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Does Rac GTPase Play a Role in EphA4 Signaling in Xenopus Embroys?

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DOES RAC GTPASE PLAY A ROLE IN EPHA4 SIGNALING IN *XENOPUS* EMBRYOS?

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ABSTRACT

In embryonic development, many cells migrate in order to correctly form new structures. One way that guidance of this migration occurs is via a repulsion mechanism involving the Eph family of receptors. Signaling through these receptors activates the repulsive mechanism that limits which tissues can interact with each other. The repulsion mechanism is not well understood, but involves the reorganization of the actin cytoskeleton and a loss of cell-cell adhesion. Activation of the EphA4 signaling pathway in Xenopus laevis embryos has been shown to cause the repulsion mechanism. The Rho family of GTPases consisting of Cdc42, Rho, and Rac, has been linked to the reorganization of the actin cytoskeleton and Rho has been demonstrated to be part of the EphA4 pathway. The hypothesis to be tested in this study is that activation of Rac is also part of the EphA4 pathway. To test this hypothesis, mutant Rac RNA coding for constitutively active Rac (caRac) was injected into Xenopus laevis embryos to determine if Rac activation can mimic EphA4 signaling. As the concentration of RNA coding for caRac increased, the degree of cellular dissociation also increased. While further experimentation must be done to make a conclusive determination, it is possible that the activation of Rac is a part of EphA4 signaling.

INTRODUCTION

In the development of vertebrates, tissue formation involves careful regulation of cell interactions. Some embryonic cells must

migrate in order to correctly form new structures. Guidance of this migration occurs via a repulsion mechanism that limits which tissues can interact with each other. One way this is done is through Eph receptors and ephrins.

The Eph family of receptors (classified as either EphA or EphB) is composed of three domains: an extracellular ligand-binding domain, a transmembrane domain, and an intracellular kinase domain (Zhou, 1998). An Eph receptor can bind to a cell-surface ligand, called ephrin, stimulating kinase activity. Eph signaling is thought to control several important processes during embryogenesis including tissue segmentation, guiding axon growth, cell migration, vasculogenesis, and potentially limb development (Holder and Klein, 1999).

Activation of one member of the Eph family, EphA4 signaling pathway, has been shown to cause a cellular dissociation and a loss of cell-cell adhesion in Xenopus laevis embryos, called the "Eph" phenotype (Winning et. al., 2001). An observed loss of adhesion was shown to be consistent with a disruption of cortical actin, which means that the reorganization of the actin cytoskeleton is an effect of Eph signaling (Bisson et. al., 2007).

The Rho family of GTPases, consisting of Cdc42, Rho, and Rac, has been linked to the
reorganization of the actin cytoskeleton (Winning et. al., 2002). GTPases are GTP-binding proteins that act like molecular switches that are either active (GTP-bound) or inactive (GDP-bound) and regulate enzyme function (Hens et. al., 2002). Rho inhibition has been shown to be a part of the activated EphA4 signaling pathway. Injecting embryos with Rho inhibitors, C3 Transferase and Toxin A, results in the "Eph" phenotype at the blastula stage (Winning et. al., 2002).

Loss of cell adhesion and cellular dissociation can be evaluated easily during the blastula stage of embryonic development. During this stage a cavity essential to development, called a blastocoel, forms in the animal pole of the embryo. If dissociation and loss of cell adhesion is occurring, the blastocoel may be abnormal or even nonexistent. In embryonic development, high activity of Rho has been shown to coincide with low activity of Rac, and vice versa (Noren and Pasquale, 2004). This has led to the hypothesis that the activation of Rac plays a role in EphA4 signaling pathway and will cause the Eph phenotype.

METHODS

IN VITRO TRANSCRIPTION OF RAC

Constructs for myc-tagged constitutively active Rac1 were kindly provided by Dr. Tim Gomez, University of Wisconsin-Madison. It was prepared by subcloning caRac into the EcoR1 site of the pCS2+ expression vector. The plasmids were linearized by XhoI digestion and the linear DNA was purified using a GENECLEAN® kit. RNA was transcribed in vitro using an Ambion mMessage mMachine SP6 transcription kit. RNA was purified by Lithium Chloride precipitation. The quantity and integrity of purified RNAs was measured by UV spectroscopy and gel electrophoresis.

