Calmodulin During Early Zebrafish Development

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Abstract

Calmodulin (CaM) mediates a diverse array of cellular responses via interactions with target proteins in a Ca^{2+}-dependent manner. CaM levels in the zebrafish, *Danio rerio*, embryo following fertilization are high (250 ng/embryo), but wane as the embryo approaches 90% epiboly (75 ng/embryo), and subsequently remain constant. This event is conversely paralleled by a 5-fold increase in calmodulin 2 mRNA starting at around four hours, or the mid-blastula transition (MBT), when embryonic transcription in zebrafish begins. In an effort to understand why high quantities of CaM, of possible maternal origin, are required during these early stages of development, we are interested in using CaM-biotin and his-CaM in an overlay assay to identify downstream effectors. CaM-biotin and his-CaM may then be immobilized on columns for use in affinity chromatography. Candidates eluted from these columns may then be subjected to MSMS for sequencing. Preliminary data have suggested that calmodulin binds the egg storage proteins lipovitellin 1 (Lv1) and lipovitellin 2 (Lv2) in the absence of free calcium. These data contradict calmodulin-Lv interactions shown in *Xenopus* to be dependent on the presence of free Ca^{2+}.

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D.rerio          MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
H.sapien         MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
M.musculus       MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADG 60
D.melanogaster   MADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDVDG 60
S.cerevisiae     MSSNLTEEQIAEFKEAFALFDKDNNGSISSSELATVMRSLGLSPSEAEVNDLMNEIDVDG 60
                 *:.:************:*****.:*:*::.**.******* .*:***::*::**:*.**

D.rerio          NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE 120
H.sapien         NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE 120
M.musculus       NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGYISAAELRHVMTNLGEKLTDE 120
D.melanogaster   NGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNGFISAAELRHVMTNLGEKLTDE 120
S.cerevisiae     NHQIEFSEFLALMSRQLKSNDSEQELLEAFKVFDKNGDGLISAAELKHVLTSIGEKLTDA 120
                 *  *:*.***::*:*::*..***:*: ***:****:*:* ******:**:*.:******

D.rerio          EVDEMIREADIDGDGQVNYEEFVQMMTAK 149
H.sapien         EVDEMIREADIDGDGQVNYEEFVQMMTAK 149
M.musculus       EVDEMIREADIDGDGQVNYEEFVQMMTAK 149
D.melanogaster   EVDEMIREADIDGDGQVNYEEFVTMMTSK 149
S.cerevisiae     EVDDMLREVS-DGSGEINIQQFAALLSK- 147
                 ***:*:**.. **.*::* ::*. :::
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Figure 1
Clustal W multiple sequence alignment was used to compare calmodulin sequences from *Danio rerio*, *Homo sapien*, *Mus musculus*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae.* Sequences were obtained from BLAST (Basic Local Alignment Search Tool). (*) = identicle residues, (:) = highly conserved residues, (.) = somewhat similar. Regions designated in color are EF-hands.

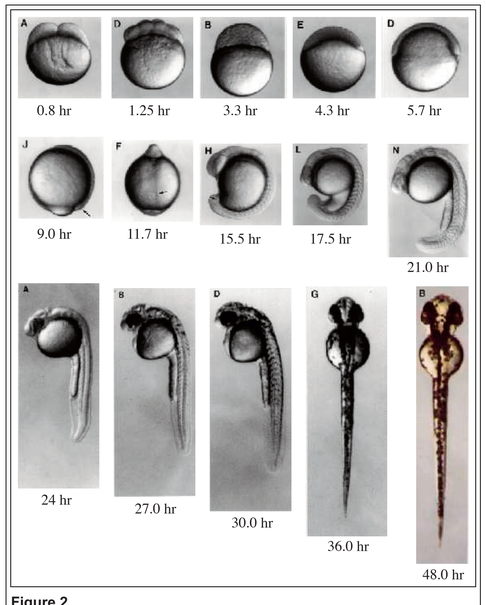

Figure 2
Danio rerio development through 48 hrs. Modified from Kimmel et al., 1955.

Introduction

The small cytosolic protein calmodulin is a highly conserved mediator of Ca^{2+} signaling, and is ubiquitously expressed in all eukaryotic cells. It is remarkably well conserved (fig. 1), and structurally resembles a dumbbell with two globular calcium binding domains separated by an alpha helix (Phe 65 to Phe 92) (Babu et al., 1998). The two Ca^{2+} binding domains each consist of an helix-loop-helix motif called an EF-hand capable of binding two Ca^{2+} ions. When these sites bind free Ca^{2+} ions the Ca2+/calmodulin complex undergoes a conformational change exposing hydrophobic residues responsible for the binding and activation of dozens of enzymes.

