Calcium/Calmodulin Dependent Protein Kinase Type-II Associates with Flightless-I to Influence its Nuclear Localization

Matthew Edward Seward
Virginia Commonwealth University

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Calcium/Calmodulin Dependent Protein Kinase Type-II Associates with Flightless-I to Influence its Nuclear Localization

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By
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B.S., James Madison University, 2004

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Richmond, Virginia
April, 2006
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<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATRA</td>
<td>All Trans Retinoid Acid</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>BamHI</td>
<td>Bacillus amyloliquefaciens H I</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxy Terminus</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium Ion</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CaMK-II</td>
<td>Ca²⁺/Calmodulin Dependent Protein Kinase Type II</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>δ</td>
<td>Delta</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DpnI</td>
<td>Diplococcus pneumoniae I</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray Ionization</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>[ethylenebis(oxyethylenenitrilo)] Tetra-acetic Acid</td>
</tr>
<tr>
<td>Fli-I</td>
<td>Flightless-I</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin Gamma</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FLAG</td>
<td>DYKDDDDK Epitope Tag</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GRABP</td>
<td>Gelsolin Related Actin Binding Protein</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen Synthase Kinase</td>
</tr>
<tr>
<td>HB</td>
<td>Homogenization Buffer</td>
</tr>
<tr>
<td>He</td>
<td>Helium</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethy) piperazine-1-ethansulfonic acid</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KN-93</td>
<td>2-[[N-(hydroxyethyl)-N-(4-methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine</td>
</tr>
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Abstract

Calcium/Calmodulin Dependent Protein Kinase Type-II Associates with Flightless-I to Influence its Nuclear Localization

By Matthew E. Seward, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, April 2006

Major Advisor: Robert M. Tombes, Ph.D., Associate Professor, Department of Biology

Ca\textsuperscript{2+}/calmodulin-dependent protein kinase type-II (CaMK-II) is a Ser/Thr protein kinase regulated by Ca\textsuperscript{2+} and Calmodulin. It is a highly conserved and broadly expressed enzyme and has a unique structure and dynamic regulation. It has the ability to remain active in the absence of Ca\textsuperscript{2+} as a result of Ca\textsuperscript{2+} dependent autophosphorylation. CaMK-II phosphorylates proteins involved in neurotransmitter secretion, long term potentiation, cytoskeletal dynamics, gene transcription, and cell motility. To support existing and identify new intracellular roles of CaMK-II, potential binding partners were identified. This was accomplished by transfecting and purifying “FLAG”-tagged CaMK-II’s (α, β\textsubscript{L}, δ\textsubscript{C}, and δ\textsubscript{E}). CaMK-II associated proteins were then identified using tandem mass spectrometry. Known binding partners were identified using this approach, including CaMK-II and calmodulin, verifying the approach’s validity. Additionally several unexpected but interesting proteins were identified, including the gelsolin related actin binding protein, Flightless-I. Fli-I is an actin binding and capping protein that also functions as a transcriptional coactivator. The CaMK-II-Fli-I interaction was confirmed
with endogenous (un-tagged) proteins. The association and localization of Fli-I are
dependent on CaMK-II’s activity state, although Fli-I is not a substrate of CaMK-II.
When CaMK-II is inhibited, Fli-I translocates to the nucleus. Conversely when CaMK-II
is artificially activated using a Ca$^{2+}$ ionophore, Fli-I returns to the cytosol. The discovery
of this reversible interaction represents a potentially new CaMK-II regulated pathway and
likely serves as a link between Ca$^{2+}$ based signal transduction pathways and regulation of
the actin component of the cytoskeleton and transcription.
Introduction

CaMK-II Overview

The role of Ca^{2+} as a secondary messenger has long been established. A component of intracellular Ca^{2+} signal transduction is the Ca^{2+}/calmodulin dependent protein kinase type-II (CaMK-II) (Hudmon and Schulman, 2002b; Schulman, 1993). CaMK-II is conserved throughout metazoans and expressed in a wide range of cell types. It is encoded by four different genes (α, β, γ, and δ) and is alternatively spliced to yield more than forty isozymes. Individual isozymes varies in expression by cell type and developmental progression and have unique localization patterns and presumably substrates (Mayer et al., 1993; Tombes et al., 2003; Tombes and Krystal, 1997; Zhou et al., 1995).

