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Characterization of LIM Kinase and Activity in Early Amphibian Embryos

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EphA4 is a cellular receptor that functions in mediating tissue interactions in embryonic development. The downstream effectors of EphA4 are mainly unknown, although previous research has indicated that the enzyme p160ROCK is one of the downstream mediators in the pathway. LIM kinase is an enzyme that is activated by p160ROCK. We have hypothesized that LIM kinase acts downstream of p160ROCK in the signaling pathway. Our results showed that LIM kinase is present and activated during development when EphA4 is active in the embryo, which is consistent with a role for LIM kinase in EphA4 signaling. Further testing is necessary to determine if LIM kinase has an active role in EphA4 signaling.

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Abstract

EphA4 is a cellular receptor that functions in mediating tissue interactions in embryonic development. The downstream effectors of EphA4 are mainly unknown, although previous research has indicated that the enzyme p160ROCK is one of the downstream mediators in the pathway. LIM kinase is an enzyme that is activated by p160ROCK. We have hypothesized that LIM kinase acts downstream of p160ROCK in the signaling pathway. Our results showed that LIM kinase is present and activated during development when EphA4 is active in the embryo, which is consistent with a role for LIM kinase in EphA4 signaling. Further testing is necessary to determine if LIM kinase has an active role in EphA4 signaling.

Introduction

LIM kinases are a special member of the LIM family proteins which contain a catalytic domain. The LIM domain consists of a cysteine-rich sequence and a unique double zinc finger motif. (Takahashi et al. 2001) The LIM domain mediates precise protein to protein interactions. LIM kinase (LIMK) is identified by two tandemly arrayed LIM domains at the N-terminus, a protein kinase at the C-terminus, and a PDZ-like motif at the interposing region. The LIM motif contains fifty to sixty amino acids and was named for the three homeodomain-containing regions; Lin-11, Isl-1, and Mec-3 (Okano et al. 1995).

LIMK catalyzes the phosphorylation of an N-terminal serine residue of cofilin, which acts as a regulator of actin dynamics. Cofilin is a type of actin-severing protein in eukaryotic cells and

acts by removing actin monomers from the ends of F-actin or by severing the actin filaments (Rosenblatt et al. 1997).

There are two genes for LIMK; LIMK1 and LIMK2. LIMK1 and LIMK2 are expressed in the adult brain and the developing neural tissues, which suggest a role in neural development and function (Nunoue et al. 1995). In *Xenopus laevis*, LIMK1 was found in developing tissue areas such as the brain, eyes, and branchial arches (Thirone et al. 2008). LIMK1 is also expressed in oocytes and during the cleavage stages of the embryo, which also suggests an early developmental role for LIMK1 (Takahashi et al. 1995).

LIMK1 is phosphorylated by Rho kinase (ROCK1) at Thr508, whereas LIMK2 is phosphorylated by ROCK1 at Thr505. ROCK1 enhances the ability of LIMK to phosphorylate cofilin. Cdc442 and Rac effecter p21-activated kinases (PAKs) also phosphorylate LIMK1, leading to cofilin phosphorylation (Maekawa et al. 1999).

ROCK1 is a kinase that is involved in the EphA4 signaling pathway. EphA4 is a member of the receptor tyrosine kinases (RTKs). Eph RTKs have certain ligands, called ephrins that form receptor-ligand complexes important in axonal growth, segmentation of the hindbrain and somites, control of cell migration, neural crest development, and vasculogenesis (Holder and Klein 1999).

The EphA4 signaling pathway is not completely known, but includes several downstream effectors including the cytoplasmic tyrosine kinase p59fyn, the small GTPase Rho, and ROCK1. The EphA4 pathway is responsible for regulating the actin cytoskeleton. EphA4 appears to inhibit RhoA which causes downstream effects in *Xenopus laevis* (Winning et al 2002). When

RhoA binds to the Rho-binding domain of ROCK, a conformational change occurs at the carboxyl terminus. The activation of ROCK leads to actin filament stabilization (Tsai and Wei 2010).

