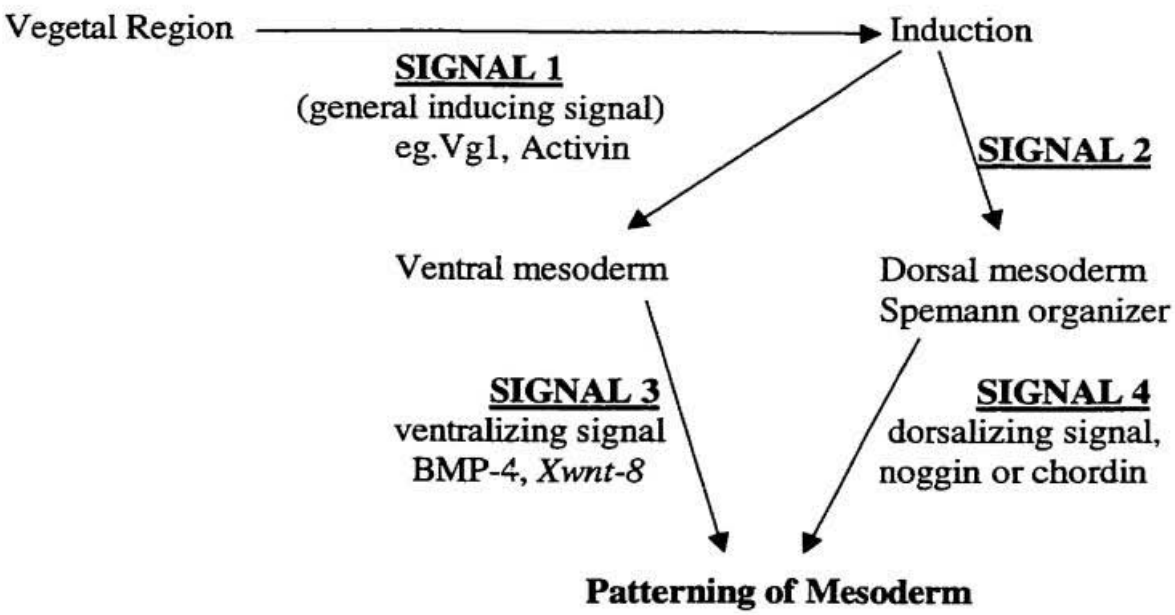

Figure 1.4 **Summary of Mesoderm induction in *Xenopus laevis***
(Wolpert *et al.*, 1998)

1.6 Fibroblast Growth Factor Receptor

The apparent importance of FGF in mesoderm induction triggered various studies to determine the mechanism of action of this growth factor. FGF has been shown to act through the transmembrane FGFR. Four FGFR genes are known to exist in mammals (Ruta *et al.*, 1988; Dionne *et al.*, 1990; Keegan *et al.*, 1991; Machael *et al.*, 1992). The known forms are:

1) FGFR *Flg* (fms-like gene FGFR1) (Ruta *et al.*, 1988): This was the first FGFR to be characterized. It was purified from chicken embryo extracts incubated with biotinylated FGF in the presence of heparin. The 92kDa protein contained a single membrane spanning region, an amino-terminal signal peptide, and three extra-cellular immunoglobulin-like domains (Ig-like). Between the first and second Ig-like domains the receptor contained a unique domain that was not present in other known growth factor receptors. This domain consists of eight consecutive acidic residues and is referred to as the 'acid box'. This is a unique feature of the FGFR and may be important for stabilization of the protein configuration during ligand-receptor interaction (Lee *et al.*, 1989).

2) FGF receptor *Bek* (FGFR2) bacterial expressed kinase (Dionne *et al.*, 1990)

This clone was obtained by screening a mouse liver cDNA expression library with anti-phosphotyrosine antibodies (Kornbluth *et al.*, 1988). The amino acid sequence of the *bek* clone and the corresponding region of *flg* are 85% identical.

3) FGFR3 (Keegan *et al.*, 1991)

To isolate this gene, a cDNA library from the human chronic myelogenous leukemia (CML) cell line was screened with the chicken V-sea gene, a receptor-like tyrosine kinase. This clone has three Ig-like regions and is activated by both aFGF and bFGF.

4) FGFR4 (Machael *et al.*, 1992)

FGFR4 has been cloned from human k-562 erythroleukemia cells. FGFR4 binds to aFGF, but not bFGF. The FGFR4 protein is somewhat unique

in that it contains a core of only four acidic residues in the acid box domain, compared to eight acidic residues in FGFR1.

The FGFR1 open reading frame consists of 800-822 amino acids, with a 90kDa protein formed. The estimated mass of the FGFR is approximately 110-150 kDa, with 6-9 potential N-linked glycosylation sites in the extracellular domain. The open reading frame consists of an 18-24 amino acid signal peptide, an extracellular domain of 346-356 amino acids, a transmembrane domain of 21 amino acids, and a cytoplasmic domain of 410-425 amino acids. The extracellular domain of the FGFR tyrosine kinases contains two or three Ig-like domains. The cytoplasmic domain consists of a juxtamembrane region, the kinase domain, which contains a 14 amino acid insertion, and a C-terminal tail (Jaye *et al.,* 1992) (See Figure 1.5).

The only forms identified to date in *Xenopus* are FGFR1 and FGFR2 (Cornell *et al.*, 1995). Studies of distribution show that FGFR1 mRNA is present throughout the early stages of development (Slack, 1994). The mRNA is more abundant in the marginal zone and animal hemisphere. FGFR2 is not

expressed in the blastula but is first detected in the anterior neural plate during gastrulation (Freisel and Brown, 1992). The expression of FGFR2 suggests that it is not involved in the establishment of mesoderm.

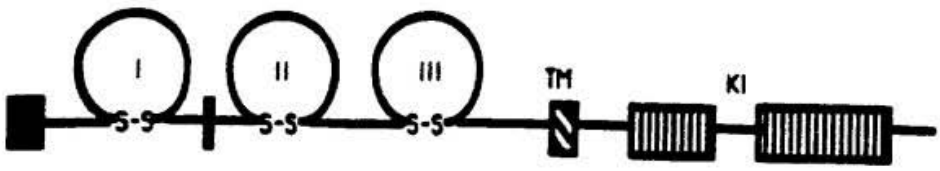

Figure 1.5 Schematic representation of the structural features of FGFR1. Sequence analysis predicts a protein with a hydrophobic leader sequence (black box), three extracellular Ig-like domains (I, II and III) with disulfide linked cystein residues (S), and a short acidic sequence (black box between I and II). This is the extracellular ligand-binding domain. There is also a single hydrophobic transmembrane domain (TM). The cytoplasmic domain contains a juxtamembrane region (believed to be important in regulation of the signal) and an intracellular tyrosine kinase domain to phosphorylate tyrosine residues. The tyrosine kinase domain is split by a 14-amino acid insert sequence (KI).

The FGFR field became more complex with the discovery that there are various alternatively spliced mRNA transcripts from at least FGFR1 and FGFR2 genes. Alternative splicing results in either 1) the inclusion/exclusion of additional amino acids or 2) the use of alternated coding exons with no net gain or loss of amino acids. In either case, the resulting proteins are structurally different (Johnson and Williams, 1993).

The significance of the large number of isoforms is not completely understood; however it is known that alternative splicing in the extracellular domain of these receptors results in altered ligand binding specificity. There are reports of the isolation of cDNAs encoding FGFR1 proteins that are missing Ig domain I (Johnson *et al.*, 1990). Analysis of this FGFR1 gene revealed the presence of an intron separating Ig domain I from the remainder of the FGFR1 coding sequence (Johnson *et al.*, 1991). Thus it appears that the presence or absence of the Ig domain is mediated by alternative splicing. The predominant form of the receptor expressed during embryogenesis is the three Ig form (Johnson *et al.*, 1990). The third Ig loop can also be spliced to form variants resulting in other FGFR forms: IIIa, IIIb, or IIIc. It has been demonstrated that the replacement of the IIIc exon by the IIIb exon reduces the relative affinities of the receptor for bFGF approximately 50-fold (Werner *et*

al., 1992). This finding suggests that the second half of the third Ig-like domain confers the specificity for binding to bFGF. Differential splicing within the third Ig-like domain provides a mechanism to change the affinity of the receptor for different ligands (Werner *et al.*, 1992) and introduces some degree of specificity.

Currently, only single cDNAs have been isolated for FGFR3 and FGFR4. Both cloned cDNAs encode three Ig domain receptor forms (Johnson and Williams, 1993). The discovery of multiple forms of the FGFR raises many important questions for future investigations. Due to the distribution of FGFR1 mRNA, this work examines only FGFR1 and its importance in mesoderm induction.

1.7 Mechanism of Action of the Receptor

The mechanism of action of the FGFR is called the receptor oligomerization model (Ullrich and Schlessinger, 1990). Binding of the ligand, FGF to the extracellular domain causes a conformational alteration of the FGFR, which induces oligomerization of receptors. Dimerization can take place between two identical receptors (homodimerization) or between different members of the same receptor family (heterodimerization) (Ullrich and

Schlessinger, 1990). Dimerization of the receptor activates the internal kinase domain and enables the receptor to autophosphorylate on tyrosine residues. Phosphorylation of dimerized receptors appears to occur via an intermolecular transphosphorylation mechanism. The phosphorylation of the internal kinase domain enables the receptor to autophosphorylate on tyrosine residues. Of the five tyrosine residues located in the cytoplasmic part of FGFR1, not including the kinase domain, tyrosine 776 (Y766) appears not to be phosphorylated, whereas Y463, Y583, Y585 and Y766 are potential phosphorylation sites (Mohammadi *et al.*, 1996). Three phosphorylation sites have been identified in the FGFR1 kinase domain, Y653,Y654 and Y730. Y653 and Y654 appear to be involved in regulation of the kinase, since mutation of Y653 and Y654 to phenylalanine residues leads to loss of kinase activity (Mohammadi *et al.*, 1996). The phosphorylation of the internal kinase domain enables the receptor to phosphorylate other cytoplasmic molecules. These molecules transduce biological responses, often involving changes in gene transcription.

Phosphorylation on tyrosine, within the FGFR, acts as a switch to allow the binding of cytoplasmic proteins containing SH2 domains (Feng and Pawson, 1994). SH2 domains are approximately 100 amino acids in length. They are non-catalytic regions, conserved among a series of cytoplasmic

signaling proteins. SH2 containing proteins bind to phosphorylated tyrosine residues on the receptor. The intracellular kinase activity of the FGFR phosphorylates these substrates. Various SH2 domains have different preferences for different tyrosine residues, adding specificity to this interaction (Feng and Pawson, 1994).

One approach used to study the function of the FGFR in *Xenopus* development was the construction of a dominant-negative form of the receptor. This construct involves deleting the FGFR intracellular kinase domain creating a non-functional mutant of the *Xenopus* FGFR. When mRNAs coding for these mutants are injected into the early embryo they are translated, and the resulting mutant proteins form non-productive dimers with endogenous wild-type receptor. The functional response of the receptor is abolished. Animal cap explants cut from embryos injected with the mutant failed to induce mesoderm when incubated with bFGF (Amaya *et al.*, 1991). In whole embryos, the injection caused specific morphological defects during gastrulation and posterior development. The absence of FGF signaling causes a reduction, although not a total depletion of mesoderm formation (Slack, 1994). There is also a severe effect on axis formation in which formation of the posterior parts is reduced. As a consequence, there is inhibition of

invagination and elongation of the dorsal mesoderm (Slack, 1994). This suggests that FGF signaling is necessary for normal gastrulation movements, particularly in the dorsal mesoderm.

The activation of the FGFR results in the phosphorylation of various intracellular proteins leading to the activation of various downstream targets (Isaccs, 1997). Signal transduction pathways lead to a variety of physiological effects. The mechanism through which the various FGFRs signal specific genes to produce different physiological effects is not completely understood. A better understanding of the mechanism through which these pathways interact will help give an insight in the above process. The stimulated FGFR is able to activate a number of known pathways. Some of these pathways in various cell systems are: 1) the Ras pathway and the Ras-dependent activation of MAPK (Figure 1.6) 2) the activation of the PI3'K pathway (Figure 1.7), also shown to activate the MAPK pathway and 3) PLCγ pathway and the Ras-independent activation of MAPK (Figure1.8).

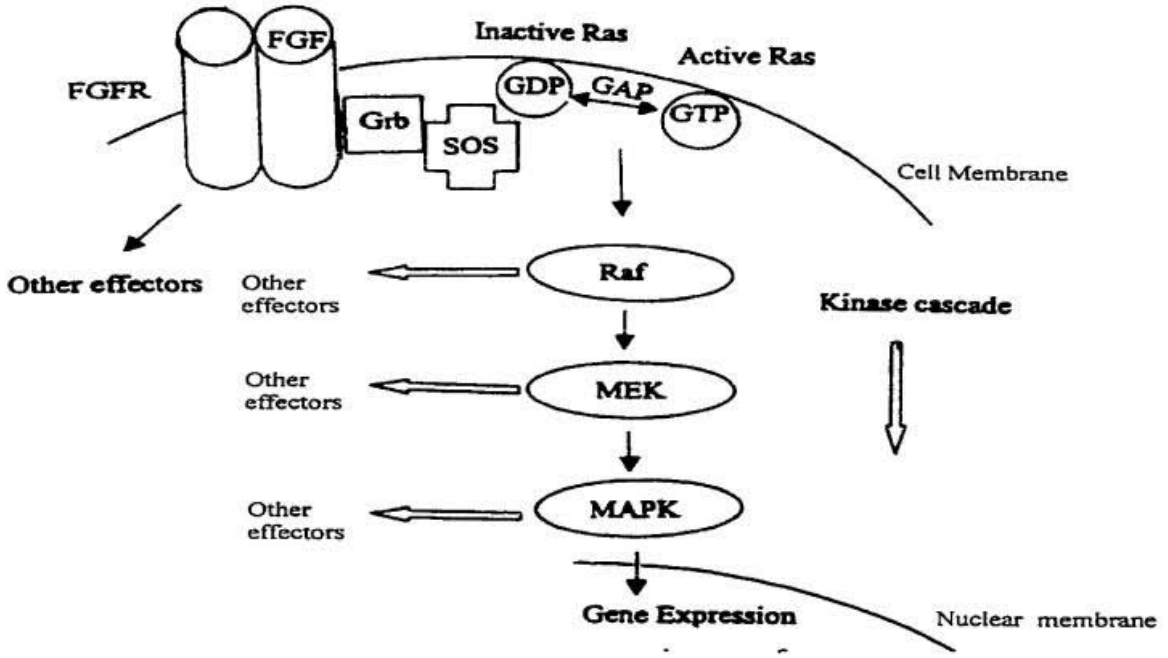

Figure 1.6 Signaling through the FGFR to Activate the Ras Pathway.

FGFR is activated when FGF binds. This leads to the dimerization of the receptor, activating the internal kinase domain. Autophosphorylation of the receptor occurs, providing binding sites for SH2 containing adapter proteins. One such protein, GRB2, complexes with the Ras-guanine nucleotide exchange factor Son of Sevenless (SOS). The recruitment of this complex to the plasma membrane is believed to bring Sos into close proximity to Ras, thus allowing it to activate Ras by catalyzing the exchange of GDP (inactive form) for GTP (active form). The activity of Ras is increased by GTPase Activation protein (GAP) leading to an increase of hydrolysis of GTP to GDP. Once activated, Ras is able to interact with and activate other proteins, which in turn stimulates signaling cascades that induce a variety of cellular responses. Ras activation culminates in the activation of Raf, which phosphorylates protein kinase (MAPK or ERK) (Smith *et al.*, 1995).

Figure 1.7 PI3'K Pathway

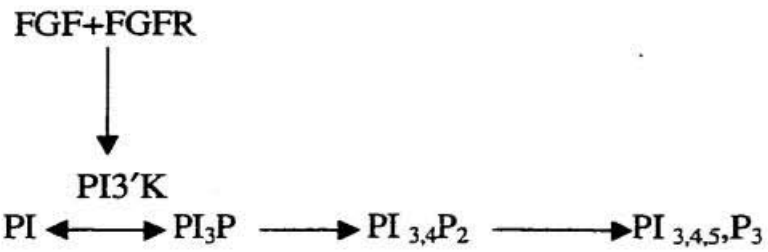

Figure 1.7 Signaling through an RTK to activate Phosphatidylinositol 3'-Kinase Pathway for inositol phosphate metabolism. FGF activates the internal kinase domain of the FGFR. This kinase is able to activate other cellular substrates. One of these substrates is PI3'K. PI3'K is important in the breakdown of phosphotidylinositol (PI) into phosphatidylinositol 3-phosphate (PI_3P). This is the beginning of the PI3K pathway, where other products are produced for example: $PI_{3,4}P_2$, phosphatidylinositol 3,4-bisphosphate; $PI_{3,4,5},P_3$, phosphatidylinostol 3,4,5-trisphosphate. These chemicals are important second messengers, which are involved in a large number of different biological pathways. This pathway and other related inositol phosphate pathways form the basis of various transduction mechanisms now known to regulate a large array of cellular processes, including metabolism, secretion, contraction, neural activity and cell proliferation (Heldman, 1996).

Figure 1.8 PLCγ Pathway

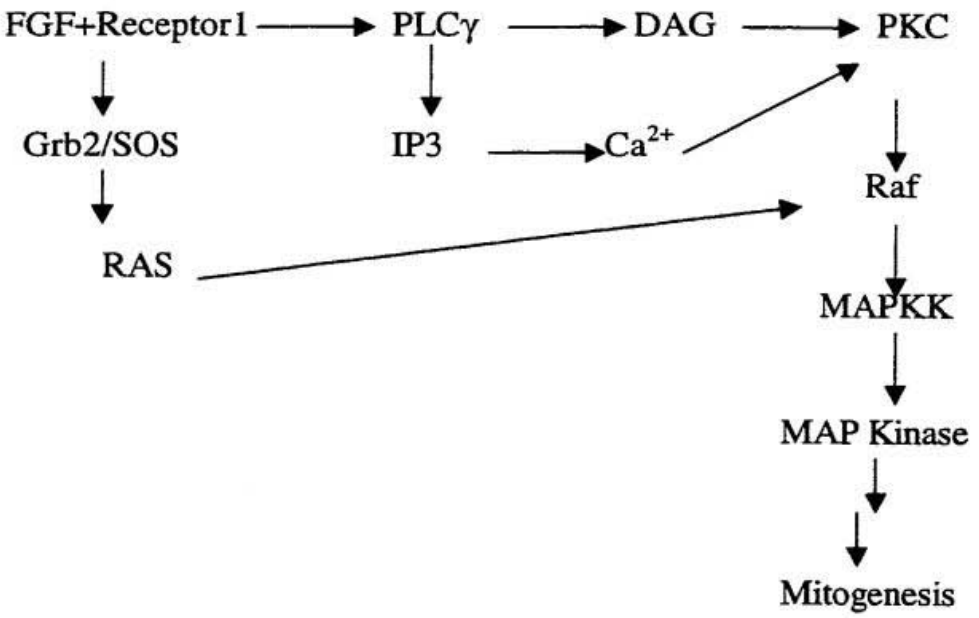

Figure 1.8 A Model for mitogenic signal transduction after growth factor stimulation (Huang, 1995). This shows two pathways used to transduce signals from the FGFR, a Ras independent and Ras-dependent activation of Raf. The PLCγ pathway produces diacylglycerol (DAG) and/or inositol triphosphate (IP3) upon activation of the receptor and phosphorylation of PLCγ. IP3 releases stores of intracellular calcium (Ca^{2+}) acting as second messenger to activate PKC. DAG also activates PKC, which is known to stimulated Raf and the MAPK pathway. The Ras-dependent pathway involves the Ras pathway shown in Figure 1.6. This figure shows that the various pathways are able to interact within the cell (Huang, 1995).