CaMK-II Structure

The CaMK-II holoenzyme consists of twelve subunits (six anti-parallel dimers) which arrange themselves in a unique fashion (Gaertner et al., 2004; Hunter and Schulman, 2005; Kanaseki et al., 1991). From the amino terminus, each subunit has three major domains; a catalytic and regulatory domain, a variable domain and an association domain. The catalytic domain comprises the first 250 amino acid residues and includes the ATP- and substrate-binding sites. The regulatory domain encodes the autoinhibitory and calmodulin (CaM) binding regions over a 30 amino acid residue region (Kolb et al., 1998; Schulman and Lou, 1989). The variable region of CaMK-II spans residues 315 to 345 up to 435, and is where alternative splicing occurs to generate isozyme variability. Splice variants are denoted by the Greek letter of the gene that they
originate from followed by a subscripted capital letter representing the specific isozyme.
The C-terminal association domain is required for the oligomerization of CaMK-II monomers into dodecamers which is essential to CaMK-II’s functionality. Dodecamers can be either homomeric or a heteromeric, consisting of either entirely one isozyme or a combination of multiple variants (Kolb et al., 1998; Lantsman and Tombes, 2005).

**CaMK-II Activation**

When the enzyme is inactive the autoinhibitory arm obstructs the active site preventing catalytic activity. When Ca²⁺ rises, the ions are bound by the Ca²⁺ binding protein CaM, which is then bound by CaMK-II, causing a relaxation of the inhibitory arm, exposing the active site (Mukherji and Soderling, 1994; Schulman and Lou, 1989). Partial activation of the enzyme increases its affinity for Ca²⁺/CaM, raising CaMK-II Ca²⁺ sensitivity and excitability (Meyer et al., 1992).

**CaMK-II Autophosphorylation**

In addition to its ability to form a unique and complex quarternary structure, CaMK-II has the ability to autophosphorylate. The most important autophosphorylation site is Thr²⁸⁷ in the regulatory domain. Autophosphorylation occurs in an intersubunit fashion between active adjacent subunits. An activated subunit can phosphorylate Thr²⁸⁷ on another active adjacent subunit, preventing the autoinhibitory arm from assuming its inhibitory confirmation. This phosphorylation frees CaMK-II of its Ca²⁺/CaM dependence allowing it to remain active in their absence because the autoinhibitory domain is occluded from the active site regardless of their presence. This autonomy is
one of CaMK-II’s most interesting features because it allows the enzyme to stay active in the absence of Ca$^{2+}$/CaM. This condition is referred to as molecular memory and makes CaMK-II more dynamically responsive to a variety of molecular conditions (Dunkley, 1992; Dupont et al., 2003; Hudmon and Schulman, 2002a; Schworer et al., 1988).

**CaMK-II Function and Localization**

CaMK-II gene products have been shown to play roles in various cellular functions and to be expressed in unique patterns. CaMK-II’s role in the brain is well established and has been shown to have regulatory roles in a number of critical neural processes. α and β CaMK-II, for example, are regulators of neurotransmitter release through binding and phosphorylation of synaptic targets and are functional in both the pre- and postsynaptic densities (Benfenati et al., 1992; He et al., 2000; Yoshimura et al., 2002). α CaMK-II is a regulator of long term potentiation and learning and memory through phosphorylation of a variety of neuronal receptors including AMPA and NMDA (Chen et al., 2001; Giese et al., 1998; Ito et al., 1991). α and β CaMK-II in nervous tissue localize to a variety of regions, varying by gene, but can be found in axons, dendrites, and cell bodies. Additional isozymes that are expressed in the brain are encoded from the δ and γ genes and are found in peripheral neural tissues, such as astrocytes and glial cells (Takaishi et al., 1992; Vallano et al., 2000). α CaMK-II is enriched in the frontal lobe of the brain and both β and δ CaMK-II are enriched in the cerebellum. It is not surprising that CaMK-II is involved in so many processes because of its expression patterns, but also because of its ability to respond to a variety of Ca$^{2+}$ signals and transduce complex
signals in an efficient manner (Chang et al., 2001; Erondu and Kennedy, 1985; Li et al., 1998).