We hypothesize that because EphA4 activation inhibits Rho and thereby ROCK1, LIMK1 and/or LIMK2 are likely inactivated in the EphA4 signaling pathway. The purpose of this project was to determine if LIMK1 or LIMK2 is expressed during the appropriate stages in development for it to be involved in the EphA4 signaling pathway. If LIMK is involved, it would need to be expressed during development when EphA4 is functioning in the embryo.

Materials and Methods

Female *Xenopus laevis* frogs were injected with 900 units of human chorionic gonadotropinm to induce ovulation. Twelve hours after the injection the frogs were squeezed so the frogs would shed the eggs. One testis from a male frog was macerated to liberate the sperm; the male frogs were sacrificed. The sperm suspension was added to the eggs and the mixture was gently shaken on a rotating platform for twenty minutes. The eggs were then dejellied using a solution of 2% cysteine, ph 7.8-8.1. The dejellied eggs were then rinsed several times in distilled water and transferred to Multiply Modified Ringer's (MMR).

At various stages, embryos were placed in microcentrifuge tubes and frozen at -70°C. Subsequently, frozen embryos were homogenized and used to prepare RNA or protein extracts. For protein samples, embryos were homogenized, then extracted once with an equal volume of freon (1,1,2 trichloro-trifluoro-ethane) to remove yolk proteins. Following this, ½ volume of Protein Sample Buffer (New England Biolabs) was added, along with 1/30 volume of

dithiothreitol (DTT). Samples were mixed and heated to 100°C for 5 minutes to denature the proteins.

RNA was isolated from embryos using an Ambion Ribopure kit. Single-stranded cDNA from stages 9, 11, and 20 was prepared from heat-denatured RNA using reverse transcriptase. Table 1 indicates reactants and amounts used for the reaction. The RNA, random decamers, and nuclease-free water were mixed together and heated at three minutes at 80°C. The tubes were removed from heat and placed on ice. The remaining RT components were added to the mixtures. The reaction tubes were then mixed and incubated at 42.1°C for two hours. The reaction tubes were incubated for ten minutes at 65°C to inactivate the reverse transcriptase and then stored at -20°C.

Amount	**Compound**
≈1.2 µg	Total RNA
2 µL	Random Decamers
to 12 µL	Nuclease Free Water
2 µL	10X RT Buffer
4µL	dNTP Mixture
1 µL	RNase Inhibitor
1µL	MMLV-RT
Total 20 µL	

Table 1: Reactants for RT-PCR reactions

In order to amplify LIMK 1 and LIMK2 specific cDNA, four oligonucleotide primers were selected. A 411 bp fragment of LIMK1 cDNA was amplified using the forward and reverse primers 5’-TTGGACTGGCACTCTGATTGC-3’ and 5’-GGATGGCGTGATAAGAAACGGAC-3’,

respectively. A 473 bp fragment of LIMK2 cDNA was amplified using the forward and everse primers 5'-TGGGGAAGGGTTTCTTTGGACG-3'and 5'-TAGTGTGCGTTTTTTGGTGGG-3', respectively. PCR was performed in the presence of dNTP as shown in Table 2. Due to unexpected bands detected at stages 9, 11, and 20, PCR was also performed with annealing temperatures 52°C and 50°C. The PCR products were separated in a 0.3% synergel/0.7% agarose gel. The gel was stained using ethidium bromide and viewed with ultraviolet radiation (Figure 2).

LIMK PCR Program		
Number of Cycles	**Temperature**	**Time**
1 cycle	95°C	5 minutes
30 cycles	95°C	1 minute
	53°C	1 minute
	72°C	1 minute
1 cycle	72°C	5 minutes
unlimited	4°C	Until quit program

Table 2: LIMK PCR program

Trans Blotting

A 10% separating gel was prepared for the 8 cell stage, stage 6, 8, 10 , and 10.5 using distilled water, 1.5 M TRIS HCL at pH 8.8, Acrylamide/Bis (30%), 10% SDS stock, 10% ammonium persulfate, and TEMED. The 4% stacking gel was prepared using Distilled water, 0.5 M Tris HCl pH 6.8, Acrylamide/Bis (30%), 10% SDS stock, 10% Ammonium persulfate, and TEMED. Embryo equivalent amounts of protein were loaded on the gel, and the gel was run at 200 volts for forty-five minutes. The gels were then equilibrated in transfer buffer for thirty minutes. The

Hybond membrane and saturated filter paper and fiber pads were saturated with transfer buffer. A sandwich was assembled consisting of the gel and Hybond membrane between two filter papers and two fiber pads was assembled and run at 197 volts for sixty minutes.