1.8 Substrates of Receptor Tyrosine Kinases

Despite the structural differences between receptor tyrosine kinases (RTK) such as Epidermal growth factor (EGF), FGF or Platelet derived (PDGF), they all share a common mechanism for activation. FGF probably acts through the same system as PDGF and EGF- although in some cases it is not certain which exact molecules are involved *in vivo*. Some of the other substrates, activated in RTK systems, are:

1) Phospholipase Cγ1 (PLC-γ1) One of the PLC isoforms cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to the second messengers diacyglycerol and inositol triphosphate, which in turn activates protein kinase C and raises the intracellular calcium level respectively (Rhee *et al.*, 1989) (See Figure 1.8). PDGF stimulates PI turnover in cells where PLC-γ1 is the principal PLC isoform, and overexpression of PLC-γ1 enhances the accumulation of inositol phosphates in response to PDGF (Margolis *et al.*, 1990)

2) SHC is expressed as two proteins of 46 and 52 kDa, each containing a C-terminal SH2 domain and an N-terminal glycine/rich sequence. Although no enzymatic activity has yet been described for the SHC proteins, overexpression of SHC has been shown to result in cell transformation. SHC proteins become tyrosine phosphorylated by activated tyrosine kinases. In addition, SHC proteins form complexes with *GRB/sem 5* gene product that links receptor tyrosine kinases and $p21^{ras}$, suggesting a role for SHC proteins in the Ras pathway (Richard, 1995).

3) Ras and related proteins of the Ras superfamily play critical roles in the control of normal and abnormal cell proliferation (reviewed in Milligan, 1992). In mammalian cells there are four true Ras proteins (encoded by Ha-Ras, N-Ras, Ki-RasA, and Ki-RasB) which, upon mutational activation, can function as independent oncogenes. These proteins relay signals from tyrosine kinases at the plasma membrane to a network of serine/threonine kinases which subsequently leads to the nucleus. The p21Ras protein is active in its GTP-bound state. This form is slowly converted to the GDP-bound form by the intrinsic GTPase activity of Ras. This activity is greatly enhanced by GTPase-activating proteins (GAPs) which subsequently lead to hydrolysis of

GTP molecule to form GDP (See Figure 1.6). Maintenance of Ras in the GTP form can lead to transformation. One class of Ras mutations, commonly found in human tumors, results in an accumulation of Ras-GTP (Milligan, 1992).

4) Phosphatidylinositol 3'-kinase (PI3'K) is a lipid kinase that phosphorylates the D3 position of phosphatidylinositol, phosphatidylinositol-4-phosphate, or PI-4,5-P2 (Whitman *et al.*, 1988) (See Figure 1.7). PI3′K exists as a heterodimeric complex that contains p85 and a 110-kD protein. The purified p85 subunit has no detectable PI3′K activity, but binds tightly to activated PDGFR or EGFR *in vitro*, suggesting that p85 acts as the regulatory subunit and p110 is the catalytic element (Otsu *et al.*, 1991). Activated mammalian PI3′K controls several important cellular functions, including cytoskeletal organization, cell division and survival maintenance (Roche *et al*, 1994). The exact role PI3′K plays in so many different cellular responses is not yet known, but it is believed that PI3′K activation leads to SHC phosphorylation resulting in recruiting more GRB/SOS to the membrane and as a result increasing the mitogenenic response (Ilasaca *et al.*, 1997). The authors overexpressed PI3′K in Cos cells and found increased MAPK activity. This stimulation was abolished with an inactive mutant of PI3′K.

5) Raf is a proto-oncogene that encodes a 68 kDa serine/threonine kinase that is phosphorylated and the kinase activity is increased after PDGF stimulation (Kolch, 1993), EGFR (Hocker *et al.*, 1998) and FGFR stimulation (Miyamoto *et al.*, 1998). There are different forms of the protein; A-Raf, C-Raf, and V-Raf showing different expression patterns (Storm and Rapp, 1990). Raf interacts directly with Ras to activated the MAPK pathway and mitogenesis (reviewed in Moodie, 1994)

6) NCK and Growth factor receptor-binding protein (GRB). These are a unique group of associated molecules due to their function. They act as adapter or linking molecules. Nck is a 45-kDa protein consisting of one SH2 domain and three SH3 domains (Lehmann *et al.*, 1990). SH3 domains are thought to mediate associations with adapter proteins or target proteins through recognition of proline-rich sequences (Pawson and Schlessinger, 1993). It has been shown that Nck is a component in a complex with the active PDGFR and the EGFR, through the SH2 domain (Yi *et al.*, 1992). Nck has also been shown to interact with a serine/threonine, Nck-associated kinase (NAK), specifically to the second of Nck's three SH3 domains (Chou and Hanafusa, 1995). A number of other Nck-associated proteins have been found in other

systems of various molecular weights (this will be described in detail in Chapter 5).

GRB is the mammalian homologue to Sem-5 gene product in *C. elegans*. Sem-5 functions by activation of Ras through a signal transduction pathway that is initiated by the *let-23* RTK, leading to vulva induction (Clark, 1992). Recently, a GRB homologue known as DRK was identified in *Drosophila*. DRK is involved in Sevenless RTK signaling during specification of the R7 photoreceptor cell in the developing *Drosophila* compound eye (Olivier *et al.*, 1993; Simon *et al.*, 1993). DRK, like GRB, has been shown to bind to SOS, a guanine nucleotide exchange factor for Ras. The 24 kDa structure of GRB consists of one SH2 domain flanked by two SH3 domains (Matuoka *et al.*, 1992). GRB associates specifically with the autophosphorylated EGF or PDGF receptors via its SH2 domain. The receptor-GRB complex binds the guanine nucleotide exchange factor, SOS through its SH3 domains. SOS promotes the conversion of GDP-p^{21ras} to GTP-p^{21ras}, allowing the tyrosine kinase to modulate Ras activity (Gale *et al.*, 1993) (See Figure1.6).

7) SHP2 Phosphatase (also called Syp, PTP1D, and PTP2C) structure consists of two SH2 domains. (Reviewed in Feng and Pawson, 1994). It appears that SHP2 may be substrate-selective and that both the catalytic and SH2 domains may contribute to this selectivity (Klinghoffer and Kazlauskas, 1995). Furthermore, it seems that SHP2 plays a role in negative feedback, in that it dephosphorlylates the PDGFR. When FGFR is activated during differention of PC12 cells, SHP2 activation appears to be important in MAPK activation, through a linkage protein called FRS2 (Hadari, 1998). The authors interrupted a complex formation between FRS2 and SHP2 by expressing a Y436F mutant FRS2 in PC12 cells. The expressing cells allowed transient but not sustained activation of MAPK, leading to a decrease FGF induced neurite outgrowth.

8) Src is one member of a family of nine tyrosine kinases that regulates cellular responses to extra-cellular stimuli. Members of the SRC family contain an SH3 and SH2 domain with a catalytic tyrosine kinase domain. (Cooper, 1993). *In vitro* experiments show that changes in Src phosphorylation induce changes in its activity. It has been shown that C-terminal phosphorylation is inhibitory and kinase domain phosphorylation is

stimulatory (Kmiecik *et al.*, 1988). Keeping Src inactive requires extensive C-terminal phosphorylation. So far, Csk (C-terminal Src kinase) is the only known kinase that specifically phosphorylates Src at its C-terminus (Reviewed in Brown, 1996).

9) FGF receptor substrate 2 (FRS2) is a recently characterized adapter molecule of 90KDa (Kouhare *et al.*, 1997). FRS2 structure is unique in that it lacks an SH2 domain, but contains a phosphotyrosine binding domain which mediates phosphotyrosine-independent interaction with amino acid residues 407-433 in FGFR1, as examined in a yeast two hybrid assay (Xu *et al.*, 1998). Activation of FGFR1 leads to tyrosine phosphorylation of FRS2 at Y196, Y306,Y349 and Y932 allowing for binding of GRB (Kouhara *et al.*, 1997). GRB, as mentioned earlier, links SOS to the activation of GTP-binding protein Ras. In addition phosphorylation of FRS2 at Y436 potentially allows binding of the tyrosine phosphatase SHP2 (Hadari *et al.*, 1998). Thus far FRS2 has been shown to be utilized by FGF R and nerve growth factor receptor (NGFR), but not by other types of RTK (reviewed in Klint and Claesson-Welsh, 1999). This would possibly imply that FRS2 has a unique signaling function, however, GRB and SHP2 operate downstream of both PDGF and EGF.

Whether FRS2 has a particular role in specific FGFR mediated biological responses remains to be shown.

1.9 Prospects for Thesis

The goal of this work was to further the understanding of the FGFR transduction pathway during mesoderm induction in the *Xenopus* embryo. FGF is involved in mesoderm induction through activation of the FGFR to differentially express specific genes involved in early embryo development. The hypothesis for this work was that FGF is involved in mesoderm induction through the phosphorylation of molecules leading to the transcriptional activation of specific molecules. My focus was to identify the components of this signal, and with this information there may be a better understanding of how the FGFR is involved in mesoderm induction in the *Xenopus* embryo.

Chapter 2

MATERIALS AND METHODS

2.1 Embryos, Dissections and Induction Assays

Xenopus embryos were obtained by artificial fertilization, handled and dissected as previously described by Godsave *et al.*, (1988) and staged according to Nieuwkoop and Faber (1975). Female *Xenopus laevis* were induced to lay eggs by subcutaneous injection of 500 I.U. of human chorionic gonadotrophin in 0.5 ml H_20. The eggs that were obtained were fertilized using *Xenopus* testes, stored in 1x Normal Amphibian Medium (NAM) (See Table1; Slack and Forman 1980), then diluted and broken-apart in H_2O. Once the eggs were fertilized and rotation occurred, the jelly coats were removed using 2.5% w/v cystein hydrochloride (Sigma Chemical Co.) pH adjusted to 7.8-8.2 with sodium hydroxide (NaOH) (Fisher). The embryos were washed thoroughly with about 1 liter of water and allowed to develop in petri dishes in 1/20 NAM.

During dissections the embryos were manipulated in NAM. NAM/2 + 1mg/mL BSA (Bovine Serum Album: Sigma Chemical Co.) was utilized to culture cut explants.

TABLE 1A. Components of NAM

	g/L(in 10x)	mM (Final in 1x)
NaCl	65	110
KCl	1.5	2
$Ca(NO_3)_2.4H_2O$	2.4	1
$MgSO_4.7H_2O$	2.5	1
EDTA (0.5M,pH8.0)	2mL	0.1
Hepes (1M,pH7.5)	100mL	10

Table 1B Components of NAM Solutions

For 100mL of 1x solution, add:

	NAM	NAM/2	NAM/20
10xNAM (from Table 1A)	10mL	5mLs	0.5mLs
Gentamycin (10mg/mL)	0.25 mL	0.25 mL	0.25 mL
Na Bicarbonate (0.1M)	1.0 mL	1.0 mL	---
Sterile H_20	88.75 mL	93.75 mL	99.25 mL

2.2 MESODERM INDUCTION EXPERIMENTS

The FGF used in this study was recombinant *Xenopus* bFGF, expressed and purified according to Kimelman *et al.* (1988) and stored at –20°C. Animal pole explants were dissected manually as described in Godsave *et al.* (1988). One hundred explants were induced either by culturing in the presence of 100ng/mL *Xenopus* bFGF diluted in NAM+BSA or incubating the animal cap directly with vegetal cells for 30 minutes. The control explants were cultured in NAM +BSA alone. After incubation on ice, the explants were solubilized and prepared for immunoprecipitation as described below for whole embryos.

2.3 IMMUNOPRECIPITATION AND WESTERN BLOTTING

To study the FGFR complexes formed during mesoderm induction, immunoprecipitations were performed using 150 stage eight embryos. The embryos were homogenized in ice-cold solubilization buffer, which contained 1% Triton, 10 mM Tris, pH 7.5,10 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl floride), 25μM/mL aprotinin, 25mg/mL leupeptin, 5mg/mL TLCK, 100μM sodium orthovanadate, 100 mM NaF, 10mM sodium

pyrophosphate. After 30 minutes of solubilization, on ice, the extract was centrifuged at 4°C and 10,000 rpm for 5 minutes, to remove the insoluble material. The supernatant was removed and placed into a new tube. The appropriate antibody was added, at the required concentration for immunoprecipitation (a list of antibodies are included in Section 2.5). The solution was incubated, rotating, at 4°C overnight.

The following day the complexes were isolated by adding Protein A- or Protein G- Sepharose (Pharmacia). The appropriate Sepharose would depend on the source of the antibody (See Table 2). This mixture was incubated at

TABLE 2 Affinities of Various Monoclonal Antibodies for Protein A or Protein G

ANTIBODY	Affinity for protein A	Affinity for protein G
Rabbit IgG	++++	-
Rat IgG 2a	-	++++
Rat IgG 2b	-	++
Rat IgG 2c	+	++
Mouse IgG 1	+	++++
Mouse IgG 2a	++++	++++
Mouse IgG 2b	+++	+++
Mouse IgG 3	++	+++

4°C for 1 hour, with rotation. The Sepharose bound to the immunoglobins of the immunoprecipitating antibody. This isolated the antibody from solution, which recognized the appropriate protein, isolating any complexes formed.

Once the Sepharose bound the immunoprecipitating antibody, the resulting complex was stable. Centrifuging the complexes for 1 minute separated the solution. The supernatant was carefully removed and discarded. The remaining beads were washed three times with 1mL of extraction buffer (10mM Tris pH 7.5, 1xTriton, 10µm vanadate, 1mM sodium pyrophosphate, and 10mM NaF), and then washed twice with 150mM NaCl. After the final wash, the supernatant was carefully removed and 35mLs of 1.5x Sample buffer (See Table 3) was added.

Table 3 Components of Sample Buffer and Triton Media

10x Triton	2x SSB
100mM Tris, pH 7.5	0.1M Tris pH 6.8
10% Triton	4.5% SDS
100mM EDTA	3.2 M β-Mercaptoethanol (β-ME)
0.2% Sodium-azide	22.2% Glycerol
Sterile Water	0.1% Bromophenol blue

Table 4 Components of SDS-PAGE Gels

	8%	10%	12%	14%
30% acrylamide *	0.9mL	1.13mL	1.36mL	1.58mL
35.8% acrylamide	1.5mL	1.87mL	2.24mL	2.64mL
RGB **	3.8mL	3.8mL	3.8mL	3.8mL
Water	3.6mL	3.0mL	2.4mL	1.8mL
20% SDS	40µl	40µl	40µl	40µl
TEMED***	5µl	5µl	5µl	5µl
10% AP****	83µl	83µl	83µl	83µl

*30% acrlyamide (19-1 acrylamide-bis acrylamide; Bio-Rad)

** RGB: Running Gel Buffer (0.5M Tris, pH 7.5)

***TEMED: N,N,N',N'-Tetra-Methylethylenediaminutese (Bio-Rad)

**** AP: Ammonium persulfate (Bio-Rad)

To dissociate the proteins present in the FGFR1 complex, the washed immunoprecipitate was boiled for 5 minutes in sample buffer (1x SSB, 1%SDS), which dissociated the antibody from the Protein A/G. The complex was run on SDS-PAGE electrophoresis to separate all of the components of the complex according to size. An 8% gel was usually used to separate the appropriate molecular weight range, but depending on the particular size of the

protein of interest, other percentage gels were prepared (as specified in Table 3).

The SDS-PAGE was run in electrode buffer at 30mA for approximately 1.5 hours then soaked in transfer buffer (See Table 5), to remove SDS from the gel (SDS will decrease the transfer of proteins). The proteins on the gel were transferred to Hybond-ECL nitrocellulose membrane (Amersham), by assembling the Western apparatus as specified by the protocol supplied by the supplier (Bio-Rad). This assembly was prepared while submersed in transfer buffer to prevent bubbles, as bubbles inhibit transfer in that area due to the fact that the voltage can not flow through. Transfer of the protein occured from the gel to the nitrocellulose toward the positive electrode with 60 volts of current for 1 1/2 hours.

Table 5 Components of Electrode Buffer and Transfer Buffer

ELECTRODE BUFFER	TRANSFER BUFFER
50mM Tris base	25mM Tris pH 8.3
383mM Glycine	192 mM Glycine
3.0 mM SDS	20% methanol
Tris base (Fisher Chemicals)	
Glycine (Bio-Rad, electrophoresis grade)	
SDS (Bio-Rad, electrophoresis grade)	

The nitrocellulose membrane was placed in Blotto (20mM Tris base pH 7.5, 137mM NaCl, 5% protein powder (powdered milk; Carnation), 0.2% azide (anti-fungal agent), 0.1% Tween-20 (Detergent; Bio-Rad Chemicals) for one hour with rocking motion. This blocked all the non-specific binding sites on the nitrocellulose. The appropriate primary antibody was then added to the membrane at the required western staining concentration, diluted with Blotto. This was incubated overnight, at room temperature, with rocking movement so that complete coverage of the membrane was obtained.

The following day, the western staining would be completed. The membrane was transferred to a large container and washed 5 times with excess volumes of TBS-T (20 mM Tris pH 7.6, 137mM NaCl, 0.1% Tween). The secondary antibody was incubated for one hour. The secondary antibody depended on the particular staining antibody used. This could be: 1) HRP (hydrogen peroxide)-labeled goat anti-rabbit (heavy chain and light chain, Amersham Co.) 2) HRP-labeled goat anti-mouse (light chain specific, Cedar Lane Laboratories Ltd.) or 3) If a biotinylated primary antibody was used, a streptavidin-HRP conjugate (Amersham) was used instead of the secondary antibody. The membrane was again washed 5 times with excess volumes of

TBS-T. The ECL (Amersham) staining involved the incubation of commercially purchased reagents using a 50-50 mixture of solution A and solution B. This was incubated for 1 minute covered with a plastic folder, and immediately exposed to ECL Hyperfilm (Amersham). The exposure time ranged from 30 seconds to 20 minutes.