Another well documented role of CaMK-II is in muscle. CaMK-II is expressed in cardiac, smooth, and skeletal muscle. \(\gamma\) CaMK-II has been shown to be expressed in vascular smooth and heart muscle. \(\delta\) isozymes regulate \(Ca^{2+}\) levels in cardiac muscle and are involved in the regulation of \(Ca^{2+}\) channels and the sarcoplasmic reticulum, all vital to maintaining regular heart beat (Abraham et al., 1996; Hoch et al., 1998; Hoch et al., 2000; Singer et al., 1997). An interesting example of CaMK-II in muscle is of \(\alpha\)KAP, a non-catalytically active form of \(\alpha\) CAMK-II which is expressed in skeletal muscles and serves as an anchoring protein (Bayer et al., 1998; Takeuchi and Fujisawa, 1997).

Developmentally, CaMK-II function is imperative, particularly in the brain. \(Ca^{2+}\) transients are fundamental to successful organismal development, which makes CaMK-II significant to the process. \(\delta\) CaMK-II isozymes have been shown to be expressed prominently during neural development (Faison et al., 2002). These isozymes are not expressed in the adult brain and are only expressed during early neural development (Bayer et al., 1999). Interestingly, \(\alpha\) CaMK-II, the primary neuronal isozyme in the adult brain is not expressed until later in brain maturation. These are prime examples of the differential expression patterns of CaMK-II genes (Brocke et al., 1995; Burgin et al., 1990).

Additionally, roles for CaMK-II have been documented in transcription (Enslen and Soderling, 1994), cytoskeletal regulation (Shen et al., 1998; Takahashi, 2001), and morphogenesis (Massé and Kelly, 1997). It is not unanticipated that it has such a diverse collection of functions, considering its intricate levels of regulation and unique structure.
Its behavior is dictated by both Ca\textsuperscript{2+}/CaM dependent and autonomous activity, pattern of isozyme expression, and homo- or hetero-oligomerization, which dictate its response to various types of Ca\textsuperscript{2+} signals. Its capability to discern between Ca\textsuperscript{2+} signal intensity, frequency, and duration and then transduce it enzymatically give CaMK-II a unique ability among protein kinases (Hudmon and Schulman, 2002b; Schulman et al., 1992).

Although much information has been established on the role of CaMK-II, many of its substrates and binding partners have not yet been identified. Discovery of some of these targets would yield insight into CaMK-II’s purpose in the cell. One approach that has been established as a method for identification of protein function is mass spectrometry.

**Tandem Mass Spectrometry**

An effective tool for identifying the complex patterns of protein-protein interaction is tandem mass spectrometry (MS/MS) (Syka et al., 2004). This technique allows for the identification of specific peptides from a complex solution. If a particular protein is isolated from cellular lysates any proteins that are associated with it are also captured. When these proteins are analyzed by MS/MS not only is the target protein detectable, so are the associated proteins. This narrows the list of candidate binding partners from the entire proteome to those detected in the analysis and yields insight into potentially uncharacterized functions.

A tandem mass spectrometer differs from a single MS by coupling together multiple mass analyzers to sum their measuring capabilities to create a more accurate analysis of the sample (Busch et al., 1988). During a MS/MS analysis a sample of
proteins in solution is reduced and denatured then digested with a protease, usually trypsin because of its efficient and consistent nature, which yields peptides of discrete size and charge. The sample is then introduced into a microcapillary HPLC which separates the peptide fragments by size. The fragmentated peptides are then introduced into a highly charged electric field at a very slow rate creating highly charged droplets, a process called electrospray ionization (ESI) (Ashcroft, 2005). The droplets pass through a field of inert gas (most often He) where they are disrupted by the collisions with the gas molecules causing the solution to evaporate and the peptides to further fragment, at which point they enter into an ion trap. A quadrupole ion trap captures peptides based on mass to charge (m/z) ratio by establishing discrete gradients of charge, which suspends the peptides in space in distinct m/z ranges by electrically focusing them. Slowly each m/z group is released by a shift of the charge gradient in the trap and the peptides are released toward the detector, an electron multiplier (March and Todd, 2005). The detector analyzes each peptide it senses and compiles a spectrum of the peptides. The scan is repeated, then the ten most abundant ions are selected and the cycle parameters are altered to screen the next ten most frequently appearing peptides. The scanning parameters are continuously redefined so only a small subset of the sample is being analyzed at any given time. This approach ensures the most accurate assessment of the proteins in the sample.