CaM action has consequences in a diverse range of cellular phenomenon. For example, metastatic cancer cells have been shown to exhibit a greater than two fold increase in transcription of the gene encoding calmodulin (Clark, 2000). Additionally cell motility and filopodial extensions are enhanced by the uncaging of Ca^{2+} within the cytosol, and blocking the activity of calmodulin negates these effects (Cheng, 2002). CaM also activates the CaM-dependent protein kinase II which has central roles in learning and memory.

Intracellular levels of calcium quickly rise in the oocyte subsequent to fertilization. These calcium ions are released from the endoplasmic reticulum (ER) downstream of a pathway that may be initiated by an intramembrane protein tryrosine kinase. This kinase may activate a signaling cascade wherein IP3 releases stored calcium from the rough ER by opening channels in its membrane. The high levels are initially required to activate proteases which cleave the proteins linking the vitellin envelope and the plasma membrane. Additionally, increased calcium concentrations stimulate protein and DNA synthesis, and activated CaM grants the cell access to the second meiotic division (Gilbert, 2000).

Our data show the levels of CaM in the developing zebrafish embryo are high following fertilization and fade as embryo exits the cleavage (rapid cell division) stages of development. The initially high level of expression may correlate with the newly fertilized egg's effort to buffer the high concentrations of Ca^{2+}. CaM may also be required for rapid cell division division and Ca^{2+} pump activation.

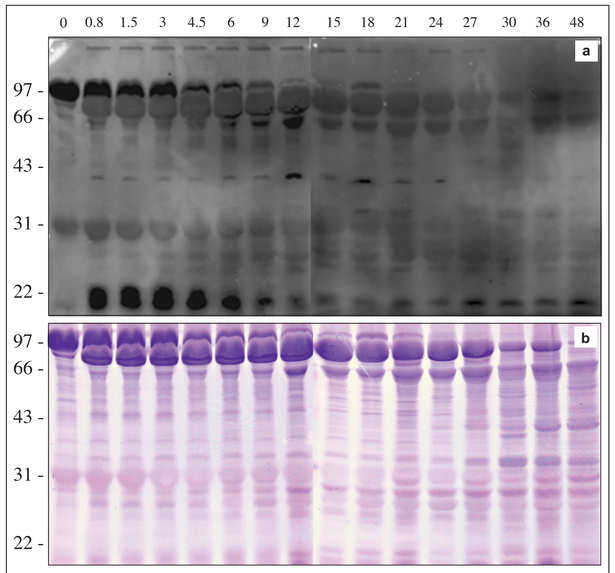

Figure 3
a) Anti-CaM (synthetic bovine immunogen) probe of progressive *D. rerio* developmental stages in the presence of EGTA. b) Coomassie stain of SDS-PAGE gel post-transfer.

15 hr - 13 somite
18 hr - 17 somite
21 hr - 25 somite
24 hr - prim 5
27 hr - prim 12
30 hr - prim 20
36 hr - prim 25
48 hr - long-pec
0 hr - stage 5 oocyte
0.8 hr - 2 cell
1.5 hr - 16 cell
3 hr - 64 cell
4.5 hr - dome
6 hr - late shield
9 hr - 90% epiboly
12 hr - 5 somite

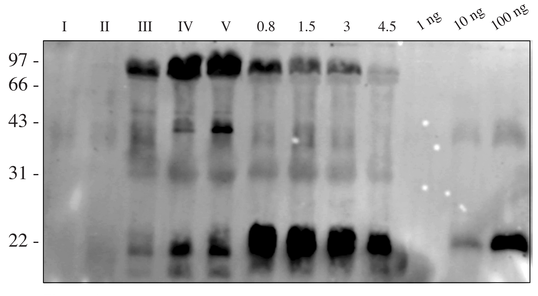

Figure 4
a) Western blot against CaM of *D. rerio* ooctyes and early developmental stages. Lanes 1ng, 10 ng, and 100 ng is are a titration of CaM purified from cod ovary.

I - stage 5 oocyte
II - stage 5 oocyte
III - stage 5 oocyte
IV - stage 5 oocyte
V - stage 5 oocyte
0.8 hr - 2 cell
1.5 hr - 16 cell
3 hr - 64 cell
4.5 hr - dome
1 ng - 1 ng CaM
10 ng - 10 ng CaM
100 ng - 100 ng CaM

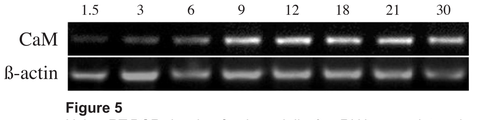

Figure 5
Using RT-PCR, levels of calmoudulin 2 mRNA were determined in sampling periods throughout the first 30 hours of development (hour of sampling indicated by lane label). B-actin was concomitantly RT-PCR amplified to ensure RNA integrity during purification, and was subsequently used as a loading control.

