THE EFFECT OF RHEB EXPRESSION IN THORACIC MUSCLE DURINGTHE MOLT CYCLE IN GECARCINUS LATERALIS

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ABSTRACT

This study aimed to detecte the role of Rheb in the molting cycle of Gecarcinus lateralis. Skeletal muscle atrophy in crustaceans is necessary for the progression of molting. The atrophy of thoracic muscles is coordinated by the action of ecdysteroid hormones. Thoracic muscle atrophy can occur during the intermolt period when hemolymph ecdysteroid levels are low. The insulin/mechanistictarget of rapamycin (mTOR) pathway, previously shown to be important for growth, development, reproduction, and metamorphosis in insects, is a likely target of ecdysteroid regulation. As complete cDNAs for ras homolog enriched in brain (Rheb) the key activator of mTOR, have been cloned from Gecarcinus lateralis. Levels of Rheb increase dramatically during molting induced by either Eyestalk Ablation (ESA) or Multiple Limb Autotomy (MLA) and showed a correlation with levels of ecdysteroid in the thoracic muscle, in both molting paradigms. Our findiings indicate that Gl-Rheb is an important activator of crustacean mTOR signaling in the molt cycle in response to ecdysteroids.

Keywords: Rheb, Gene Expression, Crustacean, Ecdysteroid, Molting, mRNA, Thoracic Muscle, Eyestalk Ablation.

1. INTRODUCTION

Molting in decapod crustaceans is controlled by molt-inhibiting hormone (MIH), an eyestalk neuropeptide that suppresses production of ecdysteroids by a pair of molting glands (Y-organs). Eyestalk-ablation (ESA) activates the YOs, which hypertrophy and increase ecdysteroid secretion. In

decapods, there is a regular pattern of atrophy and hypertrophy in skeletal muscles [1], [2].

Given the necessity, among organisms with a rigid exoskeleton, of molting in order to grow larger, withdrawal of muscles from the old exoskeleton is a necessary precursor. In particular, the basi-ischial joint at the base of the chelipeds is a constriction point for withdrawal of the large closer muscle of the propodus and so atrophy of claw muscles is a requirement for successful molting. Afterwards, expansion of the new exoskeleton and subsequent hypertrophy of the skeletal muscles completes the growth process for that molt cycle.The induction of atrophy is dependent on increased ecdysteroid levels in circulating hemolymph [1], [2].

Another fully reversible atrophy is also seen in decapod crustacean muscles. A ‘disuse’ atrophy is seen in thoracic muscles [3], [4]. Unlike the reversible claw muscle atrophy is not dependent on ecdysteroid [5] and results in great disruption of the muscle fibers, sarcomeres, and myofilaments [6], [7], [8].

One of the potential effectors of the ecdysteroid changes seen in molting is the mTOR signaling pathway. mTOR complex 1 (mTORC1) is now recognized as the pathway involved in inhibition of protein synthesis in mammalian skeletal muscle [9], [10]. TORC1 is a part of the insulin/insulin-like growth factor (IGF) pathway that is important for controlling growth in eukaryotic cells and is a serine/theonine protein kinase that regulates protein synthesis at the translational level by phosphorylating the S6 kinase (s6k) and 4E-binding protein 1 (4E-BP1) [11]. The mTOR pathway has been studied before in insects, and was shown to be important for nutrient-dependent growth [12].

The insulin/mTOR pathway is highly conserved among all metazoans and is a critical nutrient sensor for growth [11]. Of particular interest given the results of this study is the role of the protein Rheb in the insulin/mTOR signaling pathway. The Rheb family of GTP-binding proteins is a unique group within the larger Ras super family of G-proteins [13]. All Rheb proteins have guanine nucleotide binding functions, located within a series of "G boxes" and have intrinsic GTPase activity in all species examined, including *D. melanogaster*[14]. Rheb's primary function is as the critical upstream activator of mTOR, likely through its interaction with FKBP38 [15], [16].