A computer program called SEQUEST is used to analyze the experimental peptide sequences by comparing them to theoretical MS/MS spectra. The comparison is made to a database that contains hypothetical peptide sequences that could be created by protease digestion of every known protein, had they been digested in a manner similar to
how the samples were prepared. The peptides in the database are assigned a score that is representative of the likelihood of each occurring based on the theoretical digestion. The score of each theoretical peptide is compared to the actual MS results to establish the validity of the data and from the analysis of the peptides a list of proteins is generated (Hernandez et al., 2006; Yates et al., 1995).

**CaMK-II Binding Partner Screen**

An MS/MS based screen for CaMK-II binding partners was undertaken. In order to partially purify CaMK-II from whole cell lysates, the “FLAG” epitope system was used. FLAG is an eight amino acid residue epitope tag (DYKDDDK) which allows for efficient immunopurification of tagged proteins. The benefit of the FLAG system is that FLAG-tagged proteins can be easily eluted from anti-FLAG antibodies in a non-denaturing manner using a FLAG-peptide. The small peptide effectively disrupts the interaction between the FLAG tag and immobilized anti-FLAG IgG. The FLAG antibody is conjugated to agarose beads, which when stringently washed eliminates any non-specifically bound proteins leaving only FLAG-tagged CaMK-II and associated proteins. The FLAG elution approach adequately prepares samples suitable for MS/MS (Einhauer and Jungbauer, 2001).

For the binding partner screen, five FLAG-mEGFP/CaMK-II (α, β, δC, and full length and C-terminal δE) fusion vectors were constructed and transfected into either NIH 3T3 cells or into P19 neurons. NIH 3T3 cells are a well established mouse embryonic fibroblast cell line that is non-transformed, diploid, highly contact inhibited, and very transfectable (Copeland and Cooper, 1979; Jainchill et al., 1969). NIH 3T3 cells express
CaMK-II endogenously, although primarily δC. The P19 cell line is a diploid mouse embryonal carcinoma that can be induced to form neurons by treatment with retinoic acid (McBurney et al., 1982). P19 neurons express at least three different β and δ CaMK-II isozymes (Johnson et al., 2000). The transfected cells were harvested, and the FLAG tagged fusion proteins were immunopurified, eluted, and subjected to MS/MS analysis.

Experimental Approach

The CaMK-II binding partner screen generated a list of proteins which could be potential CaMK-II binding partners. It included a number of predicted proteins given CaMK-II’s reported functions and previously established binding partners, in addition to a variety of unique and unanticipated proteins.

One such unforeseen candidate was the gelsolin related actin binding protein (GRABP) Flightless-I (Fli-I). Fli-I was first identified in Drosophila melanogaster, in mutant flies that had lost the ability to fly, but has been shown to have homologs in all metazoans. It is a developmentally essential actin binding and capping protein with transcriptional coactivity (Archer et al., 2005; Lee et al., 2004). This was a result that raised particular interest given the documented role that CaMK-II plays in regulation of the actin component of the cytoskeleton.

The interaction between Fli-I and CaMK-II was pursued for this project. It has been speculated that Fli-I may serve as a link between several cellular pathways, including signal transduction pathways and regulation of the cytoskeleton through a yet to be identified protein (Davy et al., 2000; Fong and de Couet, 1999). It was hoped that it could be shown that CaMK-II is a factor in this signal transduction pathway through its
interaction with Fli-I. Several questions were initially laid out to guide the study, including:

- **Do endogenous CaMK-II and Fli-I interact?**
- **What is the nature of their interaction relative to CaMK-II activity?**
- **Does CaMK-II activity affect the localization of Fli-I?**

To answer these questions a number of approaches were taken. Antibodies against Fli-I and CaMK-II made it possible to confirm the interaction through reciprocal immunoprecipitations of endogenous proteins and also to perform immunolocalization experiments. Using different classes of CaMK-II inhibitors, media conditions, and constitutively active CaMK-II constructs I have characterized the nature of the CaMK-II-Fli-I association. I have also found that CaMK-II and Fli-I interact in a manner dependent on CaMK-II activity which also affects Fli-I’s cellular localization.
Methods

NIH 3T3 Cell Culture.