The ECL detection system works on the principle of luminescence: emission of light resulting from the dissipation of energy from a substance in an excited state. The chemical reaction occured via hydrogen peroxide catalyzing the oxidation of luminol in alkaline conditions. After oxidation, the luminol was in an excited state, which then decayed to ground state emitting light. Increased chemiluminescence can be achieved by oxidation of luminol in the presence of chemical enhancers such as phenols. This would increase the light emitted 1000 fold and extend the time of light emission. The light produced peaked after 5-20 minutes and decayed slowly, with a half-life of approximately 60 minutes. The maximum light emission was a wavelength of 428 nm (reviewed in the ECL protocol book).

The ECL western staining system has a number of advantages for use in this experimental procedure:

1) Increased sensitivity and a non-radioactive detection. This was safer than using radioactive materials and the detection of less than 1pg of antigen was proposed to be detected; this was at least 10x more sensitive than other non-radioactive methods.

2) A high ratio of signal to background, resulting in cleaner blots.

3) Results were obtained in short periods of time

4) Stripping and reprobing membranes with different antibodies was possible and enabled more information to be obtained from the same membrane.

To restain the same membrane with different antibodies, the complete removal of primary and secondary antibodies from membranes was required. This was achieved by incubating the membrane in stripping buffer (100mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and incubating at 50°C for 30 minutes with occasional agitation. The membrane was washed with excess TBS-T at room temperature for about 1 hour. The stripped blot was then incubated in Blotto for 1 hour and the staining was completed as described above. The protocol suggested sequential reprobing of membranes was possible, several times with the membranes stored wet at 4°C after each immunodetection. I found that stripping membranes resulted in an increase of background.

2.4 Nck ASSOCIATED PROTEINS

The Nck experiment was carried out as described above with a few exceptions. The FGFR complex was immunoprecipated as described. The proteins present in the complex were dissociated by boiling the beads twice for 5 minutes in the presence of 1% SDS. The supernatant containing the denatured FGFR complex proteins was diluted to a final concentration of 0.1% SDS in solublilzation buffer. This step was included because the Nck antibody required non-denaturing conditions for immunoprecipation. The diluted supernatant was incubated for 1 hour with anti-Nck. The Nck-antibody complex was isolated with protein A as described above. This then gave us a Nck-protein A complex to incubate overnight with the supernatant of an FGFR void extract. Any differences or increases in phosphorylated pattern should identify Nck-associated proteins. An FGFR-void supernatant was used, so that the Nck antibody would not bind to the intact FGFR complex and give the same pattern as the phosphorylated receptor.

2.5 ANTIBODIES

There were a number of different antibodies used for these experiments:

1) Anti-PLCγ1, used for immunoprecipitation (Upstate Biotechnology Inc.), was a polyclonal antiserum prepared against a fusion protein containing amino acids 1064-1291 of bovine brain PLCγ1. This region is located at the C-terminus of PLCγ1 gene product does not include the SH2/SH3 domains. If these domains were immunized for the production of this antibody, it may cross-react with Nck, due to sequence homology in these regions (concentration 10μl).

2) Anti-PLCγ1, used for western blotting (Upstate Biotechnology Inc.), was a mixture of DEAE-HPLC purified monoclonal antibodies directed against purified bovine brain PLCγ1 (concentration 1.0 μg/mL). Two PLCγ antibodies were utilized because the monoclonal antibody would give less background when staining the western blot. I found that if the antibody used to immunoprecipate and the antibody used to stain the

membrane were from two separate sources the staining of the heavy chain was decreased, resulting in a cleaner result.

3) Anti-FGFR1, (Upstate Biotechnology Inc.), was an affinity-purified monoclonal antibody raised against a fusion protein containing amino acids 22-325 of the extracellular domain of the human βFGFR1 (*flg*) (concentration used was 10μg/mL for immunoprecipitation and 1.0μg/mL for Western blotting).

4) Anti-phosphotyrosine (Anti-PY)(Upstate Biotechnology Inc) will recognize all phosphate groups on tyrosine. Anti-PY monoclonal antibody (from clone 4G10) was supplied as a Protein A-Sepharose purified IgG or IgG-biotin conjugate (PY-b). Concentration used for staining blots: 1.0μg/ml.

5) Anti-Nck (Transduction Laboratories) was a monoclonal antibody raised against amino acids 279-377 of human Nck. This antibody cross reacted with the *Xenopus* protein and did not display cross-reactivity with PLCγ1. The primary antibody concentration was 0.5μg/mL and the immunoprecipation concentration was 4μg/mL. This antibody showed cross-reactivity with the frog.

6) Anti-GRB2 (Transduction Laboratories), was a monoclonal antibody produced from purified GRB2 protein from rat brain (concentration used for immunoprecipation was 4μg/mL and staining was 0.5μg/mL).

7) Anti-SOS (Upstate Biotechnology Inc.), was a polyclonal antibody manufactured by immunizing rabbits against a recombinant GST-fusion protein corresponding to the C-terminal region of the murine Son of Sevenless 1 protein (concentration used for immunoprecipation was 5.0μg/mL and to immunoprecipate 5μg per sample was utilized).

8) Anti-*Xenopus* FGFR1 (produced in laboratory by Gillespie and Paterno), was prepared by immunizing rabbits with a synthetic FGFR1 peptide (Multiple Peptide System) conjugated to keyhole limpet hemocyanin (Pierce). The FGFR1 sequence (CLPKYSNGGLKKR) corresponds to the C-terminal 13 amino acids of the *Xenopus* FGFR1 (Friesel and David, 1991). Anti-XFR was purified using an FGFR1 peptide-agarose column prepared with the Prot-on kit (Multiple Peptide Systems). This antibody had the ability to immunoprecipate at 15μ/mL and western blot at a concentration of and 1.0μg/mL.

9) Anti-SHC (Transduction laboratories), was prepared by immunizing rabbits with a C-terminal peptide of the SHC protein corresponding to positions 359-473 of the intact protein. The polyclonal antibody was affinity purified on a column of the immunogen coupled to agarose. To stain western blots a dilution of 1:250 was used. To immunoprecipate 4μg of antibody was added to the supernatant.

10) Anti-SHP2 (Transduction laboratories) A 20kDa protein fragment corresponding to residues 1-177 of human SHP2 was used as an immunogen. This monoclonal antibody was purified from mouse ascites by chromatographic techniques. A dilution of 1:2500 was used to stain western blots and 4μg for immunoprecipitation.

11) Anti-human GTPase Activation protein (GAP) (Upstate biotechnology Inc.). This IgG antibody was produced using full length human GAP produced in baculovirus. This monoclonal antibody was purified using protein G chromatography. To immunoprecipate 5μg of antibody was added to the supernatant and a dilution of 1:1000 was used to stain.

12) Anti-rat PI3'Kinase (Upstate Biotechnology Inc.). The immunogen used to produce the antibody were glutathine-5-transferase fusion proteins containing (1) the N-terminal SH2 domain of rat PI3'kinase and (2) the full-length 85k subunit of rat PI3'kinase. The product produced was a

mixture of equal amounts of rabbit antiserum recognizing p85 subunit of PI3'kinase. To immunoprecipate 5μg was added to the supernatant

13) Anti-human PI3'kinase, monoclonal (Upstate Biotechnology Inc.). Two antibodies against PI3'kinase were needed because the monoclonal antibody gave a cleaner blot with less background and the amount of heavy chain identified was smaller. The monoclonal antibody was immunized using recombinant human p85 alpha expressed in *E.coli.* 1μg/ml of, protein A Sepharose affinity chromatography purified antibody was used to stain each western.

14) Anti-human SRC (Santa Cruz Inc.). This antibody was an affinity-purified rabbit polyclonal, raised against a peptide corresponding to residues 509-533 within the COOH-terminal region of the human *src* product. To immunoprecipate 1.0μg was added to the embryo extract. To stain a western blot 0.1μg/ml was used.

15) Anti-neurofilament 200 KD (Boehringer Mannheim Biochemica). To obtain this monoclonal antibody to neurofilament 200kD, dephosphorylated, Balb/c mice were immunized with the carboxyl terminal tail segment of enzymatically dephosphorylated porcine H-chain. To immunoprecipate 10µg was added to the extract.

DEVELOPMENTAL BIOLOGY 166, 101–111 (1994)

Chapter 3

Phosphorylation of Phospholipase Cγ1 and Its Association with the FGF Receptor Is Developmentally Regulated and Occurs during Mesoderm Induction in *Xenopus laevis*

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Accepted July 29, 1994

We have examined phosphorylation of phospholipase Cγ1 (PLCγ1) and its association with FGFR1 during mesoderm induction in animal pole explants and during early development in *Xenopus* embryos. In explants, PLCγ1 became associated with FGFR1 during mesoderm induction by FGF or by vegetal cells, the source of the natural inducer. Both PLCγ1 and FGFR1 were phosphorylated on tyrosine, indicating that both proteins were activated. Phosphorylation of these two proteins occurred very early during the induction process (within 0.5 hr), providing evidence that a member of the FGF family is a component of the vegetal inducing signal. PLCγ1 was also associated with FGFR1 in *Xenopus* blastulae and this association was specific to presumptive mesoderm cells. Examination of the PLCγ1 phosphorylation pattern during early *Xenopus* development and its association with FGFR1 revealed that maximum phosphorylation and association of these two proteins occurred during early- to mid-blastula stages, concurrent with mesoderm induction *in vivo*. This spatiotemporal pattern PLCγ1–FGFR1 association and phosphorylation suggests that PLCγ1 is involved in intracellular signaling during mesoderm induction in *Xenopus*. Seven additional phosphotyrosyl bands were coimmunoprecipitated with either PLCγ1 or FGFR1 from *Xenopus* blastulae; these bands may represent additional components of an FGFR1 signaling complex. One of these phosphotyrosyl bands was identified as NCK. In addition, growth factor receptor-binding protein, and son-of-sevenless two upstream regulators of RAS signaling, were co-immunoprecipitated with FGFR1.

INTRODUCTION

In *Xenopus*, experimental evidence suggests that cells located around the equator of the blastula are induced to form mesoderm in response to signals produced by neighboring vegetal cells. Although the molecular composition of the vegetal signal has yet to be defined, it most likely consists of several components and candidates include members of the FGF and the TGFβ gene families. Both FGF (Slack *et al.*, 1987; Kimelman *et al.*, 1988; Paterno *et al.*, 1989; Isaacs *et al.*, 1992) and activin (Smith, 1987; Asashima *et al.*, 1990; Smith *et al.*, 1990; van den Eijnden-Van Raaij *et al.*, 1990) have been shown to induce mesoderm in isolated ectodermal explants; however, the cellular response to these two types of growth factors is distinct (Ruiz i Altaba and Melton, 1989; Cho and De Robertis, 1990; Green *et al.*, 1990). In general, FGF induces mesoderm that is ventral in character while activin induces dorsal mesoderm, although there are some exceptions to this rule (Paterno *et al.*, 1989; Sokol and Melton, 1991; Kimelman and Maas, 1992). The requirement for each type of growth factor in mesoderm formation *in vivo* was demonstrated through the use of dominant negative mutant receptors designed to inhibit wild-type receptor activity (Amaya *et al.*, 1991; Hemmati-Brivanlou and Melton, 1992). Expression of either mutant activin receptors or mutant FGF receptors in *Xenopus* embryos interfered with the normal development of mesodermal tissue.

Mesoderm formation *in vivo* is a multistep process, the basic concept of which is described in the three signal model (Smith *et al.*, 1985). The initial inducing signal produced in the vegetal hemisphere is proposed to consist of a ventral vegetal signal that induces a large ventral mesodermal region and a dorsal vegetal signal that induces a restricted dorsal organizer region. A third signal is produced later by the organizer region and serves to dorsalize the character of the remaining mesoderm. How many different molecules make up each signal and which of the steps involves FGF or activin is unknown at present.

The response to both types of factors can be modified by the presence of other gene products, such as BMP-4, noggin, and XWNT-8. The presence of BMP-4 serves to ventralize induction by activin while having no effect on induction by FGF (Jones *et al.*, 1992). The noggin gene

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(Smith and Harland, 1992) encodes a secreted protein that can dorsalize presumptive ventral mesoderm (Smith *et al.*, 1993). Injection of Xwnt-8 cRNA into early embryos increases the dorsal character of mesoderm induced by FGF (Christian *et al.*, 1992) or activin (Sokol and Melton, 1992). However, delaying the expression of injected Xwnt-8 cDNA until after mid-blastula transition has the opposite effect and results in inhibition of dorsal mesoderm differentiation in response to activin (Christian and Moon, 1993). Thus, timing of the expression of a particular gene product can dramatically alter its effect on a developmental event. Undoubtedly, establishment of the intricate pattern of mesodermal tissues *in vivo* requires both spatial and temporal regulation of the expression of several inducing and modifying factors (reviewed in Kimelman *et al.*, 1992, Moon and Christian, 1992, Slack, 1993).

Noggin and members of the FGF, TGFβ, and Wnt gene families encode secreted proteins and therefore act at the extracellular level, with their signal being transmitted to the nucleus via transmembrane receptors. Thus, elucidation of the molecular mechanism of the cellular response during mesoderm induction requires knowledge of the intracellular signal transduction pathways mediating this process. Intracellular signaling events are best described for receptor tyrosine kinases (RTKs), such as the FGFR, during growth factor-induced mitogenesis (reviewed in Ullrich and Schlessinger, 1990). Ligand binding to the extracellular domain of the receptor results in receptor activation through dimerization and intermolecular autophosphorylation. The activated receptor can then bind a number of intracellular proteins, resulting in the formation of a signaling complex. In most cases, binding to the receptor involves association between specific RTK autophosphorylation sites and conserved src homology (SH2) domains within the substrate (reviewed in Koch *et al.*, 1991). Once bound, these substrates often become phosphorylated by the RTK, resulting in altered catalytic activity (reviewed in Iwashita and Kobayashi, 1992; Rhee and Choi, 1992). Intracellular proteins that bind to activated receptors include catalytic enzymes such as phospholipase Cγ1 (PLCγ1), the regulatory subunit (p85) of phosphatidylinositol 3′-kinase, the GTPase-activating protein of RAS as well as adaptor molecules such as growth factor receptor-binding protein (GRB2) (reviewed in McCormick, 1993, Marx, 1993) and NCK (Li *et al.*, 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992).

Recent studies have provided some information about intracellular signaling molecules involved in mesoderm induction. Whitman and Melton (1992) demonstrated the importance of RAS in signaling during induction by FGF and activin, as well as by vegetal cells. Microinjection of cRNA encoding a dominant negative mutant RAS resulted in inhibition of mesoderm induction by FGF, activin, or the natural inducing signal. Using a similar strategy, MacNicol *et al.* (1993) investigated the role of RAF-1, a serine/threonine kinase that is activated in a variety of cell lines after stimulation with growth factors (Morrison *et al.*, 1989; App *et al.*, 1991; Oshima *et al.*, 1991). The results of their experiments using a dominant negative mutant RAF-1 showed that RAF-1 kinase activity is essential for signaling by FGF but not by activin. We have previously shown that FGFR is phosphorylated upon binding FGF and that protein kinase C (PKC) is activated during mesoderm induction by FGF, although it appears that PKC is involved in the negative regulation of FGFR activity (Gillespie *et al.*, 1992).

Here we investigate the role of PLCγ1 in mesoderm induction in *Xenopus*. PLCγ1 is a substrate for several RTKs, including the FGFR, during growth factor-induced mitogenesis (Kumijan *et al.*, 1989; Meisenhelder *et al.*, 1989; Burgess *et al.*, 1990), and phosphorylation of PLCγ1 appears to enhance its enzymatic activity (Nishibe *et al.*, 1990; Kim *et al.*, 1991). PLCγ1 catalyzes the hydrolysis of phosphatidylinositol bisphosphate to inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG). IP_3 causes the release of calcium from intracellular stores while DAG is an activator of PKC (reviewed in Berridge, 1987).

Our results demonstrate that phosphorylation of PLCγ1 and association with FGFR1 occur during mesoderm induction in explants and are correlated with mesoderm induction *in vivo*. The timing of the phosphorylation event provides evidence that FGF is a component of the vegetal inducing signal. Additional components of a putative FGFR1 signaling complex are identified.

MATERIALS AND METHODS

Embryos and Cell Lines

Xenopus laevis eggs were artificially inseminated, the jelly coats removed, and the embryos cultured as described in Godsave *et al.* (1988). Embryonic stages were determined according to Nieuwkoop and Faber (1967). For the analysis of FGFR1–PLCγ1 complexes in the presumptive mesoderm (marginal zone), marginal zones were dissected from 90 embryos as described in Gillespie *et al.* (1989). The remaining tissue from the embryos was combined into a single sample and the two samples were solubilized as described below. Protein measurements of the supernatants were performed using the Bio-Rad protein assay and the same amount of total protein from each sample was used for immunoprecipitation; the protein concentration was adjusted to 2.5 mg/ml. Immunoprecipitation and Western blot analysis were carried out as described below.

MDA-468 cells were grown in 100 mm dishes in L-15 medium supplemented with 10% fetal bovine serum. For antibody testing, cells (3×10^6/plate) were washed with ice-cold phosphate-buffered saline. Subsequent extraction with solubilization buffer and immunoprecipitation were carried out as described below. One-quarter of the immunoprecipitate was used for SDS–PAGE and Western blotting.

Antibodies

The antibodies employed in this study were obtained from three different sources: Upstate Biotechnology Inc. (anti-PLCγ1, anti-FGFR1, anti-son-of-sevenless 1 (anti-SOS1), and anti-phosphotyrosine (PY and PY-b)), Transduction Laboratories (anti-NCK and anti-GRB2) and produced in our laboratory (anti-*Xenopus* FGFR1). The anti-PLCγ1 used for immunoprecipitation was a polyclonal antiserum prepared against a fusion protein containing amino acids 1064–1291 of bovine brain PLCγ1; this region is located at the C-terminus of PLCγ1 gene product and does not include the SH2/SH3 domains that might result in production of an antiserum with cross-reactivity to NCK. The anti-PLCγ1 used for Western blotting was a mixture of DEAE-HPLC purified monoclonal antibodies directed against purified bovine brain PLCγ1. The anti-FGFR1 was an affinity-purified monoclonal antibody raised against a fusion protein containing amino acids 22–325 of the extracellular domain of the human β FGFR1 (FLG). The anti-PY monoclonal antibody (clone 4G10) was supplied as a Protein A–Sepharose purified IgG or IgG–biotin conjugate (PY-b).