The purpose of this study was to determine the effects of molt induction and on the expression of Rheb an activator of mTOR signaling in *G. lateralis* thoracic muscles.Quantitative polymerase chain reaction (qPCR) was used to quantify the expression of Gl-Rheb in weighted thoracic. Molting was induced by eyestalk ablation (ESA) and multiple leg autotomy (MLA). cDNA was used to compare the expression of Rheb with hemolymph ecdysteroid level.

2. MATERIALS AND METHODS

2.1 Animals And Experimental Treatments

Adult land crabs (*Gecarcinus lateralis*) were collected in the Dominican Republic and shipped to Colorado State University, USA. Animals were maintained at 27 ºC in 75-90% relative humidity with intermolt individuals kept in communal plastic cages lined with aspen bedding wetted with 5 p.p.t. Instant Ocean (Aquarium Systems, Mentor, OH). The crab environmental chamber was maintained in 12 h:12 h light:dark cycle with twice-weekly animal feedings of carrots, iceberg lettuce, and raisins. Crabs

that have autotomized 5-8 walking limbs (MLA)were kept in the same environmental conditions in quart size (~1 l) plastic cages in playground sand wetted with 10 p.p.t. Instant Ocean. MLA crab sand cages were shielded from room illumination behind a cloth curtain, mimicking the constant darkness conditions previously shown to shorten the premolt period [17].

Precocious molting is induced by two main methods: MLA and ESA Progression of animals through the premolt period for both methods were monitored by measurement of the length of limb regenerates (every animal was missing the third walking leg on one side), called the regeneration or R index (=length of regenerate × 100 / carapace width). The R index value varies between 0 and ~24 [21], [22]. For MLA experiments, all animals had autotomized 8 walking legs. Weighted thoracic muscles were isolated from the body segment associated with intact chelipeds and unweighted thoracic muscles were isolated from body segments associated with missing chelipeds, ESA experiments were done as previously [18], [19].

2.2 RNA purification and cDNA synthesis

Total RNA was isolated from land crab tissues using TRIzol reagent (Life Technologies, Carlsbad, CA) as described previously [18]. Briefly, tissues (50-200 mg) were homogenized in 1-2 ml TRIzol and centrifuged at 12,000 × g for 15 min at 4 ºC. Supernatants were phenol-chloroform extracted and RNA in the aqueous phase was precipitated using isopropanol (0.75 ml per 1 ml TRIzol reagent). RNA was treated with DNase I (Life Technologies), extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with isopropanol, washed twice with 75% ethanol in DEPC water, and resuspended in nuclease-free water. First-strand cDNA was

synthesized using 1 µg total RNA in a 20 µl total reaction with SuperScript III reverse transcriptase (Life Technologies) and oligo-dT(20)VN primer (50 µmol/l; IDT, Coralville, IA) as described [18]. RNA was treated with RNase H (Fisher Scientific, Pittsburgh, PA) and stored at -80 ºC.

A LightCycler 480 thermal cycler (Roche Applied Science, Indianapolis, IN) was used to quantify levels of Gl-Rheb. Reactions consisted of 1 µl first strand cDNA or standard, 5 µl 2× SYBR Green I Master mix (Roche Applied Science), 0.5 µl each of 10 mM forward and reverse primers [20], and 3 µl nuclease-free water. PCR conditions were as follows: an initial denaturation at 95 ºC for 5 min, followed by 45 cycles of denaturation at 95 ºC for 10 s, annealing at 62 ºC for 20 s, and extensions at 72 ºC for 20 s, followed by melting curve analysis of the PCR product. Transcript concentrations were determined by use of the LightCycler 480 software (Roche, version 1.5) using a series of dsDNA gene standards produced by serial dilutions of PCR product for each gene (10 ag/µl to 10 ng/µl). The absolute amounts of transcript in copy numbers per µg of total RNA in the cDNA synthesis reaction were calculated based on the standard curve and the calculated molecular weight of dsDNA products.

2.3 Statistical Analyses And Software

Statistical analysis was performed using JMP 5.1.2, 6.0.0, or 8.0.2 (SAS Institute, Cary, NC). Group variances were analyzed using a Brown-Forsythe test and found to be equal ($P < 0.05$). Means for different developmental stages were compared using analysis of variance (ANOVA). All data not plotted as individual points are represented as mean ± 1 S.E. and the level of significance was set at $\alpha = 0.05$.