EMBRYO MANIPULATION AND MICROINJECTION

Adult Xenopus laevis were purchased from Nasco (Fort Atkinson, WI). Female frogs were injected with human chorionic gonadotropin hormone 12-15 hours prior to fertilization to induce ovulation. The females were squeezed to release eggs that were then kept in a 0.3 X MMR solution (1 X MMR = 100mM NaCl, 2.0mM KCl, 2.0mM CaCl2, 1.0mM MgCl2, 5.0mM HEPES). The male was anesthetized with a 5% Tricaine solution and the testes were removed and stored in 1 X MMR on ice. Testes were macerated in solution to release sperm, and then pipetted evenly over the eggs. The dish containing sperm and eggs was placed on an orbital shaker for 20 minutes to allow for fertilization. Embryos were de-jellied in a 2% Cysteine solution, pH 7.8-8.1. Embryos were rinsed and placed in 0.1 X MMR and kept cool (18°C) to prevent rapid mitosis. Fertilized embryos were place 5% Ficoll in 1 X MMR and injected at the one-cell stage using a Drummond NanoinjectII microinjector. The RNAs injected consisted of 100 pg, 250 pg, and 500 pg of RNA encoding constitutively active Rac (caRac); 2.5 ng, 5.0 ng, and 7.5 ng of RNA encoding dominant-negative Rac (dnRac). The microinjector released 9.2 µl of solution per injection. After injection, embryos were keep at 24°C for further development. At approximately halfway from stage 1 to 6, embryos that were developing properly were transferred to a solution containing 1 X MMR without Ficoll. Embryos were allowed to develop to the blastula stage before being transferred to microtubes and fixed in 10% Glutaraldehyde in 1 X PBS overnight at 4°C.

LIGHT AND SCANNING ELECTRON MICROSCOPY

In preparation for bisection, embryos were washed 5 times for 10 minutes each with 1 X PBS. Specimens were cut into halves with a scalpel blade and examined under a dissecting scope. The embryos were graded as intact, abnormal, or nonexistent based on the morphology of the blastocoel.

After bisection and evaluation, the embryos were again fixed with 10% Glutaraldehyde in 1 X PBS overnight at 4°C. In preparation for viewing under a scanning electron microscope, embryos were washed twice in 1 X PBS for 10 minutes each and postfixed in 1% osmium tetroxide for 30 minutes. Embryos were taken through a series of washes in the following order: 2 times 5 minutes in distilled water, 10 minutes in 50% Ethanol, 10 minutes in 70% Ethanol, 10 minutes in 95% Ethanol, and 2 times 5 minutes in HMDS. After the final wash, the remaining HMDS was removed and the specimens were left overnight to allow for evaporation and further drying. Specimens were then placed under a vacuum for 2 hours before being carefully mounted using hair tweezers onto double-stick carbon-permeated tape attached to polished stubs. Specimens were further stabilized with colloidal graphite. Specimens were sputter coated with gold and viewed on an AMRay 1820I scanning electron microscope.

RESULTS

The embryos injected with mutant RNA coding for caRac exhibited external lesions on the pigmented animal pole. The broken pigmentation and lesions increased in severity as the concentration of injected caRac increased from 100 pg to 500 pg (Fig. 1).

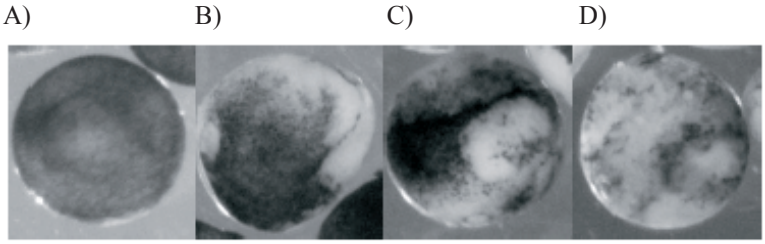
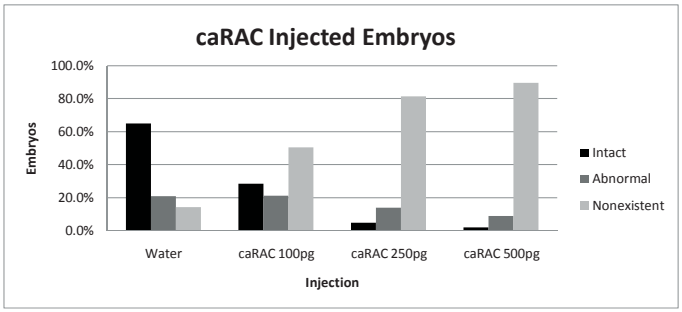


Figure 1. An external view of the animal pole using light microscopy. A. Control sample: embryo injected with water. B. Embryo injected with 100 pg caRac, exhibiting minor lesions. C. Embryo injected with 250 pg caRac. D. Embryo injected with 500pg caRac, exhibiting severe lesions and broken pigmentation.