Anti-NCK and anti-GRB2 were supplied as purified monoclonal antibodies that had been tested for cross-reactivity in frogs. Anti-NCK was raised against amino acids 279–377 of human NCK; this antibody did not display cross-reactivity with PLCγ1. Purified GRB2 protein from rat brain was the immunogen used to prepare anti-GRB2.

Anti-*Xenopus* FGFR1 (XFR) antiserum was prepared in our laboratory by immunizing rabbits with a synthetic FGFR1 peptide (Multiple Peptide Systems) conjugated to keyhole limpet hemocyanin (Pierce). The FGFR1 peptide sequence corresponded to the C-terminal 13 amino acids (CLPKYSNGGLKKR) of the *Xenopus* FGFR1 (Friesel and Dawid, 1991). Anti-XFR was affinity-purified using an FGFR1 peptide–agarose column prepared with the Prot-on kit (Multiple Peptide Systems).

Each of the UBI antibodies was tested for cross-species reactivity on Western blots containing samples from *Xenopus* embryos and the appropriate mammalian source as a positive control: a human breast carcinoma cell line, MDA-468 (Pathak *et al.*, 1979), for anti-PLCγ1 and NIH3T3 cells for anti-SOS1 and anti-FGFR1. In each case, the commercial antibody recognized a single *Xenopus* protein which comigrated with the mammalian protein (Fig. 1 for anti-PLCγ1; not shown for anti-FGFR1 and anti-SOS1).

For both immunoprecipitation and Western blotting, the antibodies were used at a concentration that produced the maximum signal. These were as follows: anti-PLCγ1, 10 μl serum (immunoprecipitation) and 1.0 μg/ml monoclonal (Western blotting); anti-FGFR1, 10 μg/ml (immunoprecipitation) and 1.0 μg/ml (Western blotting); anti-XFR, 15 μg/ml (immunoprecipitation) and 1.0 μg/ml (Western blotting); anti-SOS1, 5.0 μg/ml; anti-PY, 1.0 μg/ml; anti-NCK, 4 μg/ml (immunoprecipitation) and 0.5 μg/ml (Western blotting); and anti-GRB2, 4 μg/ml (immunoprecipitation) and 0.5 μg/ml (Western blotting).

Immunoprecipitation and Western Blotting

In vivo analysis of FGFR signaling complexes involved preparation of embryo extracts by homogenizing whole embryos in ice-cold solubilization buffer (1% Triton, 10 m*M* Tris, pH 7.5, 10 m*M* EDTA, 1 m*M* PMSF, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 5 μg/ml TLCK, 100 μ*M* sodium orthovanadate, 100 m*M* NaF, 10 m*M* sodium pyrophosphate). Each sample contained 150 embryos at the same stage of development. After a 30-min solubilization period at 2°C, insoluble material was removed by centrifugation at 10,000*g* for 5 min; the final protein concentration in the supernatants was 2.5 mg/ml. The extracts then incubated at 4°C overnight with the appropriate antibody.

The antigen–antibody complex was precipitated with either Protein A– or Protein G–Sepharose beads (Pharmacia) and washed extensively with extraction buffer. For dissociation of proteins present in the complex with FGFR1, the washed anti-XFR immunoprecipitate was boiled for 5 min in 1% SDS, 10 m*M* Tris, pH 7.4. The supernatant containing the denatured proteins was diluted to a final concentration of 0.1% SDS in solubilization buffer and incubated for 1 hr with anti-NCK. The antigen–antibody complex was then precipitated and washed as described above. The samples were subjected to SDS–PAGE followed by Western blotting onto Hybond-ECL nitrocellulose (Amersham). Bio-Rad biotinylated (0.15 μg/lane) and/or prestained (12.5 μg/lane) molecular weight standards were included on each gel.

The blots were stained with the appropriate primary antibody and either HRP-labeled goat anti-rabbit (heavy plus light chain; Gibco BRL) or HRP-labeled goat anti-mouse (light chain specific; Cedarlane Laboratories Ltd.) using the ECL kit (Amersham). To detect the biotinylated anti-phosphotyrosine (anti-PY-b) and the biotinylated standards, a streptavidin–HRP conju-

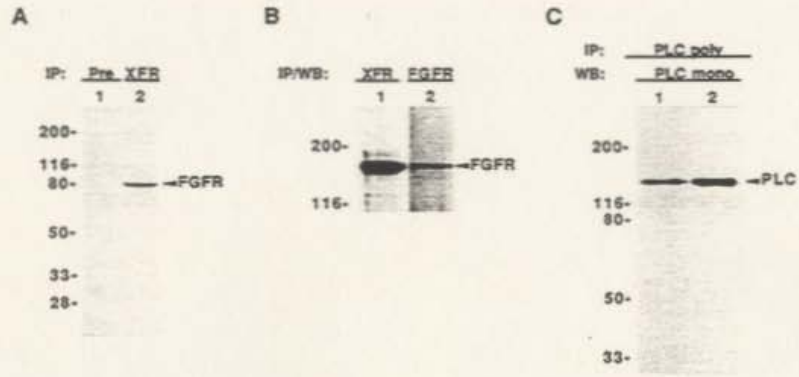

FIG. 1. Detection of *Xenopus* FGFR1 and PLCγ1. (A) Specificity of the anti-*Xenopus* FGFR1 antibody. ^{35}S-labeled *Xenopus* FGFR1 protein synthesized *in vitro* in a rabbit reticulocyte lysate system was incubated with either preimmune (lane 1) or immune (lane 2) serum and the immunoprecipitates were analyzed by SDS–PAGE and fluorography. A single labeled band of predicted molecular mass (89 kDa) was precipitated with the immune serum. Molecular weight standards are indicated on the left. (B) Comparison of the two anti-FGFR1 antibodies used in this study. Embryo extracts (150 embryos per sample) were immunoprecipitated (IP) and Western blotted (WB) with either affinity-purified anti-*Xenopus* FGFR1 (XFR, lane 1) or anti-human FGFR1 (FGFR, lane 2). The positions of the FGFR1 protein and the molecular weight standards are indicated. (C) Detection of *Xenopus* PLCγ1. A *Xenopus* embryo extract (lane 1) or a total cell extract from the MDA-468 cells (lane 2) was immunoprecipitated with anti-PLCγ1 polyclonal antiserum then Western blotted with anti-PLCγ1 monoclonal antibody. The positions of the PLCγ1 protein and the molecular weight standards are indicated.

gate (Amersham) was used instead of the secondary antibody. Generally, the exposure times ranged from 1 to 5 min. For reprobing, the blots were stripped in 2% SDS, 0.1 *M* 2-mercaptoethanol, 62.5 m*M* Tris–HCl, pH 6.7, at 50°C for 30 min; for most experiments, blots could be successfully stripped and reprobed one or two times.

In Vitro Translation

^{35}S-labeled *Xenopus* FGFR1 protein was synthesized *in vitro* using a coupled transcription/translation system (Promega): 50 μCi of [^{35}S]methionine (1000 Ci/mmol; Amersham) and 1 μg of pcDNA1neo containing the entire coding region of *Xenopus fgfr1* (Paterno *et al.*, unpublished) was added to the TNT reticulocyte lysate system in a final volume of 50 μl and incubated at 30°C for 90 min. Immunoprecipitations were carried out as described above using 4 μl of translation products and 20 μl of preimmune or immune serum in 1 ml of solubilization buffer. The immunoprecipitates were analyzed by SDS–PAGE and fluorography (Paterno *et al.*, 1989).

Mesoderm Induction

The FGF used in this study was recombinant *Xenopus* bFGF, expressed and purified according to Kimelman *et al.* (1988) and then stored at −20°C. Animal pole explants were dissected manually as described in Godsave *et al.* (1988). Explants were induced either by culturing in the presence of 100 ng/ml *Xenopus* bFGF diluted in normal amphibian medium (NAM; Slack and Forman, 1980) plus 1mg/ml bovine serum albumin (BSA) or by direct contact with vegetal cells. Control explants were cultured in NAM plus BSA alone. Following a 30-min incubation on ice, explants were solubilized and prepared for immunoprecipitation and Western blot analysis as described above for whole embryos. Each sample consisted of 100 explants.

RESULTS

PLCγ1 Is Associated with FGFR1 during Mesoderm Induction by FGF or by Vegetal Cells

Traditionally, studies of RTK substrates such as PLCγ1 have taken advantage of the fact that these substrates are physically associated with activated receptors and thus can be coimmunoprecipitated with anti-receptor antibodies. In addition, coprecipitated substrates often contain phosphotyrosine indicating that they have been phosphorylated by the RTK. We employed this strategy to examine FGFR signaling complexes in *Xenopus* embryos. A number of different antibodies were utilized in this study and all commercial antibodies were tested for cross-species reactivity. For recognition of *Xenopus* FGFR1, two different antibodies were utilized. The initial experiments (Figs. 2 and 3) employed a commercially available monoclonal anti-FGFR, directed against the extracellular domain of the human FGFR1 protein. However, during the course of this study, we prepared a polyclonal XFR, directed against a synthetic peptide corresponding to the C-terminus of *Xenopus* FGFR1 protein. Affinity-purified anti-XFR specifically recognized *Xenopus* FGFR1 protein synthesized *in vitro* using a rabbit reticulocyte lysate system (Fig. 1A) and both human and *Xenopus* antibod-

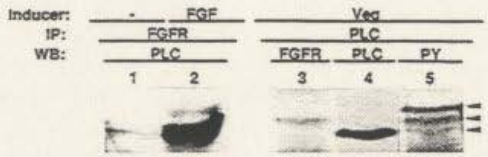

FIG. 2. Phosphorylation of PLCγ1 during mesoderm induction by FGF or by vegetal cells. Ectodermal explants (100 per sample) were incubated on ice for 30 min in the absence (−) or presence (FGF) of a saturating concentration of *Xenopus* bFGF (100 ng/ml; Gillespie *et al.*, 1989) or in contact with vegetal cells (Veg). Explants were solubilized in the presence of phosphatase inhibitors and the supernatant examined for co-immunoprecipitation of PLCγ1 with FGFR1. The antibodies used for immunoprecipitation (IP) and Western blotting (WB) are indicated above each lane. The blot containing the Veg-induced sample was probed sequentially with anti-PY (lane 5), anti-FGFR (lane 3), and anti-PLC (lane 4). The positions of the PLCγ1 and FGFR1 proteins are indicated.

ies recognized comigrating proteins from *Xenopus* embryo extracts (Fig. 1B). The *Xenopus* antibody displayed greater sensitivity for detecting *Xenopus* FGFR1 protein than did the human antibody (Fig. 1B, compare lanes 1 and 2), therefore, all subsequent experiments were carried out with the *Xenopus* antibody.

PLCγ1 was detected using commercially available antibodies. For staining Western blots, we used a mixed monoclonal antibody and for immunoprecipitation a PLCγ1-specific polyclonal antibody was used. This combination of antibodies identified a single band of the appropriate molecular mass (144 kDa) from *Xenopus* embryo extracts (Fig. 1C, lane 1) and this protein comigrated with PLCγ1 from a human breast carcinoma cell line, MDA-468 (Fig. 1C, lane 2).

To investigate the possibility that PLCγ1 is involved in intracellular signaling during mesoderm induction, we examined association of PLCγ1 with FGFR1 during mesoderm induction by FGF *in vitro* and by vegetal cells, the source of the natural inducing signal. Explants were incubated for 30 min on ice in the absence or presence of exogenous FGF or in contact with vegetal cells. Extracts were then analyzed for co-immunoprecipitation of PLCγ1 with FGFR1.

PLCγ1 co-immunoprecipitated with FGFR1 in explants treated with FGF, but not in untreated explants (Fig. 2, lanes 1 and 2), suggesting that PLCγ1 is involved in signal transduction during mesoderm induction by FGF. The blot containing the immunoprecipitate from explants induced by vegetal cells was first stained with anti-PY and then stripped and reprobed with anti-FGFR and finally with anti-PLC. Both FGFR1 and PLCγ1 were present in the immunoprecipitate (Fig. 2, lanes 3 and 4) and by overlaying the three autoradiographs, we were able to determine that FGFR1 and PLCγ1 corresponded to the middle and lower phosphotyrosyl bands, respectively, in lane 5. The apparent molecular mass of the FGFR1 and PLCγ1 proteins was 158 and 144 kDa, respectively, which is in close agreement with the reported size for these two proteins (Ryu *et al.*, 1987; Ueno *et al.*, 1992).

These results demonstrate that FGFR1 was phosphorylated and associated with PLCγ1 during induction by vegetal cells and suggest that FGFR1 phosphorylated PLCγ1. In addition, activation of FGFR1 was detected within 30 min after exposure to the vegetal cell inducing signal, providing direct evidence that the FGF signaling pathway is activated during the initial stages of mesoderm induction *in vivo*.

The third phosphotyrosyl protein detected in the immunoprecipitate containing FGFR1 and PLCγ1 (Fig. 2, lane 5, top arrow) has an apparent molecular mass of 180 kDa. Attempts to identify this protein were unsuccessful and complicated by the fact that this phosphotyrosyl protein was not always detected in our samples (see Figs. 4–7).

Phosphorylation of PLCγ1 and Association with FGFR1 Is Developmentally Regulated and Is Correlated with Mesoderm Induction in Vivo

The results in Fig. 2 demonstrate that phosphorylation of PLCγ1 occurs during mesoderm induction *in vitro*. Mesoderm induction *in vivo* is thought to occur dur-

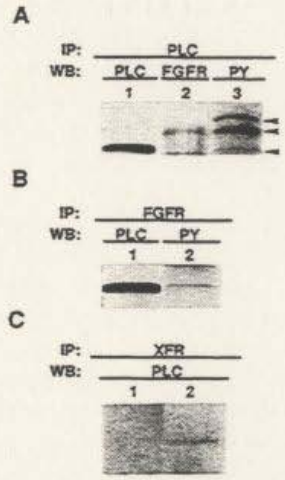

FIG. 3. Association of PLCγ1 with FGFR1 in *Xenopus* blastulae and in marginal zone cells. (A) Coprecipitation of FGFR1 with PLCγ1. Extracts from *Xenopus* blastulae (150 per sample) were immunoprecipitated (IP) and the blots stained (WB) with the indicated antibodies. The middle and lower phosphotyrosyl bands (see arrows, lane 3) comigrated with FGFR1 and PLCγ1, respectively. (B) Coprecipitation of PLCγ1 with FGFR1. A *Xenopus* blastula extract (150 embryos) was immunoprecipitated with anti-FGFR and the blot stained with anti-PY (lane 2). The blot was stripped and reprobed with anti-PLC (lane 1). (C) Association of PLCγ1 with FGFR1 in presumptive mesoderm. Marginal zones (lane 2) were dissected from 90 embryos and the remaining tissue (lane 1) was pooled into a separate sample. Soluble extracts were immunoprecipitated with anti-XFR and the blot stained with anti-PLC.

ing blastula stages and takes place in the equatorial region of the embryo known as the marginal zone. To investigate a possible interaction between FGFR1 and PLCγ1 during mesoderm induction *in vivo*, we examined coimmunoprecipitation in blastula stage embryos and whether association of these two proteins took place specifically in the presumptive mesoderm (marginal zone). Embryo extracts from *Xenopus* blastulae were subjected to immunoprecipitation with either anti-PLC (Fig. 3A) or anti-FGFR (Fig. 3B) followed by Western analysis with anti-FGFR, anti-PLC or anti-PY. The FGFR1 co-immunoprecipitated with PLCγ1 (Fig. 3A, lanes 1 and 2) and vice versa (Fig. 3B, lane 1), demonstrating a physical association between PLCγ1 and FGFR1 *in vivo*. In addition, both FGFR1 and PLCγ1 were phosphorylated on tyrosine (Fig. 3A, lane 3 and 3B, lane 2). Whether this association between FGFR1 and PLCγ1 is restricted to presumptive mesodermal cells was determined by comparing the levels of FGFR1-associated PLCγ1 in the presumptive mesoderm (marginal zone) to that present in the remainder of the embryo. Marginal zones were dissected from *Xenopus* blastulae and the remaining tissue combined into a separate sample. Soluble extracts from the two samples were subjected to immunoprecipitation with anti-XFR and examined for the presence of PLCγ1. Although the recovery of FGFR1-associated PLCγ1 was low, PLCγ1 was detectable in marginal zone cells (Fig. 3C, lane 2) but not in the remainder of the embryo (Fig. 3C, lane 1).

The presence of phosphorylated FGFR1 in *Xenopus* blastulae indicates that it is activated during this stage of development and the fact that the associated PLCγ1 contains phosphotyrosine is consistent with phosphorylation by FGFR1. Specific association of PLCγ1 with FGFR1 in presumptive mesoderm cells is consistent with our *in vitro* induction results and suggests that PLCγ1 is involved in intracellular signaling during mesoderm induction *in vivo*.

A phosphotyrosyl protein migrating slower than FGFR1 was detected in the immunoprecipitate (Fig. 3A, lane 3, top arrow). This protein has the same molecular mass as the unidentified phosphotyrosyl band observed in Fig. 1 (lane 5, top arrow) and probably represents the same protein.

Mesoderm induction *in vivo* is known to occur prior to stage 11 (mid-gastrula); therefore, we examined the phosphorylation pattern of PLC-γ1 in embryos during early development. The phosphorylation state of PLCγ1 was determined by immunoprecipitating PLCγ1 from embryo extracts followed by Western blotting with anti-PY. Five different stages of early development representing first cleavage (stage 2), early-, mid-, and late-blastula (stages 7, 8, and 9, respectively), and mid-gastrula (stage 11) were examined. As shown in Fig. 4A, five phosphotyrosyl bands of apparent molecular mass 158, 144, 112, 66, and 48 kDa were observed at mid-blastula stage (lane 3). Some phosphorylation of the same five bands was also observed in early blastulae (stage 7, lane 2). Note that the heavy chain of the immunoprecipitating antibody could also be detected; this nonspecific staining of the heavy chain was seen only when whole serum was used for immunoprecipitation and was probably due to the relatively large quantity of IgG present (100 μg compared to 10–15 μg when affinity-purified or monoclonal antibodies were used).