3. RESULTS

3.1 Effects Of ESA/MLA On Hemolymph Ecdysteroid Levels In The Land Crab

The Rheb gene (ras homolog enriched in brain), an activator of mTORwere obtained from RT-PCR and RACE [20]. cDNAs containing the complete ORF of Gl-Rheb.qPCR was used to analyze levels of land crab *Gl-Rheb* in cDNA from thoracic muscle (TM), as animals progressed through the molt cycle, and compared with the corresponding hemolymph ecdysteroid hormone levels. Induction of molting used one of two classical methods: MLA and ESA. Titers of hemolymph ecdysteroid levels increased in response to both methods of molt induction (Fig. 1A and Fig. 2A). As noted, ESA induced a rapid increase that was then followed by a slower, but prolonged increase than the intact levels (Fig. 1A). MLA-induced changes in hemolymph ecdysteroid levels were slower than the changes seen in ESA, and only increase was observed in mid-premolt (R=15) in those animals. However, late in premolt (R=22) there was an even greater peak in ecdysteroids than the intact. Although postmolt hemolymph levels are not possible to measure in ESA animals due to mortality, the levels observed in MLA animals in the 10 days immediately following molt were very low (Fig. 2A).

Animals were ES-ablated at Day 0, hemolymph and thoracic muscle tissues were collected from intact (Day 0) and at 1 day and at 3days and at 7 days and at 14 days and 20 days post-ESA. The Gl-Rheb mRNA levels were quantified by quantitative reverse transcriptase-polymerase chain reaction (qPCR). Means of ESA animals that were significantly different shared same letters indicate significant difference between molt stages. All data are

presented as means ± s.e.m. Sample sizes varied with molt stage (Intact Day 0, n = 12; Days 1, 3, and 7, n = 10; Day 14, n = 8; Day 20, n = 9).