Does Rac GTPase Play a Role in EphA4 Signaling in Xenopus *Embryos?*

A)



B)

	Quality of Blastocoel		
Sample	Intact	Abnormal	Nonexistent
Water	64.8% (59)	20.9% (19)	14.3% (13)
100pg caRAC	28.4% (23)	21.0% (17)	50.6% (41)
250pg caRAC	4.6% (3)	13.8% (9)	81.5% (53)
500pg caRAC	1.8% (1)	8.8% (5)	89.5% (51)

C)

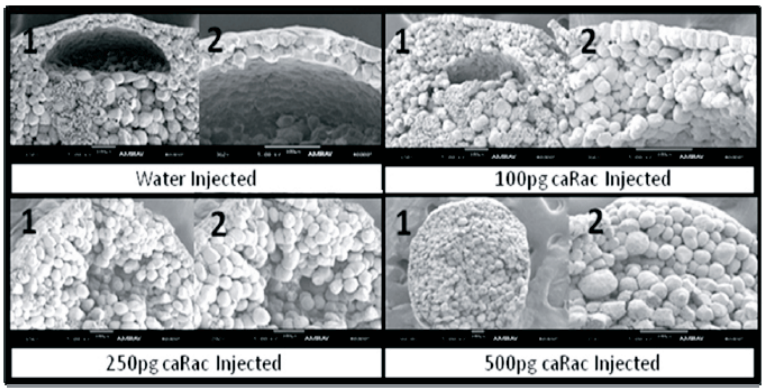


Figure 2. Various representations of data gathered from the internal morphology of injected embryos. A. Graph displays the percentage of embryos vs. the graded quality of the embryos for each injection sample. B. Table presents the percentage and the quantity (in parenthesis) of embryos from each injection sample. C. Embryos prepared for scanning electron microscopy. 1) Image of the bisected internal animal pole. 2) Image of cells making up the roof of the blastocoel. Much appreciated assistance with scanning electron microscopy provided by Dr. Glenn Walker and Rae Labadie.

The control sample of water-injected embryos had no disruption of external pigmentation and had intact blastocoels (Fig. 1A and 2). The caRac injected embryos showed that as the concentration of RNA coding for caRac increased from 100 pg, to 250 pg, to 500 pg, the number of embryos with an intact blastocoel decreased, while the number of embryos with an abnormal or nonexistent blastocoel increased (Fig. 2A and 2B). The roof of the blastocoel was assessed to determine the extent of cellular adhesion and dissociation (Fig. 2C2). The roof of the blastocoel of water injected embryos was composed of angular cells that fit tightly together. In contrast, the roof of the blastocoel in caRac injected embryos exhibited rounded cells (Fig. 2C).

DISCUSSION

It is possible that Rac activation is part of the EphA4 signaling pathway. Increasing concentrations of constitutively active Rac caused an increasing loss of cell adhesion. This was indicated by the lesions and broken pigmentation apparent on the external animal pole of the embryos. Loss of cell adhesion is associated with the reorganization of the actin cytoskeleton exhibited by the activation of the EphA4 signaling pathway. However, further experimentation is required to make a conclusive determination. A trial should be performed to examine the effects of injecting embryos with RNA coding for dominant-negative Rac. Furthermore, a rescue experiment should be conducted to determine if inactive Rac can rescue embryos from effects of active EphA4 signaling, the "Eph" phenotype. Lastly, if Rac is determined to play a role in EphA4 signaling, co-injections with RNAs encoding mutant versions of other proteins involved in the EphA4 pathway, such as ROCK and Rho, can be done to establish the order of signaling within the pathway.

A study by Hens et al. in 2002, agreed that Rac signaling is important for cell adhesion. They suggested that both inactive and active Rac in Xenopus embryos caused some degree of cellular dissociation. Inactive Rac injected cells were far less adherent to one another than active Rac injected cells. The similar results obtained from the expression of both the Rac mutants were explained as a possible Rac activity gradient that required for proper cellular effects to occur.

The study of Eph receptors has seen a number of advances when it comes to understanding the role of GTPases in guiding cell migration and regulating cell shape. However, much is still needed to fully comprehend the details of Eph/ephrin signaling pathways and morphogenesis.

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