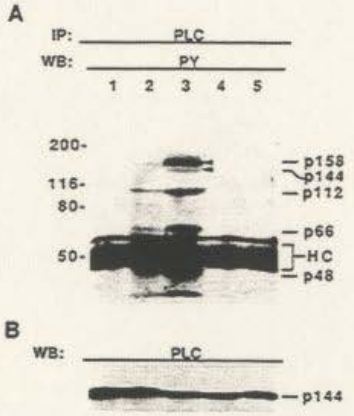

FIG. 4. Stage-specific phosphorylation of PLCγ1. Embryo extracts (150 embryos per sample) from the two-cell (lane 1, stage 2), early blastula (lane 2, stage 7), mid-blastula (lane 3, stage 8), late blastula (lane 4, stage 9), or mid-gastrula stage (lane 5, stage 11) were immunoprecipitated (IP) with anti-PLC and the Western blot (WB) was stained with anti-PY (A). Five phosphotyrosyl bands were detected at mid-blastula (lane 3) with some phosphorylation occurring at early blastula (lane 2). The apparent molecular mass of these phosphotyrosyl bands is shown on the right and the positions of the molecular weight standards on the left. The positions of p158 and p144 are indicated with arrowheads and the heavy chain (HC) of the immunoprecipitating antibody with a bracket. (B) The same blot reprobed with anti-PLC.

To determine which of these represented PLCγ1 and to determine whether we were observing stage-specific phosphorylation or stage-specific expression of PLCγ1, the blot was stripped and restained with anti-PLC (Fig. 4B). By overlaying the autoradiographs, we were able to determine that PLCγ1 corresponded to the 144-kDa phosphotyrosyl band. The expression level of PLCγ1 protein was constant throughout these stages of development (Fig. 4B), demonstrating that PLCγ1 is specifically phosphorylated during early to mid-blastula stage.

The association of PLCγ1 with FGFR1 during early embryonic development was also examined. Samples representing the same developmental stages presented in Fig. 4 (first cleavage, early-, mid-, and late-blastula and mid-gastrula) were immunoprecipitated with anti-XFR and stained for PLCγ1 (Fig. 5A); a parallel set of samples was stained with anti-PY (Fig. 5B). The blot from Fig. 5A was stripped and reprobed with anti-XFR

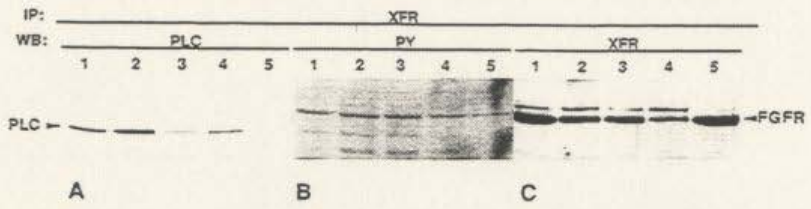

FIG. 5. Association of PLCγ1 with FGFR1 during early development. (A) Coprecipitation of PLCγ1 with FGFR1 during early development. Embryo extracts (150 embryos per sample) from the two-cell (lane 1, stage 2), early blastula (lane 2, stage 7), mid-blastula (lane 3, stage 8), late blastula (lane 4, stage 9), or mid-gastrula stage (lane 5, stage 11) were immunoprecipitated (IP) with anti-XFR and the Western blot (WB) was stained with anti-PLC. The position of the PLCγ1 protein is indicated. (B) Phosphorylation pattern of FGFR1 and PLCγ1 during early development. A parallel set of embryos (150 per sample) was immunoprecipitated with anti-XFR and the Western blot stained with anti-PY. (C) FGFR1 expression during early development. The blot from (A) was stripped and reprobed with anti-XFR. The position of the FGFR1 protein is indicated.

(Fig. 5C). The expression level of FGFR1 protein was relatively constant throughout the stages examined (Fig. 5C); however, a peak of PLCγ1 association with FGFR1 was observed at early blastula (Fig. 5A, lane 2) followed by a rapid decline with none detectable by mid-gastrula (stage 11; Fig. 5A, lane 5). Since the expression level of PLCγ1 protein was also constant throughout these developmental stages (Fig. 4B), these data demonstrate that PLCγ1 association with FGFR1 was developmentally regulated. Phosphorylation of PLCγ1 was observed at early- and mid-blastula (Fig. 5B, lanes 2 and 3) with little or none at late-blastula and mid-gastrula (Fig. 5B, lanes 4 and 5). Similarly, the peak of FGFR1 phosphorylation occurred at early- and mid-blastula stages (Fig. 5B, lanes 2 and 3), followed by a steady decline (Fig. 5B, lanes 4 and 5). Some association and phosphorylation of PLCγ1 and FGFR1 was also observed at first cleavage (Figs. 5A and 5B, lane 1) and it is unclear at the moment what this represents.

These results show that FGFR1–PLCγ1 association and phosphorylation levels peak during early- to mid-blastula stages, coincident with the onset of mesoderm induction *in vivo*.

NCK, GRB-2, and SOS1 Are Associated with the FGFR1 in Blastula Stage Embryos

Our interest was to identify other proteins that may form part of an FGFR1 signaling complex in *Xenopus* blastulae, particularly the phosphotyrosyl proteins that coprecipitated with PLCγ1 (Fig. 4A). First we compared the pattern of phosphotyrosyl proteins that coprecipitate with FGFR1 to those observed with PLCγ1. Embryo extracts were immunoprecipitated with either anti-XFR or anti-PLC followed by Western blot analysis with anti-phosphotyrosine. For this and all subsequent experiments, the anti-phosphotyrosine used was a biotin conjugate (anti-PY-b), as the signal-to-noise ratio was significantly increased with this antibody.

The pattern of phoshotyrosyl proteins precipitated by the two antibodies was virtually identical (Fig. 6A, lanes 1 and 2) with the exception of a 100-kDa band unique to the FGFR1 immunoprecipitate (see arrowhead in Fig. 6A). In addition to the five phosphotyrosyl bands seen in Fig. 4A, we observed four more phosphotyrosyl bands of apparent molecular mass 87, 71, 62, and 58 kDa; the appearance of additional bands was probably due to the increased sensitivity of the anti-PY-b.

By overlaying autoradiographs of blots stained with specific antibodies onto the corresponding phosphotyrosyl profile, we were able to determine that p158 and p144 phosphotyrosyl bands corresponded to FGFR1 and PLCγ1, respectively. This comparison was done on five separate occasions, an example of which is shown in Fig.

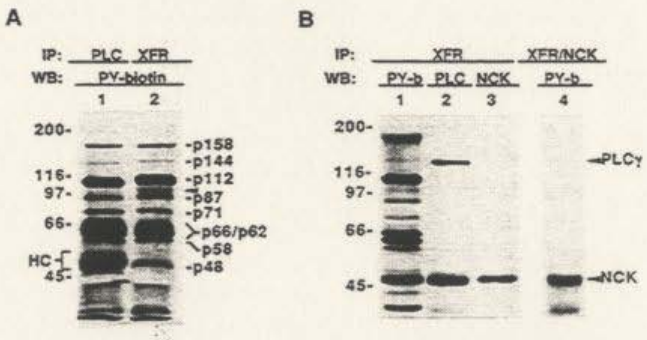

FIG. 6. Analysis of phosphotyrosyl proteins associated with FGFR1 in *Xenopus* blastulae. Extracts from *Xenopus* blastulae (150 per sample) were immunoprecipitated (IP) and the blots stained (WB) with the indicated antibodies. (A) Comparison of the phosphotyrosyl bands associated with FGFR1 to those associated with PLCγ1. The apparent molecular mass of the phosphotyrosyl bands is indicated on the right and the molecular weight standards are on the left. The arrow indicates a phosphotyrosyl band (100 kDa) that is unique to the FGFR1 immunoprecipitate. (B) Detection of phosphorylated PLCγ1 and NCK in the FGFR1 complex. A *Xenopus* blastula extract (600 embryos) was separated into four equal samples and each was immunoprecipitated with anti-XFR; the immunoprecipitate from one sample (lane 4) was boiled in 1% SDS and the denatured proteins were reprecipitated with anti-NCK prior to Western blotting. Molecular weight standards are shown on the left and the positions of the PLCγ1 and NCK proteins are indicated.

FIG. 7. Association of GRB2 and SOS1 with FGFR1 in *Xenopus* blastulae. (A) Extracts from *Xenopus* blastulae (150 per sample) were immunoprecipitated (IP) with anti-XFR (lanes 1-3) or anti-GRB2 (lane 4); the Western blot of the anti-XFR immunoprecipitate was stained (WB) sequentially with anti-PY-b (lane 1), anti-XFR (lane 2), and anti-SOS1 (lane 3), while that of the anti-GRB2 immunoprecipitate was stained with anti-XFR. Molecular weight standards are shown on the left and the positions of the FGFR1 and SOS1 proteins are indicated. (B) A *Xenopus* blastula extract (150 embryos) was immunoprecipitated with anti-XFR and the Western blot stained with anti-PY-b (lane 1); the blot was stripped and reprobed with anti-GRB2 (lane 2). Molecular weight standards are shown on the left and the position of the GRB2 protein is indicated.

7A, lanes 1 and 2 (FGFR1), and Fig. 6B, lanes 1 and 2 (PLCγ1).

The molecular mass of the fastest migrating phosphotyrosyl protein, p48, was similar to that reported for NCK, an SH2/SH3 domain containing protein that has been reported to associate with RTKs in a ligand-dependent manner (Li *et al.*, 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992; Lee *et al.*, 1993). We investigated the possibility that p48 was NCK through the use of a NCK-specific antibody. As shown in Fig. 6B, lane 3, a single band that comigrates with p48 was detected with anti-NCK. Note that the anti-PLC mixed monoclonal antibody also recognizes NCK (lane 2), as has been previously reported (Meisenhelder and Hunter, 1992; Park and Rhee, 1992). To confirm that the FGFR1-associated NCK was indeed phosphorylated on tyrosine, the immunoprecipitated FGFR1 complex was dissociated by boiling in 1% SDS and the solubilized proteins were reprecipitated with anti-NCK; the blotted sample was then stained with anti-PY-b. As expected, under these conditions only NCK was recovered; the NCK protein contained phosphotyrosine and comigrated with p48 (Fig. 6B, lane 4). These results demonstrate that, in *Xenopus* blastulae, phosphorylated NCK protein was associated with FGFR1 in a putative signaling complex.

While continuing with efforts to identify the remaining phosphotyrosyl proteins, we decided to turn our attention to signaling pathways known to be important for mesoderm induction in *Xenopus*. One such pathway involves the activation of RAS (Whitman and Melton, 1992). These authors demonstrated that microinjection of cRNA encoding a dominant negative mutant RAS resulted in inhibition of mesoderm induction by FGF, implicating RAS as a downstream effector of FGFR. Activation of RAS by the epidermal growth factor receptor (EGFR) is mediated by SOS which binds to EGFR through the adaptor molecule GRB2 (reviewed in Marx, 1993; McCormick, 1993). A similar signaling mechanism has been demonstrated for the platelet-derived growth factor receptor (PDGFR) with the exception that GRB2 does not bind to the PDGFR directly, but interacts through the phosphotyrosine phosphatase, SYP (Li *et al.*, 1993). Given that different RTKs have several substrates in common, we examined the possibility that GRB2 and SOS1 were associated with FGFR1 in *Xenopus* blastulae.

Both SOS1 and GRB2 were immunoprecipitated with anti-XFR (Fig. 7A, lane 3 and 7B, lane 2); in addition, FGFR1 could be immunoprecipitated with anti-GRB2 (Fig. 7A, lane 4). However, neither FGFR1-associated SOS1 or GRB2 was found to contain detectable levels of phosphotyrosine (Fig. 7A and B, lane 1). These data demonstrate the presence of GRB2 and SOS1 in a putative FGFR1 signaling complex and are consistent with a report by Whitman and Melton (1992) that RAS activity is required for mesoderm induction by FGF.

DISCUSSION

We have shown that PLCγ1 became associated with FGFR1 during mesoderm induction by FGF or vegetal cells *in vitro*, demonstrating that PLCγ1 is part of the signaling pathway(s) activated by these two inducers. In addition, association of PLCγ1 with FGFR1 in explants occurred within one half hour after exposure to FGF or to vegetal cells, the source of the natural inducer. These results show that signaling through FGFR1 occurs very early during mesoderm induction by the natural signal and provide the first direct evidence that a member of the FGF family is a component of the vegetal inducing signal. The identity of the vegetal inducing signal has remained elusive to date. Attempts to implicate FGF or activin by including neutralizing antibodies or follistatin in vegetal induction assays have led to the conclusion that neither basic FGF nor activin are major components of the natural inducing signal (Slack, 1991). However, if the vegetal signal consists of several types of inducing molecules and/or several members of one family, it may not be possible to completely inhibit induction in this manner. A requirement for activin at some stage during mesoderm formation *in vivo* has come from experiments using a truncated form of the activin receptor (Hemmati-Brivanlou and Melton, 1992). This truncated receptor inhibits activin induction in explants and, more importantly, mesoderm formation *in vivo*. Evidence that at least one of the FGFs is involved in mesoderm formation comes from experiments with a dominant negative mutant construct of FGFR (Amaya *et al.*, 1991). This mutant receptor was shown to interfere

with wild-type receptor activity and expression of this mutant receptor in *Xenopus* embryos resulted in deficiencies in organized mesodermal tissue. The question that remained to be answered was when during the multistep process of mesoderm formation is FGF involved? FGF could be a component of the vegetal inducing signal or be expressed/released in the inducing or responding cells at some point downstream in the cascade of events initiated by the vegetal signal. Amaya *et al.* (1993) used their dominant negative FGFR construct to investigate how disruption of FGF signaling affects the expression of early mesodermal markers and their results suggest that FGF is involved in events that occur prior to gastrulation. Our data extend these findings by providing evidence that FGF is actually part of the initial inducing signal.

While the exact role that PLCγ1 plays during *Xenopus* development remains to be determined, we have shown that FGFR1–PLCγ1 association as well as the phosphorylation pattern of both proteins was temporally and spatially correlated with mesoderm induction *in vivo*. If recruitment of PLCγ1 into the FGFR1 signaling complex increases its enzymatic activity as has been demonstrated in other systems (reviewed in Rhee and Choi, 1992), then one might expect to detect changes in the levels of IP_3 and DAG, both products of PLCγ1 catalytic activity. Indeed IP_3 levels were shown to increase significantly during early *Xenopus* development and this increase coincided temporally with the onset of mesoderm induction (Maslanski *et al.*, 1992). Although consistent with the data reported here, we cannot exclude the possibility that the increase in IP_3 production results from catalytic activity other enzymes. DAG is known to activate PKC and our previous work has shown that PKC activity was increased in FGF-treated explants (Gillespie *et al.*, 1992). In addition, our results suggested that PKC was part of a feedback loop that negatively regulated mesoderm induction by FGF. Thus, PLCγ1 may be involved in negative modulation of mesoderm induction, acting to limit intracellular signaling initiated in response to FGF.

The phosphotyrosine content of PLCγ1 was consistently low relative to the level of PLCγ1 protein that was detectable (see Figs. 3, 4, and 6). This may reflect a low level of PLCγ1 activation which would be consistent with the low level of PKC activation detected in FGF-treated explants (Gillespie *et al.*, 1992). Alternatively, FGFR1 stimulation of PLCγ1 activity may occur independently of tyrosine phosphorylation, as has been reported for the EGFR (Hernandez-Sotomayor and Carpenter, 1993).

We have demonstrated that NCK is present in the putative FGFR1 signaling complex and that, like PLCγ1, it is phosphorylated during early-to-mid-blastula stages. Thus, NCK may have a crucial role in the FGF signaling cascade regulating mesoderm differentiation during embryonic development. NCK was originally isolated as a cDNA encoding a novel SH2/SH3 containing protein from a human melanoma library (Lehmann *et al.*, 1990) and later shown to be an oncoprotein (Li *et al.*, 1992) which becomes phosphorylated in response to activation of PDGFR and EGFR in cultured cells (Li *et al.*, 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992). NCK is composed primarily of SH2 and SH3 domains; therefore, it has been postulated that NCK functions as an adaptor molecule for coupling RTKs to an as yet unidentified downstream effector. It is conceivable that the NCK-binding effector in *Xenopus* blastulae is one of the phosphotyrosyl proteins present in the putative FGFR1 signaling complex. We are currently investigating this possibility.

GRB2 and SOS1 were also associated with FGFR1 in *Xenopus* blastulae although neither were phosphorylated on tyrosine. This is in agreement with the results of a recent study using COS-1 cells, in which SOS was shown to be phosphorylated on serine and threonine but not on tyrosine in response to insulin stimulation (Baltensperger *et al.*, 1993). Like NCK, GRB2 is composed of SH2 and SH3 domains and evidence suggests that it functions as an adaptor molecule. GRB2 represents the mammalian homologue of SEM-5 from *Caenorhabditis elegans* and DRK from *Drosophila melanogaster*. Both SEM-5 and DRK are important signaling molecules involved in distinct differentiation events during the development of these two organisms and both act downstream of an RTK in a pathway that leads to activation of RAS. SEM-5 functions in signal transduction through the *let-23* RTK during vulval induction in *C. elegans* (Clark *et al.*, 1992) while DRK is involved in Sevenless RTK signaling during specification of the R7 photoreceptor cell in the developing *Drosophila* compound eye (Simon *et al.*, 1993; Olivier *et al.*, 1993). DRK, like GRB2, has been shown to bind to SOS, a guanine nucleotide exchange factor for RAS (Buday and Downward, 1993; Gale *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Rozakis-Adcock *et al.*, 1993; Simon *et al.*, 1993). A requirement for RAS activity during FGF-induced mesoderm differentiation in *Xenopus* has been demonstrated by Whitman and Melton (1992). Thus, the association of GRB2 and SOS with FGFR1 may link FGF action to RAS activation in *Xenopus* and provide another example of GRB2/SOS involvement in regulating cellular decision events during embryonic development.

Several additional phosphotyrosyl bands were observed in association with FGFR1 in *Xenopus* blastulae; these probably represent additional components of a signaling complex. Therefore, it will be important not only to identify these phosphotyrosyl proteins but also to determine their specific function, along with that of PLCγ1, GRB2, SOS1, and NCK, possibly through the use

of dominant negative mutant constructs. Such experiments are currently underway in our laboratory.

During the past few years, a wealth of information has accumulated concerning the spatiotemporal expression pattern of potential inducing molecules and their receptors (for review, see Kimelman *et al.*, 1992). While this type of information is extremely important, it does not allow us to determine when mesoderm induction actually takes place. Our results show that it is feasible to detect activation of a particular signaling molecule, thus enabling us to map when and where specific inducing molecules become functionally active. This methodology is not only useful for the study of signaling through the FGFR but will also be important for examining *in vivo* activation of other types of receptors involved in mesoderm induction, once the appropriate antibodies become available.

The authors thank Joy Greenland for technical assistance and Drs. Gary Paterno and Ken Kao for critical reading of the manuscript. This work was supported by grants to L.L.G. from the Medical Research Council of Canada and from the National Cancer Institute of Canada with funds from the Canadian Cancer Society.