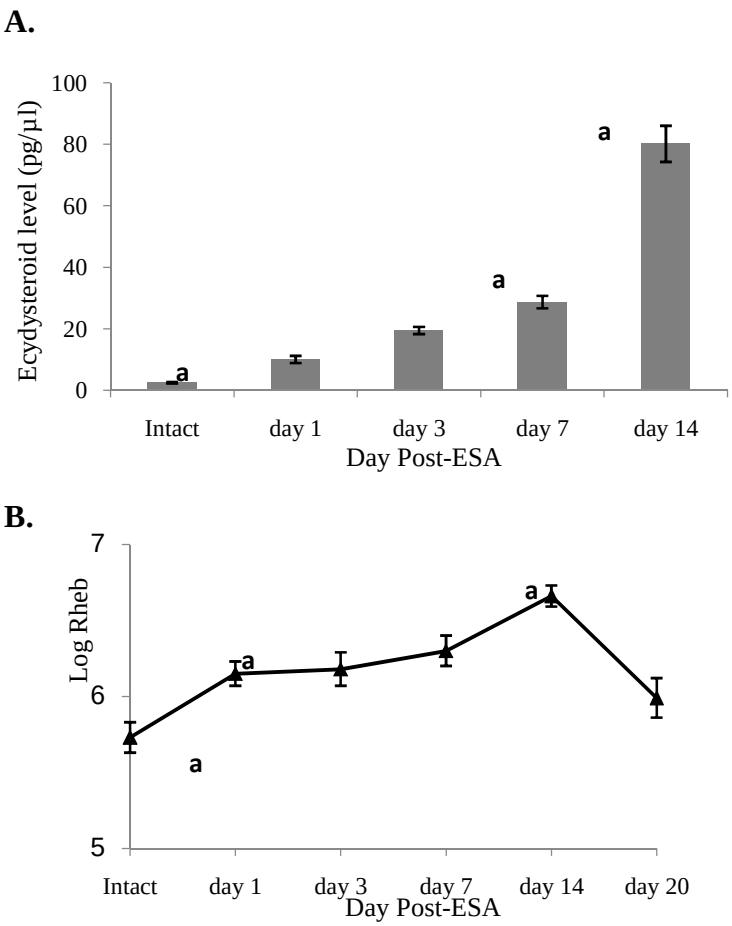


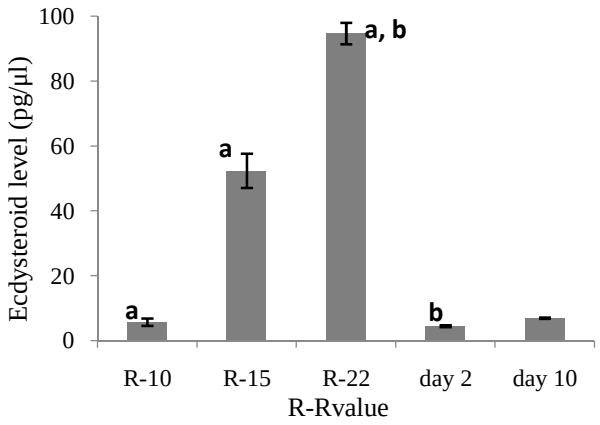
Fig.1Standard error bar demonstrating the effects of eyestalk ablation (ESA) on hemolymph ecdysteroid titer (A) and thoracic muscle expression of Gl-Rheb(B) in *G. lateralis*.

3.2 Effects Of ESA On Gene Expression In Thoracic Muscle Tissues

Only small, transient effects were observed on the expression of our "housekeeping" gene, Gl-EF2in thoracic muscle after ESA [18]. ESA had a strong effect on expression of Gl-Rheb, the important regulator of mTOR activity, in thoracic muscle (Fig. 1B). Thoracic muscle shows significant

increases in Gl-Rheb expression by 1 day post-ESA, however, much lower levels of *Gl*-Rheb in thoracic muscle in intact animals contribute to an immediate significant induction in Gl-Rheb expression. This induction in thoracic muscle is slow but sustained, reaching a new significant peak above the intact level at 14 days post-ESA.Thoracic muscle, despite the larger increase in expression, significantly declines by 20 days post-ESA from its 14 day peak (Fig. 1B).

A.



B.

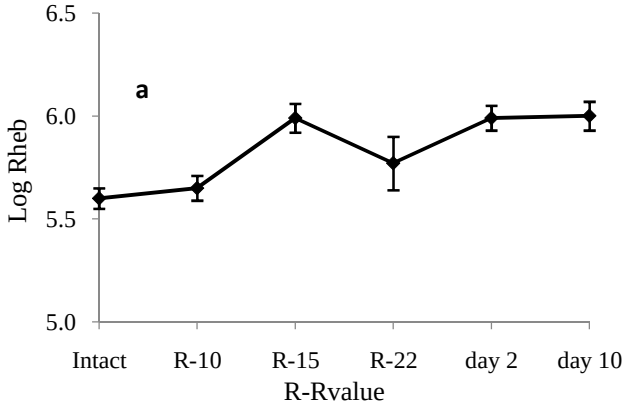


Figure 2. Standard error bar showing the effects of multiple leg autotomy (MLA) on hemolyph ecdysteroid titers (A) and thoracic muscle expression of Gl-Rheb (B) in G. lateralis.

Hemolymph ecdysteroid levels were quantified by ELISA. Gl-RhebmRNA levels at early premolt (10-R), mid premolt (15-R), late premolt (22-R), 2 days postmolt, and 10 days postmolt were quantified by quantitative reverse transcriptase-polymerase chain reaction (qPCR). All data are presented as mean ± 1 S.E. Sample sizes varied with molt stage (Intact, N=15; R-10, N=10; R-15, N=11; R-22, N=10; 2 and 10 days post-molt, N=13). Means that are significantly different from each other shared same letter

3.3 Effects Of MLA On Gene Expression In Thoracic Muscle Tissues

Animals in the multiple limb autotomy experiment showed changes over the molt cycle for the Gl-Rheb (Fig. 2B). For the MLA experiment intact intermolt animals were compared with animals that had begun progressing through premolt as measured by their R-index value. Since MLA is a natural process in land crabs, animals survive to undergo a normal ecdysis, and it is therefore possible to monitor expression after defined time periods after ecdysis (time postmolt).