NIH 3T3 cells were maintained in DMEM with 10% FBS, L-Glutamine, Penicillin, and Streptomycin at 37°C in a 5% CO₂ incubator in plastic tissue culture dishes (Nunc, Rochester, NY). Serum free experiments were in DMEM (Gibco/Invitrogen, Carlsbad, CA) with 0% FBS, L-Glutamine, Penicillin, and Streptomycin.

P19 Embryonal Carcinoma Cell Culture.

Undifferentiated P19 cells were maintained in 10% FBS, L-Glutamine, Penicillin, and Streptomycin at 37°C in a 5% CO₂ incubator. They were induced to differentiate by treatment with 5x10⁻⁷M all trans retinoic acid (ATRA) at 1x10⁵ cells/ml in DMEM, 5% FBS, in bacteriological petri dishes for 4 days. The induced cells formed embryoid bodies, which were then plated at 2-5 x 10⁵ cells/cm² on tissue culture dishes. Dishes were pre-coated with EHS laminin (0.1-0.5 μg/ml) in neurobasal medium containing N2 supplement (Invitrogen) on day 0.

Drug Treatment.

The CaMK-II inhibitors, KN-93 and mAIP were used at a final concentration of 10 μM and were added directly to cell cultures. The Ca²⁺ ionophore, ionomycin was used at a final concentration of 1 μM. It was prepared as 1mL of 2 μM stock in serum free media then added to 1 mL of cells in culture.
**Cell Harvest.**

Cells were harvested by trypsin-EDTA and resuspended in Homogenization buffer with 30mM 4-(2-Hydroxyethyl) piperazine-1-ethansulfonic acid (HEPES, pH 7.4), 2.6 mM EGTA, 20mM MgCl₂, 80mM β–glycerol phosphate, 0.1 μM okadaic acid, and 10 μg/mL of chymostatin, leupeptin, aprotinin, pepstatin, and soy bean trypsin inhibitor. The cells were sonicated on ice with two five seconds burst from a probe sonicator (Misonix, Farmingdale NY) and centrifuged at 4°C for 15 minutes at 12,000 x g. Protein concentrations were quantitated in triplicate with the BCA protein assays (Pierce, Rockford, IL) then immediately aliquoted and frozen at -80°C.

**FLAG-tag Fusion Vector Construction.**

The four full length CaMK-II isozymes in this study (α, β, δ, and ε) had been previously cloned into the EGFP-C1 vector (Clontech) as well as a C-terminal δₑ construct, which is a truncated construct lacking the first 308 amino acid residues, the sequence that codes for the catalytic domain. The GFP sequence had a point mutation to prevent the dimerization of GFP, by changing Leu²²¹ to a Lys²²¹. Monomeric EGFP (mEGFP) tagged CaMK-II sequences were amplified through PCR, using Hi-Fidelity Platinum Taq polymerase (Invitrogen). The amplification was primed at the C-terminal end of the gene, with a primer containing a BamHI cut site. The α and β were specifically primed by, CCCGGATCCTTACTGGGGCAGGAGG and CCCGGATCCTCCTCAGCGGAGG, respectively. The three δ constructs were primed with a γ specific primer because each was a chimera with the γ association domain with the primer CCCGGATCCTCCTCAGCGGGCGGCA. The N-terminal
side of the GFP sequence of all five constructs was targeted by a primer with a KpnI cut site, CGCGGTACCATGGTGAGCAAGGGCGAGGAG. The PCR products were ligated into the pCDNA-3.1' vector, which had previously had the FLAG-2AB sequence cloned into it at the HindIII site of its multiple cloning region (the plasmid was provided courtesy of Dr. Mark Mayhew at the University of Virginia). Five vectors were created; FLAG/mGαWT, FLAG/mGβWT, FLAG/mGδcWT, FLAG/mGδeWT, and FLAG/mGδe-C-terminal. The constitutively active FLAG construct (FLAG/mGδeCon) was generated by site directed mutagenesis, substituting Asp287 for Thr287 of a FLAG/mGδeWT construct (GCATCGTCAGGAGGACGTGGAGTGTTTGCG) using Pfu Ultra high fidelity long read polymerase (Stratagene, LaJolla, CA). The plasmids were confirmed by restriction digest, immunoblots, CaMK-II activity assays, and DNA sequencing and showed that all five had been accurately constructed.