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July 7, 1999

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Re: Phosophorylation of Phosopholipase Cγl and its Assocation with FGF Receptor is Developmentally Regulated and Occurs during Mesoderm Induction in Xenopus laevis Volume 166 pp. 101-111 (1994) Developmental Biology

For: Thesis submission to: Memorial University of Newfoundland, St. John's NF, Canada

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Chapter 4 Results: part II

As stated previously, a complex is formed with substrates of other RTK and the activated receptor. These substrates can be co-immunoprecipated with the receptor, which often contain phosphorylated proteins, indicating that they have been phosphorylated by the receptor. The goal subsequent to publishing the *Developmental Biology* paper was to identify the remaining components associated with the FGFR1. With this information a correlation may be made between the proteins involved in the complex and their relationship with FGFR1 during early embryonic development. The co-immunoprecipated phosphorylated pattern of PLCγ1 and FGFR shows eight phosphotyrosyl bands. This banding pattern is the same regardless of the immunoprecipating antibody (See Figure 6A in Chapter 3).

These were the associated proteins identified with the activated FGFR1 joined to the receptor by SH2 and/or SH3 bonds. Using this molecular weight pattern as a guide and the extensive information available on signaling molecules, a number of different antibodies against relevant signaling proteins

were investigated. By overlaying autoradiographs of the blots stained with the specific antibodies onto the phosphotyrosine pattern and reciprocating the immunoprecipitation a number of these bands were identified.

4:1 GTPase-Activating Protein

Ras-GAP appears to be a negative regulator of p21*ras* by stimulating its weak intrinsic GTPase activity (Egan and Weinberg, 1993). This occurs when there is activation of a tyrosine receptor, activating exchange factors, such as GRB2/Sem5 and SOS. This initiates the exchange of guanine-nucleotides, converting Ras-GDP to Ras-GTP (reviewed and in Egan Weinberg, 1993).

GAP is generally considered to be involved in the signaling from tyrosine kinases. In cells stimulated with EGF or transformed by pp60v-*src*, Ras-GAP becomes phosphorylated on both tyrosine and serine residues and forms complexes with two phosphorylated proteins of 62 and 190 kDa (Ellis *et al.*, 1991). GAP is upregulated with PDGF stimulation in fibroblast cell lines (Vetter and Bishop, 1995) and has a reported molecular weight of 120 kDa. GAP appears to be a relevant second messenger in RTK and there was a similar molecular weight protein in this complex, so the existence of GAP was investigated as a possibility to occur within the FGFR1 complex.

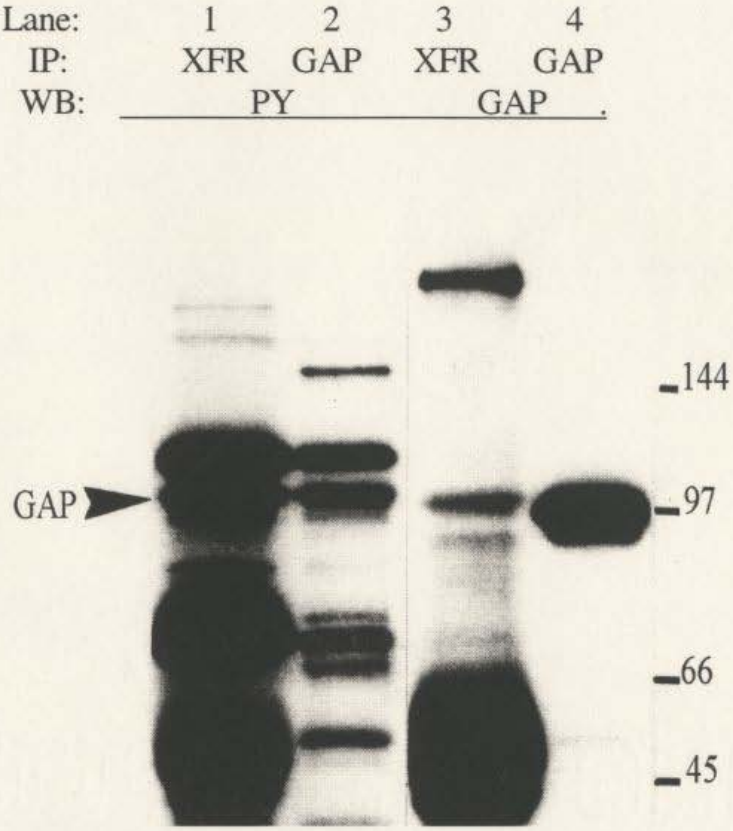

FIG. 4.1. Association of GAP with FGFR1 in *Xenopus* blastulae. A *Xenopus* blastula extract (150 embryos) was immunoprecipated (IP) with anti-Xenopus FGFR1 (XFR) and the western blot (WB) was stained with GAP. This blot was stripped and reprobed with anti-phosphotyrosine (PY). The relative position of the molecular weight standards is shown on the right and the position of GAP protein is indicated on the left.

Figure 4.1 shows that both FGFR1 and GAP immunoprecipate the same phosphorylated complex (lane 1 and 2). Lane three and four show that if the experiment was reversed, a similar banding pattern exists. Lane four shows that GAP is detected in our extracts. The GAP band in lane 3 and 4 aligned with the phosphotyrosine-banding pattern. This suggests that GAP is associated with the FGFR1 and GAP is phosphorylated.

4:2 PI3′ Kinase

PI3' -kinase (PI3' K) is an enzyme that phosphorylates at the D-3 position of the inositol ring of phosohatidylinositol (PI), PI-4-phosphate, and PI-4, 5-biphosphate (PI-4, 5-P2) (Heldman, 1996). PI3'K exists as a heterodimer of 85kDa (p85) and 110 (p110) subunits. p85 may serve as regulator of the catalytic subunit, p110, by acting as the link between PI3'K and the ligand-activated receptor (Freund, 1995).

PI3'K has been shown to be involved in other signaling pathways of receptor kinases such as NGFR (Jackson, 1996) and PDGF (Heldman, 1996). PI3'K has known SH2 domains (Escobedo, 1991). Due to the characteristics

of this enzyme I investigated the possibility that PI3′K may occur within the FGFR1 complex.

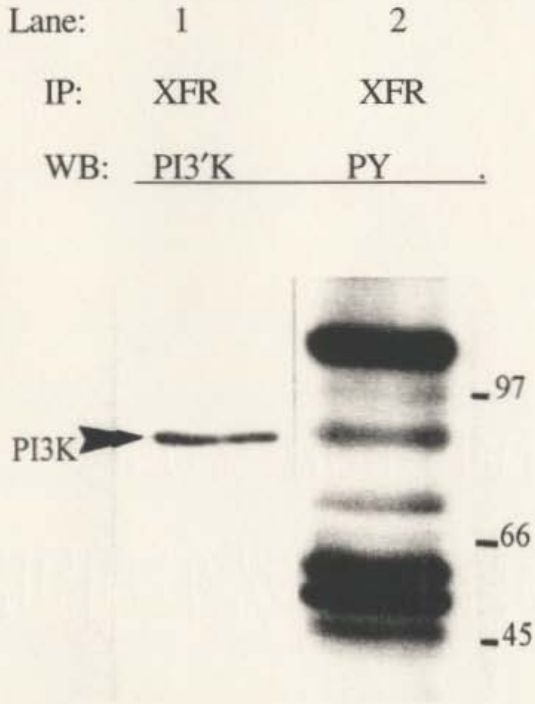

FIG. 4.2. Association of PI3′K with FGFR1 in *Xenopus* blastulae. A blastula extract (150 embryos) was immunoprecipitated (IP) with anti-*Xenopus* FGFR1 (XFR) and the western blot (WB) was stained with anti-PI3′K. The blot was stripped and reprobed with anti-phosphotyrosine (PY). The relative position of the molecular weight standards is shown on the right and the position of the PI3′K protein is indicated.

the FGFR1 (lane 1), suggesting that PI3'K is associated with the activated receptor, and this band does appear to be phosphorylated (lane 2). This suggests that PI3'K is the unidentified 87kDa phosphorylated band in the FGFR1 complex.

4:3 SHP2 Phosphatases

Phosphotyrosine phosphatases (PTPases) are enzymes that remove phosphate groups from protein tyrosine residues. SHP2 (also named PTP1D, Syp or SH-PTP2) has been identified as a 72kDa phosphotyrosine phosphatase comprising two SH2 domains. SHP2 has been shown to be associated with the activated EGFR complex (Wong *et al.*, 1997). Roche *et al* (1996) examined the role of SHP2 and other effector proteins in the mitogenic response induced by PDGF in fibroblasts. SHP2 has been shown to be involved in the insulin receptor complex with other proteins including GRB2, Nck and PI3'K (Kasuga, 1996). The insulin-induced binding of SHP2 to the complex

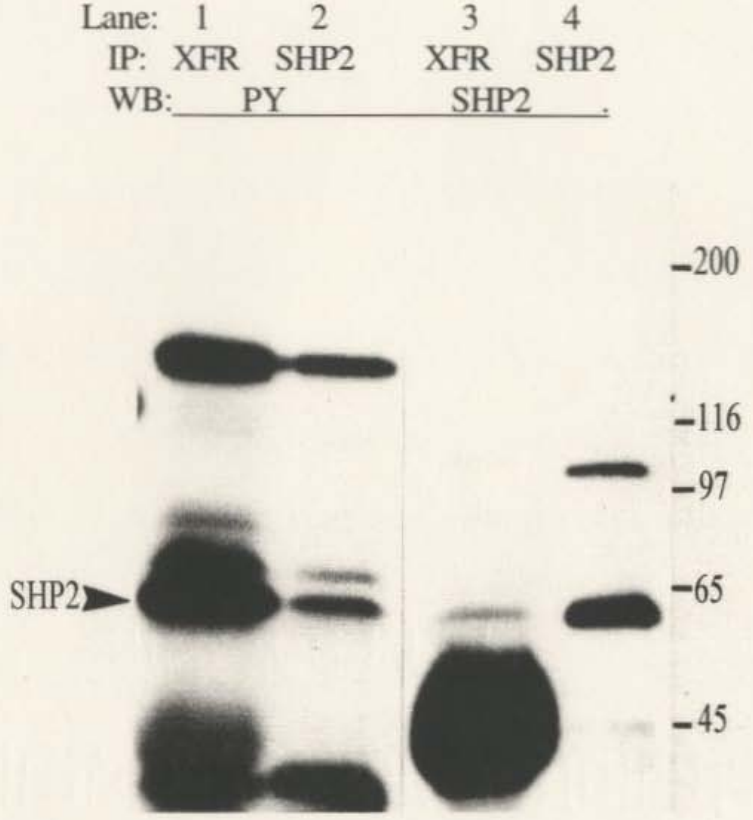

FIG.4.3. Association of SHP2 with FGFR1 in *Xenopus* blastulae. An extract of 150 *Xenopus* embryos, at blastula, was immunoprecipated (IP) with anti-FGFR1 (XFR) or anti-SHP2 and the western blot was stained with anti- SHP2, then stripped and reprobed with anti-phosphotyrosine (PY). The relative position of the molecular weight markers is shown on the right and the position of the SHP2 is indicated on the left.

generates the signal to activate the Ras protein. Upon FGFR stimulation in PC12 cells, SHP2 activation leads to MAPK activation (Hadari, 1998). It has been shown to be involved in other tyrosine kinases such as receptor for macrophage colony-stimulating factor (M-CSF) (Rohrschneider and Carlberg , 1997), platelet endothelial cell adhesion molecule-1 (PECAM-1) (Fujiwara *et al.*, 1997). SHP2 was studied in the FGFR1 system to determine if it was one of the unknown components.

Figure 4.3 shows a similar western pattern with either SHP2 or FGFR1 antibody and stained with anti-SHP2 (lanes 3 and 4). SHP2 appears to be associated with the FGFR1 complex because if you immunoprecipate with SHP2 and stain with anti-phosphotyrosine a similar pattern exists (lane 2). The same blot was stripped and reprobed. When the autorads were overlapped, the corresponding SHP2 band is phosphorylated. This information suggests that the phosphatase SHP2 is associated with the activated FGFR1 complex, and is phosphorylated.

4:4 Nck-Associated Protein

Because of Nck structure (i.e. three SH3 and one SH2 domains), it is assumed to be an adapter or linking molecule but the exact protein-protein interaction is poorly characterized (Lehmann *et al.*, 1990). Nck has been shown to be within the activated FGFR complex, as shown in Chapter three. The objective of these experiments was to identify which protein in the FGFR1 complex was associated with Nck.

Figure 4.4 shows that there are a number of Nck-associated proteins in this system. As shown in Figure 4.4 (lanes 1 and 3) three of the FGFR1-associated phosphotyrosyl bands bound to the Nck-beads. None of these proteins bound to the beads containing a control antibody (lane 4), confirming the specificity of the interaction with Nck. The exact identity of these proteins is unknown, but there are a number of identified Nck-associated proteins in the literature to date. This information is discussed in the Chapter five.

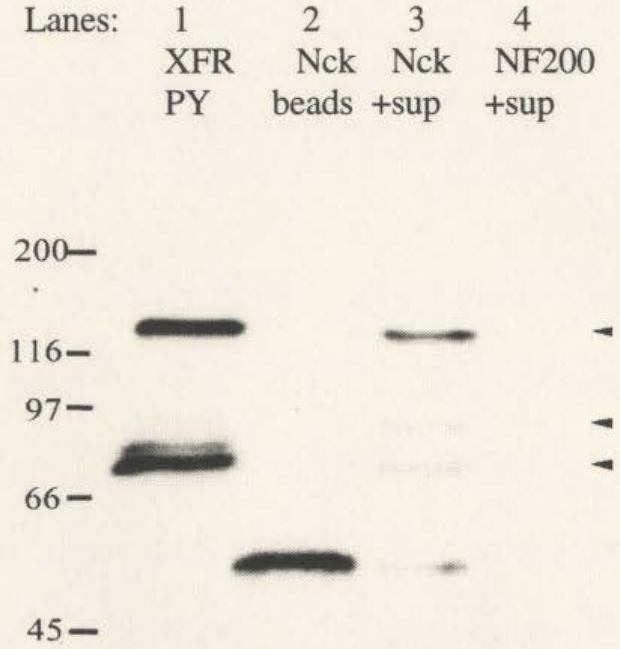

FIG.4.4. Nck-Associated proteins within the FGFR1 complex formed in *Xenopus* blastula. Lane 1 shows an extract of 150 embryos immunoprecipated with anti-FGFR1 (XFR) and the western blot stained with anti-phosphotyrosine (PY). Lane 2 shows the purified Nck that was isolated from Sepharose beads. Lane 3 shows another sample of Nck, as isolated in lane two, incubated with the supernatant (sup) of a FGFR-void *Xenopus* extract. Lane 4 shows the control anti-neurofiliment (NF200) on Sepharose beads incubated with supernatant. The whole blot is stained with anti-phosphotyrosine. The relative position of the molecular weight markers is indicated on the left and the position of the Nck associated proteins are indicated on the right.

4:5 Non-Associated Effectors

SRC and SHC are proteins that have been identified in other RTK systems including FGFR1 (Richard, 1995). These proteins were not identified as components of the FGFR1 complex (See Figures 4.5 and 4.6)

SRC and family members exist in an increased phosphorylation state when fibroblasts are exposed to PDGF (Ralston and Bishop, 1985). SRC activity increased in EGF stimulated cells (Landgren *et al.*, 1995). Stover *et al.* (1995) has proposed that SRC may phosphorylate sites within the EGFR to which SRC then binds. These sites are not phosphorylated by the receptor itself. A large number of substrates have been identified in response to SRC activation, that are similar to proteins phosphorylated in cells stimulated through RTK and are implicated in mitogenetic signaling pathways. These include PLCγ1, RasGAP, SHP2, SHC and the p85 subunit of PI3′K (reviewed in Brown, 1996). This complex contains a number of these proteins, which suggests that SRC might be involved in this FGFR1 signaling pathway.

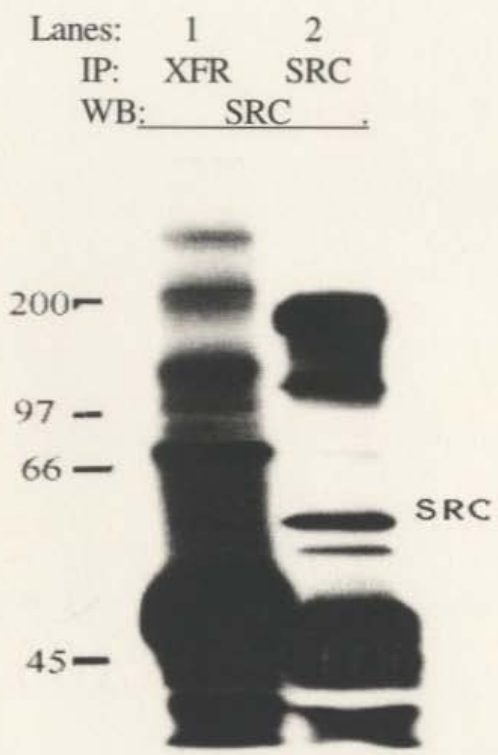

FIG.4.5 SRC is not Associated with the FGFR1 in *Xenopus* Extracts.

Extract (150 embryos) were immunoprecipated with either anti-FGFR1 (XFR) or SRC. The western blot was stained with SRC. The relative position of the molecular weights is indicated on the left and the position of SRC is indicated on the right.

The pattern shown in figure 4.5 is very distinct. The SRC protein is indicated in lane 2. The pattern in lane 1 is very different from the phosphorylated pattern and the SRC protein does not appear to be associated. The phosphorylated state of SRC was not tested.

SHC is an adaptor protein that is widely expressed in all tissues and contains an SH2 domain, a collagen-like domain but no obvious catalytic domain (Pelicci *et al.*, 1992). SHC is activated with FGFR stimulation in L6 myoblast cells (Klint *et al.*,1995). GRB2 was not found to directly interact with FGFR1, rather an indirect interaction via SHC has been identified (Klint *et al.*, 1995). SHC was also found to be tyrosine-phosphorylated upon interleukin-2 stimulation in CTLL-20 cells and upon activation, interacted with GRB2 to activate the Ras GTP/GDP exchange (Ravichandran, 1994). GRB-SOS was found in our complex, yet GRB2 or SOS was not phosphorylated (See Chapter 3). We investigated the possibility that SHC acted as an adapter molecule for the GRB2-SOS complex in our system.

Figure 4.6 shows no specific staining of the SHC antibody in the FGFR1 immunoprecipated lane, yet there is staining in the SHC immunoprecipated lane. The staining pattern is different than the phosphorylated pattern observed with the immunoprecipated FGFR. This indicates that the SHC antibody is recognizing a protein, but SHC is not in our complex. The white band at 50kDa is the negative staining of the heavy chain of the immunoprecipating antibody.