Immunoblotting

The non-specific binding sites were blocked by immersing the membrane in 5% (w/v) blocking agent (Amersham) in PBS-T (1X PBS, 0.1% Tween 20) for one hour. The membrane was then washed with PBS-T for fifteen minutes, followed by two further washes in PBS-T for five minutes each. The primary antibody used recognizes active (phosphorylated) LIMK1 and LIMK2. The primary antibody was diluted to 1:100 in PBS-T. The membrane was incubated at room temperature in the dilute primary antibody for one hour on an orbital shaker at sixty RPM. The membrane was washed three times with PBS-T as before, and a secondary antibody (anti-mouse IgG conjugated to alkaline phosphatase) was diluted 1:2000 in PBS-T. The membrane was incubated at room temperature in the diluted secondary antibody for one hour on an orbital shaker. The membrane was then washed three times as before with PBS-T, followed by incubation in Enhanced chemifluorescent (ECF; Amersham) solution (an alkaline phosphatase substrate) for approximately twenty minutes. The ECF substrate was drained off from the membrane and the blot was placed on an absorbent surface for twenty minutes to dry. The membrane was scanned on a Fuji FLA-3000 fluorimeter using a 570 nm filter.

Results

The Western Blot analysis (Figure 1) indicated a phosphorylated LIMK1/2 protein band at the 8-cell stage, stage 6.5 (64 cells), 8 (early blastula), 10 (early gastrula), and 10.5 (mid-gastrula). A previous northern blot (Winning and Sargent 1994; Figure 2) indicated RNA bands for EphA4 at stages 10.5, 11, 12, 15, 20 and 23.

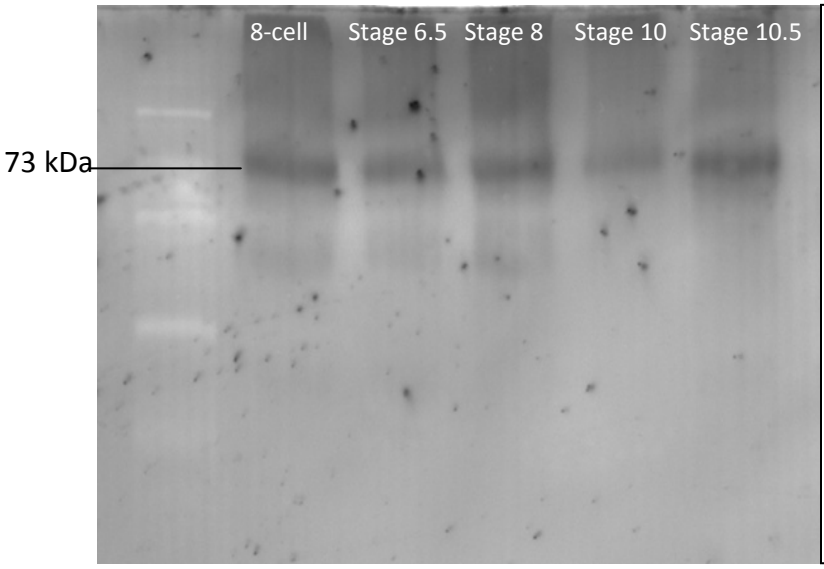

Figure 1 Western Blot Analysis. RNA isolated from the 8 cell stage, stage 8, stage 10, and stage 10.5 of embryos were used. Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and incubated in the p-LIMK-1/2 antibody. Sample proteins were fluoresced using ECF substrate and visualized using a 570 nm filter.