Discussion

Western analysis of calmodulin in early zebrafish development suggests that a large part of the extant calmodulin is in association with a ~ 100 kDa protein that behaves biologically as lipovitellin. This is evidenced by its molecular weight, signature degradation into lower molecular weight forms as development ensues, its abundance in the oocyte (the major protein constituent), and previously published data showing lipovitellin as a CaM target in *Xenopus* (Molla et al., 1983). This interaction in *Xenopus* was, however, dependent on the availability of free calcium. Our protein preparation, and subsequent sample treatment, maintained concentrations of EGTA, indicating that the vitellogenin-calmodulin is calcium independent. CaM ran in the gels in its inactive form (no bound Ca^{2+}) and not as it would travel bound Ca^{2+}, at a lower molecular weight. Vitellogenin is generated by the liver and sequestered into ooctyes by receptor mediated endocytosis. Perhaps calmodulin is maternally donated to the oocyte by piggybacking on lipovitellins. Western analysis or sequencing of the 100 kDa protein will have to be conducted before we can be sure of its identity, additional RT-PCR experiments against the other two calmodulin genes will also have to be conducted before we can be confident that the protein is not transcribed from maternally donated mRNA.

Future Directions

- We have expressed and purified a [His]tag-[S]tag-CaM fusion protein. A thrombin cleavage site between the His-tag and S-tag allow for a [S]tag-CaM cleavage product that may be detected with S-protein-AP in a far western. This far western will be used to identify candidates for transient CaM downstream effectors during development.
- Use affinity chromatography columns with [His]tag-[S]tag-CaM to pull down Ca^{2+}-dependent effector candidates for sequencing.
- RT-PCR all CaM genes to determine origin of immuoreactive CaM.

Materials and Methods

- **Purification of calmodulin**: Modified Gopalakrishna protocol (1982). In brief, 5 g cod ovary was homogenized in 40 mL buffer I (40mM Tris pH 7.5, 5 mM EGTA) and centrifuged 12,000 g for 15 min. Supernatant was set at 90° C for 5 min, and spun again for 12,000 g for 15 min. $CaCl_2$ was added to supernatant to a final 6 mM. A phenyl sepharose column was equilibrated in binding buffer (20 mM Tris pH 7.5, 0.15 M NaCl, 1 mM $CaCl_2$), and supernatant was poured over column, column was washed with 4 bed volumes binding buffer, and eluted with elution buffer (20 mM Tris pH 7.5, 0.15 M NaCl, 4 mM EGTA).
- **Western analysis**: Protein preparations of zebrafish embryos throughout development were resolved by SDS-PAGE and transfered to a PVDF membrane for western analysis, transfer was conducted in phosphate buffer (25 mM KH2/K2HPO4 pH 7.0) overnight at a constant 20V in a transblot apparatus. After transfer the membrane was fixed for 45 min in 0.2% gluteraldehyde in phosphate buffer, and washed with TBS, proteins were visualized by ponceau staining. Standard protocol was used for subsequent steps using a 1º rabbit anti-calmodulin Ab (1:150) and 2º GAR (1:5,000) Ab. Chemi-luminescence was visualized by a Storm Phosphorimager.
- **RT-PCR:** Dechorionate 40 embryos, place in baked homogenizer, remove egg water and add 400ul Trizol Reagent. Homogenize and transfer to sterile eppendorf. Incubate 5 min at RT, add 80ul chloroform and shake hard for 15 sec. Incubate 2-3 min at RT and centrifuge at 10K RPM for 15 min at 4º C. Take top phase, aquious to new tube precipitate by adding 200 ul Isopropanol. Incubate at RT 10min, centrifuge at 10K RPM 10 min at 4º C. Remove fluid. Wash pellet with 500 uL cold 70% EtOH (use DEPC water),vortex and spin 5 min at 4º C. Remove EtOH, air dry briefly and resuspend in 50ul DEPC H20. Aliquot into 2 20 ul aliquots and feeze immediately at -70º. Dilute remaining 10 ul 5X, and continue immediately to reverse transcription. RT reaction and PCR carried out according New England BioLabs cat# E6500S - First strand cDNA synthesis kit.

References

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