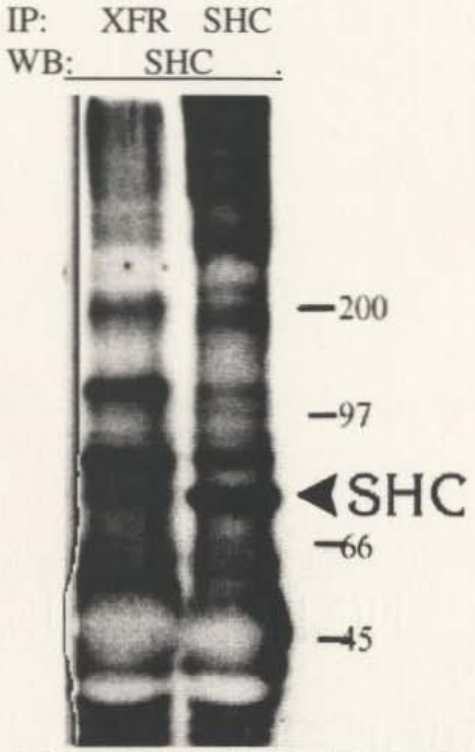

FIG.4.6 SHC is not Associated with the FGFR1 in *Xenopus* Extracts.

Extract (150 embryos) were immunoprecipated with either anti-FGFR1 (XFR) or SHC. The western blot was stained with SHC. The relative position of the molecular weights is indicated on the right and the position of SHC is indicated on the left.

These results have been published (See Appendix):

Ryan,P.J. and Gillespie,L.L.G. (1998) Identification of Phosphorylated Proteins Associated with the Fibroblast Growth Factor Receptor Type 1 during Early *Xenopus* Development. *Biochemical and Biophysical Research Comm.* 244:763-767.

Chapter 5

Discussion

As mentioned earlier, FGF binds to the extracellular region of the FGFR, which leads to the dimerization of the receptor and rapid autophosphorylation of tyrosine residues (reviewed in Klint and Claesson-Welsh, 1999). Phosphorylation leads to the recruitment of various cellular target proteins through the respective SH2 regions. These SH2-containing proteins can bind to the receptor and a stable complex is formed. The identification of all of the proteins associated with the FGFR during early embryonic development in *Xenopus* was the basis of this work. The results are summarized in figure 5.1.

We have identified all but one of the FGFR-associated phosphotyrosyl proteins. Proteins present in the complex include PLCγ1, Ras-GAP, PI3′K, SHP2, Nck as well as three Nck-associated proteins: NAP65, NAP81, NAP123 and an unknown protein of molecular mass of 73kDa.

The first protein we investigated was PLCγ1. Explants were cut at stage eight (mid-blastula) and incubated with bFGF or the natural inducer, *Xenopus* vegetal pieces. The association is the same in both cases.

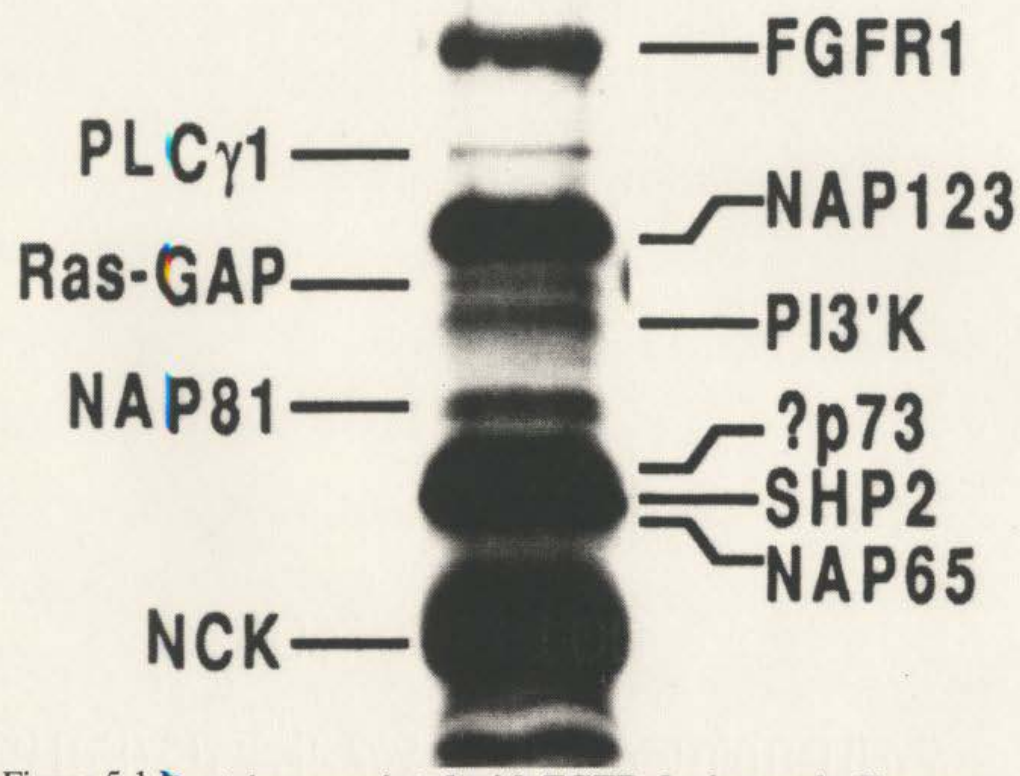

Figure 5.1 **Proteins associated with FGFR during early *Xenopus* Development.** A *Xenopus* blastula extract (150 embryos) was immunoprecipated with FGFR1 and the western blot was stained with anti-phosphotyrosine. The identity of each phosphotyrosyl band is indicated. NAP is the abbreviation for Nck-associated protein and the number following is the estimated molecular mass. The unidentified phosphorylated band is indicated by ?, followed by its estimated molecular mass.

This showed that PLCγ and FGFR are associated in a complex within 30 minutes of activation. Both FGFR and PLCγ are phosphorylated. The early activation of FGFR suggests that the FGF signaling may be important in the initial inducing signal during mesoderm induction in *Xenopus laevis*. It is now suspected that a member of the TGFβ family, Vg1, is the natural inducer of dorsal mesoderm from the vegetal zone (Kessler and Douglas 1995). It is speculated that FGFs are required in the animal hemisphere as competence factors, providing low level of stimulation of the tyrosine kinase signal pathway (reviewed in Isaacs, 1997). FGF activity is necessary for the full range of responses to the vegetal inducing signal, including the activation of *Xbra* transcription in the marginal zone of the late blastula.

The activation of the FGFR and PLCγ1 is stage specific *in vivo*. The location of the *in vivo* signal has been shown to be in the marginal zone. These spatio-temporal similarities with mesoderm induction provide further evidence of the importance of the FGFR activation in this process.

Phosphorylation of Y766 in FGFR-1 allows binding to, and subsequently, tyrosine phosphorylation of, PLCγ1 (Mohammaddi *et al.*, 1991). Tyrosine phosphorylation of PLCγ1 is known to be accompanied by

its activation, leading to hydrolysis of phosphatidylinositol bisphosphate (PI) to inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG) (Rhee *et al.*, 1989). IP_3 causes the release of calcium from intracellular stores that can initiate gene transcription. DAG is an activator of protein kinase C (PKC). PKC is activated during mesoderm induction by FGF (Gillespie, 1992) and is involved in the negative regulation of the FGFR, since pretreatment of explants with phorbol 12-myristate 13-acetate (a PKC activator) inhibited FGF induction (Gillespie *et al.*, 1995). PKC has been shown to be involved in other negative feedback loops. One such example is the regulation of Kit/SCF-R by direct phosphorylation on serine residues in the receptor (Blume *et al.*, 1994).

PKC can also directly phosphorylate Raf-1 and increase the kinase activity (Kolsh *et al.*, 1993). Raf acts upstream of MEK in the MAPK pathway to induce mitogenesis. The role of Ras is to recruit Raf to the plasma membrane (Leevers, 1994). Ras associates with the serine kinase c-Raf-1 in a GTP-dependent manner, and this complex phosphorylates and activates MAPK/ERK kinase (MEK). MEK then activates members of the mitogen-activated protein (MAP) kinase family. Overexpression of a *Xenopus* MAP kinase phosphatase blocks mesoderm induction by FGF, and

causes characteristic defects in mesoderm formation in intact embryos (Smith *et al.*, 1995). Expressing different levels of activated MEK mimics the induction of mesoderm by FGF, in a dose-dependent manner. Through these two experiments Smith *et al.* (1995) have identified the activation of MAP kinase as the only signal transduction event required for all aspects of FGF-induced ventral mesoderm formation. This links PLCγ to the activation of MAP kinase pathway and the development of ventral mesoderm. It also shows that activation of PKC may have a dual purpose- acting to negatively regulate the FGFR signaling and activating the MAPK pathway.

If a mutant FGFR, lacking Y766 was expressed in L6 cells or PC12, it has been found that aFGF is able to induce DNA synthesis in the L6 cells or induce differentiation of PC12 cells (Peters *et al.*, 1992). This indicates that PLCγ1 activity is not essential for FGF-induced mitogenesis in these cells. However, other studies suggested that PLCγ may play a role in PDGF-induced mitogenic signaling (Valius and Kazlauskas, 1993). A mutant PDGF failed to activate PLCγ, PI3′K or Ras and was unable to initiate DNA synthesis. This activity was restored with association of PLCγ. To account for both of these findings it is possible that receptor kinases may induce multiple signals to activate mitogenesis. The tyrosine kinase generates two

signals that interact with activated Raf, one is the activation of Ras through SOS/GRB and the other involves the PLCγ1 activation of Raf through PKC. Blocking one signaling pathway in a multipathway signal transduction system may not result in a significant difference in the final response if the remaining pathways can deliver a sufficiently strong signal. This possibility is supported by recent studies implicating MAPK as a critical component of mitogenic signaling pathway. MAPK can be activated by both a Ras-dependent and Ras-independent mechanisms (Burgering *et al.*, 1993).

Although PLCγ1 has been implicated in the mitogenic response of other growth factors, the role of this protein in FGF-induced cellular responses is unclear. Thus, cells expressing the mutant FGFR-1 lacking Y766, still respond to FGF stimulation with increased mitogenicity, although not as efficiently as cells expressing the wild type receptor (Klint *et al.*, 1995). This is in agreement with results from Huang *et al.* (1995) showing the level of Raf and MAP activities were reduced in FGF-stimulated L6 myoblasts expressing the Y766F mutant FGFR-1, indicating that PLCγ1 via PKC modifies the phosphorylation status and thereby affects the activity of Raf. In *Xenopus*, a point mutation in the FGFR changing Y766 to phenylalanine does not affect the ability of animal cap explants to

differentiate into mesoderm (Muslin *et al.*, 1994). These results suggest that PLCγ1 activation by the FGFR is not required for FGF-stimulated mesoderm induction. It is possible that multiple pathways exist and deleting the PLCγ1 pathway does not abolish the response.

Both SOS and GRB2 were co-immunoprecipitated with FGFR, and FGFR could be immunoprecipated with anti-GRB (Chapter 3). However, neither FGFR-associated SOS nor GRB was found to contain detectable levels of phosphotyrosine. This indicates that GRB and SOS are present in the FGFR signaling complex but are not tyrosine phosphorylated. This is consistent with a report by Whitman and Melon (1992). The authors injected a dominant inhibitory mutant Ras, called p21(Asn 17)$^{Ha\text{-}ras}$, into *Xenopus* embryos to study the role of Ras with different inducing factors. They found that Ras activity is required for mesoderm induction by FGF. They also identified a constitutively active Ras mutant partially mimicking the mesoderm inducing activity of FGF and activin. These results are also in agreement with Baltensperger (1993), where in COS cells, SOS was shown to be phosphorylated on serine and threonine, but not on tyrosine in response to insulin, leading to Ras activation by insulin. The association of GRB and SOS with FGFR1 links FGF action to p21^{ras}. This association is believed to

bring GRB and SOS in close proximity to the plasma membrane and consequently in contact with Ras. An analogous system exists in both *Caenorhabditis elegans* and *Drosophlia melanogaster* with Sem-5 and Drk as the GRB homologue respectively (Clark, 1992; Oliver *et al.*, 1993). As discussed in the introduction, both are important signaling molecules involved in distinct differentiation events during the development of these two organisms and both act downstream from Ras. The identification of SOS and GRB in this complex provides a link between FGFR activation and the Ras pathway.

SHP2 has been identified in our complex with the FGFR. This is consistent with a report by Hadari and Schlessinger (1998). They have demonstrated that in PC12 cells the FGF-induced tyrosine phosphorylation of SHP2 leads to recruitment of an additional GRB-SOS complex by a lipid-anchored docking protein called FRS2. In cells expressing a mutant FRS2, binding of GRB and SHP2 was impaired resulting in weak and transient MAPK response which failed to induce neuronal differentiation of PC12 cells. Their experiments showed that the catalytic activity of SHP2 is essential for a sustained MAPK response and for potentiation of FGF-induced neurite outgrowth. A targeted deletion of the mouse SHP2 gene,

removing most of the N-terminal SH2 domain, showed that the kinetics of FGF-stimulation of the Ras-pathway depended on SHP2 (Saxton *et al.*, 1997). Genetic studies in *Drosophila* and biochemical studies with mammalian cells suggest that SHP2 and its *Drosophila* homologue Corkscrew play a positive role in the control of MAPK activation, cell growth and differention (Bennett *et al.*, 1996; Herbst *et al.*, 1996). It is possible that the existence of SHP2 acts to positively regulate the FGFR response by the recruitment of additional GRB/SOS and increases the activation of the MAPK pathway.

PI3′K was identified as the 87-kDa protein band in our FGFR complex. There is no known role for activation of the PI3′K pathway in *Xenopus,* although our data are consistent with various other RTK systems. PI3′K has been found to be important in the activity of PDGF, EGF and insulin (reviewed in Chiarugi, 1997). It has been suggested that PI3′K is involved in mediation growth factor-induced mitogenicity, motility responses and growth factor receptor internalization and trafficking (Blume *et al.*, 1994). Overexpression of PI3′K in COS cells activated MAPK in a G-coupled receptor dependent fashion, and expression of a catalyically inactive mutant of PI3′K, abolished the stimulation of MAPK (Ilasaca *et al.*, 1997).

The authors suggest that the activation of MAPK by PI3′K requires a tyrosine kinase that, in turn phosphorylates SHC and induces an association with GRB2 leading to a Ras-dependent activation of MAPK.

We have demonstrated that Nck is present in the complex and is phosphorylated during early-to-mid-blastula stages (see Chapter 3). Thus, Nck may have an important role in the FGF cascade regulating mesoderm differentiation during embryonic development. Nck, as described earlier is believed to be an adapter molecule for coupling RTKs to an, as yet unidentified downstream effector. The Nck-associated proteins, are indicated in figure 5.1 as NAP123, NAP81, and NAP65, have not yet been identified.

Several proteins that associate with Nck have been identified in other systems. 1) Lussier and Larose (1997) have identified a 70-75 kDa gamma2 isoform of the serine/threonine casein kinase I (CKI-gamma2) by using yeast two-hybrid system. 2) Quilliam *et al.* (1996) have identified two proteins by screening a bacterial expression library with Nck SH3 domains; Wiskott-Aldrich syndrome protein (WASP, a putative CDC42 effector) and a serine/threonine protein kinase (PRK2). It is interesting to note that PRPK2 binds to the SH3 region of PLCγ and Rho in a GTP-dependent manner. The authors suggest that PRPK2 may coordinately mediate signal transduction

from activated receptor and Rho and that Nck may function as an adapter to connect receptor-mediated events to Rho protein signaling. The Rho family of GTPase induces transcriptional activation from the *c-fos* serum response element via the serum response factor (SRF) (Hill *et al.*, 1995). 4) A 125kDa protein called NAP1 (Nck-associated protein 1) was isolated using bacteria expressing glutathione S-transferase fusion proteins (Kitamura *et al.*, 1997). The authors suggest that NAP125 is in a multimolecular complex that may mediate some of the biological effects transmitted by Rho GTPase. 5) A novel mammalian serine/threonine kinase that interacts with the SH3 domains of Nck, called Nck Interacting Kinase (NIK), that likely acts upstream of MEKK1 (MEK-kinase 1), has also been identified (Su *et al.*, 1997). Other proteins such as 6) p130cas (Schlaepfer *et al.*, 1997) and 7) SAM68 (Lawe *et al.*, 1997) have also been found to be associated with Nck. Our NAPs could be one of these known proteins, or in combination with other novel proteins. With further study and the development of novel protein antibodies, these molecules can be identified.

Future experiments could identify the specific role for the components of our complex in the development of mesoderm. These experiments would involve the injection of deleted fragments of the target proteins and then

testing the developing embryo for any changes in gene expression or FGF-induced effect on explants of animal caps. There is interesting recent information now known about various FGFR variants. One such example is the FGFR VT- isolated from a *Xenopus* blastulae library in our lab (Gillespie *et al.*, 1995). This isoform is a result of a two amino acid deletion in juxamembrane region causing differences in regulation of the receptor. It would be interesting to see if the FGFR complex would change when this variant is injected into the developing embryos. How do they change? Are there any differences in the complex between the two receptor isoforms? Other experiments could study any resulting change in regulation of the receptor as a result of any changes in the complex components.

These data indicate that FGFR1 may signal through at least three pathways: PI3'K, PLCγ and the Ras pathway. Evidence shows that each of these pathways leads to the activation of the MAPK pathway. Smith *et al.*, (1995) have shown that the activation of MAPK pathway is the only signal transduction event required for FGF-induced mesoderm induction and different levels of activated MEK mimic the dose-dependent mesoderm induction. This suggests that the FGFR complex is important in the formation of mesoderm in early Xenopus development. The components of

this complex act to strictly regulate the FGFR signal pathway providing both positive and negative regulation. Our results enable us, to map when and where specific inducing molecules become functionally active with FGFR stimulation, during early embryonic development. Our results suggest multiple pathways to tranduce signals from the receptor tyrosine kinases to the activation of MAPK kinase mitogenesis. If multiple paths do exist, it is important to know whether the different pathways relay signals to the nucleus independently, or whether they converge at some point to activate a common downstream element. The identification of key molecules that receive multiple signals from the cell surface and relay them to the nucleus is of great interest. Identification of all components of FGFR complex will give a better understanding of the FGF pathway leading to future advancements in FGFR signaling, and a better understanding of the role of FGF in developing embryo.

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Appendix

These data have been published during the writing of this thesis: Identification of Phosphorylated Proteins Associated with the Fibroblast Growth Factor Receptor Type 1 during Early *Xenopus* Development. *Biochemical and Biophysical Research Comm.* 244:763-767.