**FLAG-transfection.**

Approximately 80% confluent cells in 100mm tissue culture dishes were transfected with 4 μg of FLAG plasmid and diluted with 8μg of pBS-KS+ using lipofectamine 2000 in Optim-MEM and 3% FBS (Invitrogen, Carlsbad, CA). Four hours post-transfection, cells were either refed with DMEM/10%FBS or trypsinized and replated at 50% of their original confluence in DMEM/10%FBS. Cells were harvested 18-24 hours later as described above.
FLAG Immunoprecipitations.

1 mg of total protein from FLAG-transfected cell lysate was precleared with 50μL of mouse IgG conjugated to agarose at 1mg/mL (Sigma, St. Louis, MO) overnight at 4°C with rocking. The supernatant was then combined with 50μL M2 agarose anti-FLAG at 0.6mg/mL (Sigma) and incubated for 2-5 hours with rocking at 4°C. The beads were then washed twice with 0.5mL HB/protease and phosphatase inhibitors/0.1%NP-40, followed by one wash with 0.5 mL of sterile 30mM Tris pH 7.4. Immunoprecipitated proteins were then eluted twice with 100 μL sterile 30mM Tris and 0.2 mg/mL FLAG-elution peptide (Sigma) for 15 minutes on ice. Samples were immediately frozen until MS/MS analysis.

Tandem Mass Spectrometry.

Samples for MS/MS were prepared by reduction and alkylation. A portion of the FLAG eluent was incubated in 10 mM DTT for 1 hour at 37°C to reduce the sample then alkylated, with 5 μM Iodoacetic acid for 30 minutes at room temperature in the dark and then again with DTT to quench the Iodoacetic acid. The sample was then digested with Endoproteinase Lys-C, which cleaves peptides on the C-terminal side of Lys residues, and Trypsin, which cleaves at the C-terminal side of Arg and Lys residues, at 200 μg/mL for one hour at 37°C. The mass spectral analysis was performed on a Finnigan LTQ-FTMS machine and analyzed using the accompanying software and scored using the SEQUEST approach (ThermoElectron Corp, San Jose, CA). These protocols are the standard techniques used at the University of Virginia Mass Spectrometry Facility and can be found in greater detail at:
Immunoprecipitations.

100 µg of NIH 3T3 lysate protein was incubated with 1 µg of primary antibody overnight at 4°C. 1.5 µg biotinylated secondary antibodies were added to the solution and incubated for 2 to 5 hours at 4°C. They were then incubated with 30 µL of pre-washed streptavidin-Magnesphere (Promega, Madison WI) or Protein-G Dynabead (Dynal/Invitrogen, Carlsbad, CA) paramagnetic beads for 1-2 hours at 4°C with rocking. The Immunoprecipitates were then washed 3 times with 500 µL cold HB in a magnetic separation stand and were then resuspended in 20 µL of HB. Immunoprecipitates for CaMK-II activity assays were frozen until use or were combined with SDS sample buffer containing 30 mM DTT and boiled for SDS-PAGE.

Immunoblots.

Immunoprecipitates were separated on 4-15% or 10% SDS-PAGE gels using the Mini-Protean II gel electrophoresis system (Bio-Rad, Hercules CA). Proteins were transferred to 0.45 µm nitrocellulose sheets for 1 h at 100V and blocked with Tris Buffered Saline, 0.01% Tris-20, and Sodium Azide (TBSTA), containing 5% BSA and 2% pre-immune serum of the secondary antibody host for 1 hour. Blots were then incubated overnight with primary antibodies at 1 µg/ml in 2% BSA/TBSTA. Blots were washed three times with TBSTA and incubated for 2-5 hours with alkaline phosphatase-conjugated secondary IgG at 2 µg/ml in 2% BSA/TBSTA. The blots were washed once in
0.1M NaCl and 5mM MgCl₂ at pH 9.4 and developed with 0.25 mg/mL of both nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

**Total Protein Stain.**

Samples to be stained for total protein were separated on 10% polyacrylamide gels. The gel was then fixed for 30 minutes at room temperature in 50% methanol and 10% acetic acid. It was then incubated overnight at room temperature in undiluted SYPRO Ruby protein gel stain (Molecular Probes/Invitrogen, Carlsbad, CA) in an opaque developing tray. The gel was washed twice for thirty minutes in a 10% methanol and 7% acetic acid solution then rinsed twice with dH₂O and visualized on a UV-transilluminator (UVP, Upland California).