Expression of Gl-Rheb in land crabs was greatly affected in thoracic muscle over the molt cycle of the MLA experiment (Fig. 2B).Thoracic muscle showed increased expression of Gl-Rhebbut the progression was slower increases beginning only at ~R=10 and rising gradually from there. The thoracic muscle premolt showed significant increases peakedat R=15 over the intact level of Gl-Rheb. Thoracic muscle expression of Gl-Rheb in postmolt continued to rise slowly, peaking over the intact levels at 10 days postmolt(Fig. 2B).

4. DISCUSSION

The highly conserved insulin/IGF/mTOR signaling pathway is found in all metazoans and has an important role as a nutrient sensor critical for growth and development [11].

It was clear thta the components of this pathway would be regulated during the molt cycle of crustaceans; Rheb was cloned from three decapod crustacean species [20]. The important domains found in other Rheb proteins “G boxes” necessary for GTP-binding and GTPase activity [23], [24].

In this studyESA method was used to examine the Rheb transcriptional response of animals in the intermolt phase of the molt cycle to acute activation of the molting gland (YO). As previously reported, it was observed that ESA resulted in large increases in hemolymph ecdysteroid concentrations (Fig. 1A). Induction of molting by the more natural process of MLA method showed a more gradual increase in hemolymph ecdysteroids (Fig. 2A), than that seen for ESA (above) consistent with a more gradual entrance into premolt [5].In this experiment, the effect on thoracic muscle expression of Gl-Rhebwas slightly different, however, the premolt peak expression was only 1.7-fold over intact, at R=15, with the true peak (2-fold over intact) seen 10 days postmolt.

Since Rheb is a GTPase and active as an effector of mTOR in its GTP-bound state [25], [26], [27], we would expect cellular processes to turn greater transcription of Rheb into a greater pool of active Rheb, capable of activating mTOR. This is what was seen with exogenous expression of Rheb via transfection of human cells greater expression of Rheb even in the presence of inhibitors greatly increased all read-outs of mTOR activity [15], [16]. Because of this, we believe that Gl-Rheb transcriptional changes in the

molt process are indications of up-regulation of mTOR (kinase) activity. This observed up-regulation of mTOR activity and its well understood downstream effectors of p70S6kinase and 4E-BP1, important for increasing translation of mRNA into protein [11], indicates that we have identified at least part of the mechanism by which this overall increase in protein synthesis occurs [18].

Correlations with Gl-Mstn transcript levels are complex, however. In the previous study [18], in both molt induction methods, thoracic muscle Gl-Rheb is significantly and positively associated with transcript levels of Gl-Mstn. Given that no atrophy is necessary in thoracic muscles for successful molting [8], [28], this divergent regulation of Gl-Rheb is perhaps not surprising.

We hypothesize that in thoracic muscles, growth to happen in the postmolt period is being primed during premolt by increases in Gl-Rheb expression.Given the increases in Rheb for a period of weeks, it may be expected that there would be increases in global protein synthesis. However, the changes in Gl-Mstn and Gl-Rheb in this ‘disuse’ are coordinated rather than antagonistic and so the concurrent changes in protein synthesis cannot be arrived at with certainty without further study of the changes in protein synthesis.

5. CONCLUSION

Based on the results of this study, Gl-Rheb is regulated in different manners in atrophy processes in thoracicmuscle. The increase noted in Gl-Rheb transcript levels in premolt thoracic muscle is correlated with elevated hemolymph ecdysteroid titers. This supports our hypothesis that ecdysteroids are causing an increase in mTOR signaling components, and

particularly the mTOR activator Rheb, during premolt, probably through interaction with nuclear receptors and increased transcription. Some of this regulation may be mediated by Gl-Mstn, indicating that the acute response to ecdysteroid increase in ESA may involve Mstn/Smad-dependent increased transcription. Because of this, we may hypothesize that the cellular machinery of mTOR component regulation of growth/muscle remodeling in the context of thoracic muscle atrophy is by the direct action of ecdysteroid on the process. Further experiments to dissect the causation of this pathway, however, arenecessary.

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