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 244, 763–767 (1998)
ARTICLE NO. RC988326

Identification of Phosphorylated Proteins Associated with the Fibroblast Growth Factor Receptor Type I during Early *Xenopus* Development

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Received February 10, 1998

Signaling through the FGF receptor (FGFR) is required for mesoderm induction in *Xenopus*. Some of the downstream signaling molecules implicated in this developmental process include Ras, Raf and MAP kinase. In a previous report, we demonstrated that PLCγ1, Grb-2, SOS and Nck were associated with activated FGFR1s in a signaling complex in *Xenopus* blastulae. In addition, several unidentified phosphotyrosylproteins were present in the FGFR1 complex. Here we identify three of these proteins as Ras-GAP, the p85 of PI3'K and SHP2, while demonstrating that c-Src and Shc were not associated with the FGFR1. Furthermore, we show that three additional phosphotyrosylproteins from the FGFR1 complex specifically bound to the adaptor molecule Nck. © 1998 Academic Press

The family of fibroblast growth factors (FGFs) are involved in a number of cellular activities, including mitogenesis, cell differentiation and angiogenesis (reviewed in 1, 2). The cellular response to FGF is mediated by high affinity cell surface FGF receptors (FGFRs). Functional FGFRs are integral membrane proteins composed of an extracellular ligand-binding domain, a transmembrane domain and an intracellular domain containing a split tyrosine kinase domain (1). FGF binding to the extracellular domain of the FGFR leads to activation of the receptor which in turn binds and phosphorylates a number of intracellular substrates, altering their catalytic activity and initiating intracellular signal transduction cascades.

FGFs induce mesoderm differentiation in explants from *Xenopus* blastulae (3-5) and FGFR signaling has been shown to be required for this developmental event (6). Activation of FGFR1s during mesoderm induction in *Xenopus* results in the formation of a signaling complex containing at least ten detectable proteins (7). One of these was shown to be phospholipase C gamma 1 (PLCγ1) whose catalytic activity leads to activation of protein kinase C (PKC). PKC activity had previously been reported to increase in FGF treated explants and to result in inhibition of mesoderm induction by FGF (8). Thus, PLCγ1 may be involved in negative modulation of mesoderm induction, acting to limit intracellular signaling initiated in response to FGF.

Additional components of the FGFR1 signaling complex formed during mesoderm induction in *Xenopus* blastulae were identified as Nck, Grb-2 and Son-of-sevenless (SOS) (7). Further identification of the FGFR1-associated phosphotyrosylproteins will be important for elucidation of the molecular mechanisms involved in signal transduction during FGF-induced mesoderm differentiation. In this report, we show that the FGFR1 in *Xenopus* blastulae is associated with the GTPase-activating protein (Ras-GAP), the regulatory subunit (p85) of phosphatidylinositol 3'-kinase (PI3'K) and the protein tyrosine phosphatase SHP2/Syp/SH-PTP2/PTP1D (SHP2) as well as three phosphotyrosyl proteins that interact with the adaptor molecule Nck.

MATERIALS AND METHODS

Embryos. *Xenopus laevis* were purchased from Nasco. Eggs were artificially inseminated, the jelly coats removed and the embryos cultured as described (7). Embryonic stages were determined according to Nieuwkoop and Faber (9).

Immunoprecititation and Western blotting. The antibodies employed in this study were as follows: anti-PI3'K, anti-GAP, anti-Src, anti-Shc and biotinylated anti-phosphotyrosine (PY) purchased from Upstate Biotechnology Inc.; anti-Nck and anti-SHP2 from Transduction Laboratories; anti-NF from Sigma; anti-*Xenopus* FGFR1 (XFR) was produced and affinity-purified in our laboratory. Protein A-Sepharose was obtained from Pharmacia.

Analysis of FGFR1 signaling complexes involved the preparation

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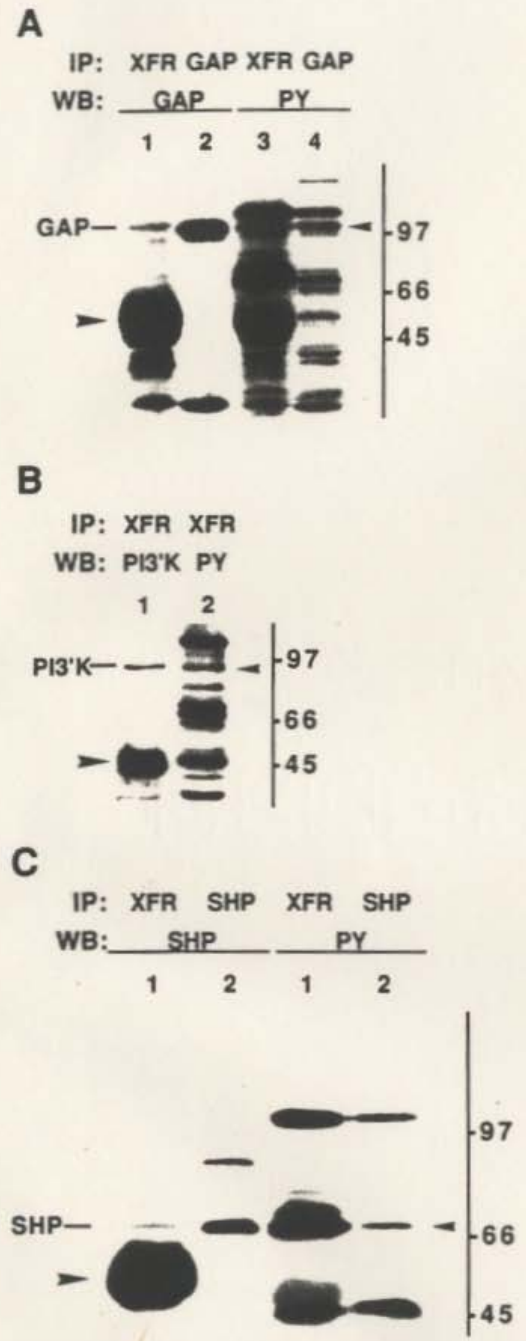

FIG. 1. Association of phosphorylated Ras-GAP, PI3'K and SHP2 with the FGFR1 in Xenopus embryos. (A) Co-precipitation of Ras-GAP with the FGFR1. Extracts from *Xenopus* blastulae (150 per sample) were subjected to immunoprecipitation (IP) with either anti-FGFR (XFR; lanes 1 and 3) or anti-GAP (lanes 2 and 4). Western blotting (WB) was performed with anti-GAP (lanes 1 and 2), after which the blot was stripped and re-stained with anti-phosphotyrosine (PY; lanes 3 and 4). The position of the GAP protein is shown and the position of the corresponding phosphotyrosyl band is indicated by a small arrow. Molecular weight standards are shown on the right. A large arrow indicates the heavy chain of the immunoprecipitating antibody which, in some cases, was recognized by the secondary antibody during Western blotting. (B) Co-precipitation of the p85 of PI3'K with the FGFR1. Embryo extracts were subjected to immunoprecipitation with anti-XFR and the blot stained with anti-PI3'K (lane 1). The blot was stripped and re-stained with anti-PY (lane 2). The position of p85 is shown and the corresponding phosphotyrosyl band is indicated by a small arrow. The large arrow indicates the same as in (A). (C) Co-precipitation of SHP2 with the FGFR1. Extracts were prepared and analyzed as in (A). The position of SHP2 is shown and the corresponding phosphotyrosyl band is indicated by a small arrow. The large arrow indicates the same as in (A).

of embryo extracts, immunoprecipitation and Western analysis as described (7). Each sample contained 150 stage *Xenopus* blastulae. For re-staining, the blots were first stripped in 2% SDS, 0.1M 2-mercaptoethanol, 62.5 mM Tris-HCl, pH6.7 at 50°C for 30 min.

For the analysis of Nck-associated proteins, Nck was isolated from the anti-XFR immunoprecipitate as follows: the anti-XFR-Protein A-Sepharose beads were boiled for 5 min in 100ul of 1%SDS, 10mM Tris, pH7.4. The supernatant containing the denatured proteins was diluted to a final concentration of 0.1%SDS in solubilization buffer (7) and incubated for 1h with anti-Nck at 4°C. The antigen-antibody complex was precipitated with Protein A-Sepharose and the beads were washed extensively with ice-cold solubilization buffer (1% Triton, 10mM Tris pH7.5, 10mM EDTA, 1mM PMSF, 25ug/ml aprotinin, 25ug/ml leupeptin, 5ug/ml TLCK, 100uM sodium orthovanadate, 100mM NaF, 10mM sodium pyrophosphate). The Nck-bound beads were either prepared directly for PAGE or incubated for 1h with FGFR1-depleted blastula embryo extract. Depletion of the extract was accomplished by pre-incubation with anti-XFR coupled to Protein A-Sepharose and was performed to ensure that we were not simply purifying FGFR1 complexes. The Nck beads were washed as above and prepared for Western analysis. Control beads, consisting of Protein A-Sepharose with bound neurofilament H subunit monoclonal antibody (Sigma), were incubated with FGFR1-depleted embryo extract, washed and prepared for Western analysis exactly as described for the Nck beads.

RESULTS AND DISCUSSION

In a previous report, we demonstrated that Grb2, SOS, Nck and PLCγ1 were associated with the FGFR1 during the blastula stage of *Xenopus* development (7). Several additional phosphotyrosyl-containing proteins were also associated with the FGFR1 in a signaling complex and our aim was to identify these additional phosphotyrosylproteins.

Receptor tyrosine kinases (RTKs) have been shown to associate with a number of signaling molecules (reviewed in 10), including proteins with enzymatic activity such as PLCγ1, Ras-GAP, SOS, c-Src and the protein tyrosine phosphatase SHP2, as well as a number of proteins composed mainly of SH2 and SH3 domains that function as adaptor molecules to couple catalytically active proteins to the RTK. Examples of such adaptors include Nck, Shc, Grb2 and the p85 subunit of PI3'K. Therefore, we began our investigation of FGFR-associated phosphotyrosylproteins by examining known signaling molecules. The strategy that we employed to identify these proteins was to immunoprecipitate FGFR1 from *Xenopus* blastulae under non-de-

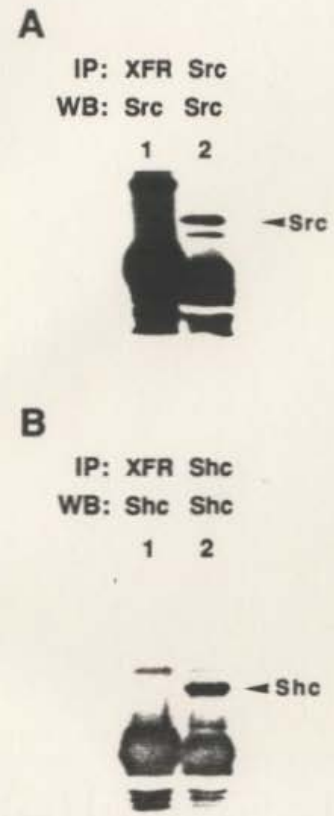

FIG. 2. Src and Shc do not associate with the FGFR1 in Xenopus embryos. Extracts from *Xenopus* blastulae (150 per sample) were prepared and analyzed as described in the legend to Fig. 1. The antibodies used for IP and WB are indicated above each lane. The position of the Src (Panel A, lane 2) or Shc (Panel B, lane 2) proteins isolated from embryo extracts is indicated. No corresponding proteins were co-precipitated with the FGFR1 (Panel A and B, lane 1).

naturing conditions, followed by Western blotting with an antibody that recognizes the RTK substrate in question, or that recognizes phosphotyrosine.

Using a monoclonal antibody against Ras-GAP, we were able to demonstrate that Ras-GAP is present in *Xenopus* embryos at this stage of development (Fig. 1A, lane 2) and that the Ras-GAP protein is associated with the FGFR1 (Fig. 1A, lane 1). The blot was stripped and re-stained with anti-phosphotyrosine and, by overlaying the films, we were able determine that Ras-GAP was phosphorylated on tyrosine (Fig. 1A, lanes 3 and 4). Similarly, we were able to show that the p85 of PI3′K was associated with the FGFR1 in *Xenopus* embryos (Fig. 1B, lane 1) and that this protein was phosphorylated on tyrosine (Fig. 1B, lane 2). The protein tyrosine phosphatase, SHP2, was also present in *Xenopus* embryos (Fig. 1C, lane 2) and co-precipitated with the FGFR1 (Fig. 1C, lane 1). Re-staining the blot with anti-phosphotyrosine revealed that FGFR1-associated SHP2 was phosphorylated (Fig. 1C, lane 1).

Several of the remaining phosphotyrosyl bands migrated in the molecular weight range of c-Src and Shc. Both of these proteins have been shown to interact with the FGFR1 in other systems (11, 12), however, antibodies against c-Src and Shc failed to demonstrate any association between either of these proteins and the FGFR1 (Fig. 2A and B, lane 1), even though both of these proteins were present in the embryo (Fig. 2A and B, lane 2).

Nck is a 47kDa protein that functions as an adaptor molecule for a number of different proteins (13-21) and that is present in the FGFR1 complex in *Xenopus* blastulae (7). Nck has recently be shown to play an important role in specification of mesodermal fate in *Xenopus* (13). Microinjection into embryos of RNA encoding Nck containing a non-functional third SH3 domain resulted in ventralization of dorsal mesoderm and affected the migration of mesodermal cells during gastrulation, thus implicating Nck binding proteins in these developmental processes. Therefore, we examined the possibility that one or more of the remaining phosphotyrosylproteins in the *Xenopus* FGFR1 complex is a Nck-associated protein. Nck immunoprecipitated from *Xenopus* embryos and bound to Sepharose beads (Fig. 3, lane 2) was used for affinity precipitation from a blastula embryo extract. As shown in Fig. 3 (lanes 1 and 3), three of the FGFR1-associated phosphotyrosyl bands bound to the Nck beads. None of these proteins bound to beads containing a control antibody (lane 4), thus confirming the specificity of the interaction with Nck. These results suggest that Nck functions as an

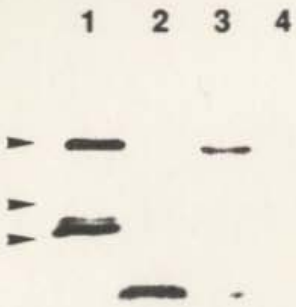

FIG. 3. Nck-associated phosphotyrosyl proteins in the FGFR1 complex. The FGFR1 complex (lane 1) was dissociated and Nck was isolated using anti-Nck and Protein A-Sepharose beads. The Nck beads were then either extracted for Western analysis (lane 2), or incubated with an FGFR1-depleted embryo extract, as described in Methods and Materials. The Nck-bound phosphotyrosyl proteins (lane 3) were analyzed by Western blotting with anti-phosphotyrosine. The Nck-associated phosphotyrosyl bands are indicated by arrows. In lane 4, a neurofilament H subunit antibody was used instead of anti-Nck as a control for non-specific interactions.

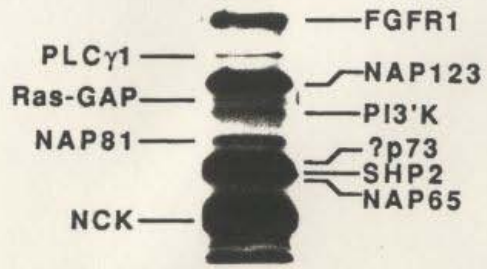

FIG. 4. FGFR1-associated phosphotyrosyl proteins. The identity of each individual FGFR1-associated phosphotyrosyl band is indicated. As demonstrated in a previous report (7), the two upper phosphotyrosyl bands represent FGFR1 and PLCγ1, while the fastest migrating band represents Nck. The phosphotyrosyl bands corresponding to each of the following: Ras-GAP, p85 of PI3'K, SHP2 and the three NAPs (*N*ck-associated *p*rotein), are indicated. The number that follows each NAP is the estimated molecular mass. The unidentified phosphotyrosyl band is indicated by a ? followed by its estimated molecular mass. The triplet encompassing p73, SHP2 and NAP65 sometimes runs together, as shown here, but this region is clearly composed of three distinct bands, as seen in Fig. 1A and 1B.

adaptor to link these three proteins to the FGFR1 or to each other.

The estimated molecular mass of these three *N*ck-*a*ssociated *p*roteins (NAPs) was calculated to be 65kDa (NAP65), 81kDa (NAP81) and 123kDa (NAP123). Several proteins that associate with Nck have been identified in other systems; these include the Wiskott-Aldrich syndrome protein (WASP; 14), a 66kDa protein; PKC-related kinase 2 (Prk2), a 130 kDa protein that is a potential effector of Rho protein signaling (15); NAK, a 65kDa serine/threonine kinase (16); SAM68, a nuclear RNA binding protein (17); p130cas, a Crk associated substrate that has the structural features of an adaptor protein (18); p125Nap1, a Nck-associated protein isolated from rat (19); SOS (20); the γ2 isoform of casein kinase I, a 70-75kDa protein (21) and NIK, a 140kDa Ste20-related kinase (22). Whether NAP65, NAP81 or NAP123 represent the *Xenopus* homologue of any of these known Nck-associated proteins must await the availability of antibodies that recognize the *Xenopus* proteins.

We have succeeded in identifying all but one of the FGFR1-associated phosphotyrosyl proteins and our findings, which include those from a previous report (7), are summarized in Fig. 4. Proteins present in the FGFR1 signaling complex include PLCγ1, Ras-GAP, PI3'K, SHP2, Nck as well as three Nck-associated proteins: NAP65, NAP81 and NAP123. The estimated molecular mass of the unknown phosphotyrosyl protein was 73kDa.

Previously, we showed that Grb2 and SOS were also contained in the FGFR1 complex, although neither one is phosphorylated (7). Thus, in *Xenopus* blastulae, the FGFR1 signals through at least three pathways: PI3'K, PLCγ1 and the Ras pathway. Nothing is known about the role of PI3'K signaling in early *Xenopus* development. Studies of PLCγ1, on the other hand, have suggested that PKC, a downstream effector of PLCγ1, is involved in negative regulation of FGFR activity (8, 23). The importance of Ras and downstream signaling molecules, such as Raf and MAP kinase, in mesoderm induction has been demonstrated through the use of dominant negative and constitutively active constructs (24-26). SOS and Ras-GAP function as positive and negative regulators of Ras, respectively, and it is interesting to note that both are associated with activated FGFR1s in *Xenopus* blastulae. Taken together, these data suggest that early developmental events such as mesoderm induction and mesoderm patterning may require a fine balance of positive and negative regulation of FGFR1 activity and downstream signaling pathways.

ACKNOWLEDGMENTS

This work was supported by a grant from the Natural Sciences and Engineering Research Council to L.L.G. and G.D.P.

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