**Calmodulin Overlay.**

The samples for calmodulin overlays were transferred to nitrocellulose, and then blocked with TBS containing 0.1% Tween-20, 2mM CaCl₂ for 30 minutes at room temperature. Blots were then placed in TBSTA/2mM CaCl₂ with 1μg/mL of biotinylated calmodulin (Calbiochem, San Diego, CA) and incubated overnight at room temperature. The blots were then washed three times in TBS/2mM CaCl₂/0.1% Tween-20 for 10 minutes at room temperature. Blots were then transferred to TBS/2mM CaCl₂/0.1% Tween-20 and 2μg/mL alkaline phosphatase-conjugated streptavidin (Jackson Immunology Labs, West Grove, PA) and incubated at room temperature for 1-2 hours. All blots were washed three times as above and developed in 0.25 mg/mL of NBT and BCIP each in 0.1M NaCl and 5mM MgCl₂ at pH 9.4.
CaMK-II Activity Assays.

Total CaMK-II activity was assessed by measuring phosphate incorporation into an artificial substrate. The kinase reactions were carried out in a solution of 20 mM HEPES (pH 7.4), 0.1 mM dithiothreitol, 15 mM magnesium acetate, 20 mM β-glycerophosphate, 0.5 μM PKA inhibitor peptide, 0.1 μM okadaic acid, 40 μM sodium orthovanadate, 0.5 mCi [γ-32P-ATP], 35 μM autocamtide-2 (peptide substrate), 1 μM calmodulin, 1 mM EGTA, and either none or 3 mM Ca\(^{2+}\). The reaction ran for 10 minutes at 30°C, then 20 μl was pipetted onto P81 phosphocellulose paper squares that were air dried for 1 min and washed five times in 500 mL 1% phosphoric acid. The samples were quantitated by Cerenkov counting in a Beckman LS6000IC scintillation counter. The sequence of autocamtide-2 is KKALRRQETVDAL and the assay conditions were previously optimized for detection of CaMK-II activity (Tombes et al., 1999; Tombes et al., 1995).

Imunolocalization.

Cells were grown on glass cover slips that had been preincubated with 1 μg/mL of fibronectin and then fixed in methanol at -20°C for 3 min. Fixed cells were blocked overnight at 4°C in TBSTA containing 5% BSA and 2% appropriate pre-immune serum. Cells were then incubated for 1 hour at 37°C with 0.2 μg/mL of primary antibody in TBSTA/2% BSA in a humidified chamber solution then washed in TBSTA three times for 5 minutes. The cover slips were then incubated with 1 μg/mL of Texas Red conjugated secondary antibody in TBSTA/2% BSA for 30 minutes at 37°C in a
humidified chamber and washed as described above. They were then mounted onto glass slides and stored until imaged.

**Imaging.**

All phase contrast and fluorescent images were taken on an Olympus IX-70 inverted microscope using a 12-bit camera (Olympus, Melville, NY). Fluorescent images were taken with two second exposures using a 595 nm dichroic long pass filter (Chroma, Rockingham, VT) illuminated by a mercury arc lamp. The images were processed using Olympus Microsuite version Five.

**Antibodies.**

The mouse anti-flightless-I antibody was a purified monoclonal, mouse IgG, at 1mg/mL, from Covance, Berkeley, CA. The mouse anti-β CaMK-II primary antibody was obtained from Zymed/Invitrogen Carlsbad, CA. The goat anti-δ CaMK-II, rabbit anti-GFP and goat anti-Tropomodulin-3 were purchased from Santa Cruz Biotechnology, Santa Cruz CA. The monoclonal mouse anti-cofilin and anti-integrin was bought from BD Biosciences/Pharmingen in San Diego, CA. The goat anti-mouse, texas red conjugated, donkey anti-mouse conjugated to biotin, donkey anti-goat biotin conjugated, and biotinylated donkey anti-rabbit secondary antibodies were all purchased from Jackson Immunological Laboratories, West Grove, PA.
Results

Identification of CaMK-II Binding Partners.

The first half of this study was to identify protein binding partners of CaMK-II using MS/MS. Five FLAG-tagged CaMK-II (α, βE, δC, δE, and δE C-terminal) constructs were transfected into NIH 3T3 cells and one into P19 neurons. The bait DNA was diluted 1:3 with an empty pBlueScript vector, which kept the transfection efficiency high but allowed for the tagged CaMK-II to mix with the endogenous population. This helped to correct for the artificial nature of the experimental conditions.