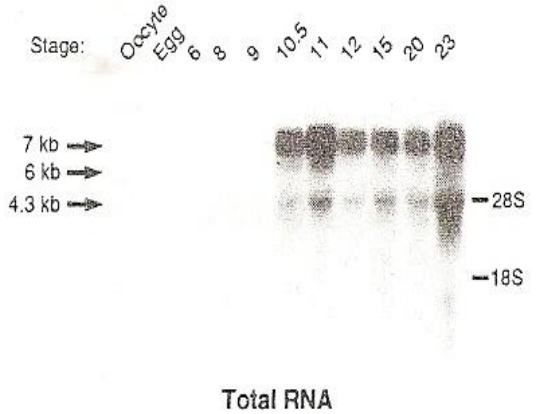

Figure 2. EphA4 northern blot of total RNA from oocyte, unfertilized egg and various embryonic stages, probed with Pag cDNA (Winning and Sargent 1994).

The expression pattern for LIMK1/2 was further analyzed using RT-PCR (Figure 3). LIMK1 and LIMK2 mRNAs were detected during stages 9, 11, and 20. Using the specific primers LIMK1 and LIMK2 produced the expected band sizes for each stage. Extra bands were also consistently present for LIMK1 and LIMK2 at stages 9, 11, and 20 at annealing temperatures 50°C, 52 °C, and 53 °C.

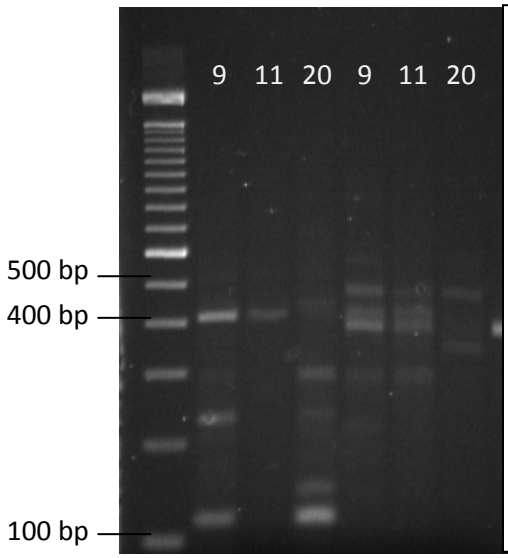

Figure 3. RT-PCR Analysis. Random primed cDNA from stages 9, 11, and 20 were used as templates. PCR conditions were as follows: denaturation at 95°C for 60 seconds, followed by annealing at 53° C for 60 seconds, and extension at 72°C for 60 seconds (for 30 cycles).

Discussion

The Western Blot indicated that LIMK is phosphorylated and therefore active at the 8-cell stage, stage 6.5, 8, 10, and 10.5 (Figure 1). Although our results indicate that LIMK is present, we cannot differentiate between LIMK1 and LIMK2 on the western blot because the antibody, p-LIMK-1/2 recognizes both LIMK1 and LIMK2, and the proteins co-migrate. Further analysis of LIMK is necessary to determine which kinase is present at each stage in development. EphA4 expression begins at stage 10.5 (Figure 2), when LIMK1/2 is also active.

These data are consistent with our hypothesis that in order for LIMK1/2 to be involved in the EphA4 pathway, it must be expressed in development when EphA4 is expressed. This is true of the proteins at stage 10.5; furthermore, LIMK1 and LIMK2 RNAs appear to be present at least up to stage 20 (Figure 3). The RT-PCR analysis indicated expected bands around 411 and 473 for LIMK1 and LIMK2, respectively. Extra bands were present, however, for LIMK1 and LIMK2 at stages 9, 11 and 20. The extra bands are possibly the result of the primers binding to unknown sequences in the cDNA mixture. More analyses are necessary to determine the cause of the bands.

Future Directions

Two experiments are necessary in order to determine whether LIMK1 or LIMK2 is inactivated in the EphA4 signaling pathway. The first would be to express a constitutively-active LIMK1 or LIMK2 at the same time that EphA4 is active to determine whether or not LIMK activity can rescue the embryos from the effects of EphA4. In this experiment, the introduced LIMK activity would override the inactivation of the endogenous LIMK, preventing the effects of EhA4 signaling. The second experiment would be to create a mutated inactive LIMK1 and LIMK2 (separately) to determine whether inactivating LIMK can produce the EphA4 phenotype. Introducing inactive LIMK would reproduce the effects of EphA4 signaling, producing the effects of signaling without Epha4 activity.

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