Four hours post-transfection the cells were sub-cultured to ensure that the cells were motile and actively progressing through the cell cycle. They were harvested 16-24 hours later. The tagged proteins were isolated with antibodies against the FLAG-tag and eluted two times using a FLAG-elution peptide. The FLAG IP and elution process effectively cleared non-specifically bound proteins from the lysates during the first elution, which was confirmed by total protein stains and GFP Western blots (Figures 1A and B).

The eluents of the IPs were then analyzed by MS/MS and yielded a number of potential binding partners. In total eleven MS/MS experiments were conducted. The first was with the FLAG/mGδE-C-terminal construct, which lacked the catalytic domain and was carried out in transfected P19 neurons. In this experiment CaM and CaMK-II were detected, which served as an internal control supporting the validity of the approach. Additionally several previously identified CaMK-II binding partners were detected including β-tubulin and β- and γ-actin further corroborating the legitimacy of
the data (Easley, et al., 2006). Experiments two and three were duplicate experiments of each other with FLAG/mGβEWT. The results from the experiments were nearly identical, further substantiating the results as authentic. The fourth, fifth, and sixth experiments were performed in the same fashion as the initial three, except with FLAG/mG δCWT, FLAG/mG δEWT, and FLAG/mGΔαWT, respectively. The βE, δC and δE isozymes were selected because they are all endogenously expressed in NIH 3T3 cells. α CaMK-II is not typically present, but it represented an effective point of comparison, as did δE-C-terminal because it is effectively catalytically inactive which is why they were chosen for the screen.

The results from these six experiments yielded many previously unidentified binding partners including a number of actin capping and binding partners, such as Fli-I (and two of its associated proteins LRRFIP-I and -II), tropomodulin-3, and cofolin. These actin capping proteins were of particular interest because of the documented but not specifically characterized role of CaMK-II in cytoskeletal regulation. Other proteins of interest identified in the screen included myosin heavy and light chain and vimentin (table 1).

Four additional MS/MS experiments were performed using less stringent harvesting and immunoprecipitating conditions. Low salt HB, which was similar to the composition of standard HB except for the lack of 80 mM β–glycerol phosphate, was used to reduce the ionic strength of the buffer. FLAG/δE-C-terminal and FLAG/mGδEWT were transfected into NIH 3T3 cells and harvested under both normal and low salt conditions and analyzed by MS/MS. Although it was suspected that additional proteins could be identified because their interaction with CaMK-II had been
disrupted by the stringency of the preceding experiments, no additional candidates were detected by this method. The final experiment was a negative control experiment using FLAG/GFP with no cDNA cloned into it and harvested under the standard conditions. The results from this experiments were subtracted from the data to correct for proteins that may have non-specifically bound either GFP or the FLAG-tag.

A large volume of data was generated from the various MS/MS experiments. It was not possible to initially pursue all of the potential binding partners identified in the screen. It was necessary to select either one or several specific targets to characterize experimentally. Additionally, several pieces of the data were dismissed as artifacts of the experimental conditions. These included peptides corresponding to human keratins that were detected in all of the screens because they were assumed to be contamination that was introduced during the preparation of the samples. Other results were generated by only one or two peptides and were given a very low score of legitimacy and were also dismissed. Certain results, such as various immunoglobins and several stress response proteins were disregarded as false positives. A list of all of the identified proteins can be found at http://www.cellmigration.org/resource/discovery/#binding.

A review of the current literature made Fli-I the first choice for detailed analysis from our screen. The interaction of CaMK-II and Fli-I had the most potential for further analysis. The commercial availability of a quality antibody against this protein helped to establish it as the subsequent focus of the study.
Interaction of Endogenous Fli-I and CaMK-II.

The identification of Fli-I as a potential CaMK-II binding partner from our MS/MS screen was in an artificial system due to transfection and the over expression of CaMK-II. It was essential to confirm this as a legitimate interaction and not an artifact of the experimental conditions. This was done by using reciprocal immunoprecipitations of untreated NIH 3T3 lysates.

When β and δ CaMK-II antibodies were used to immunoprecipitate endogenous proteins it was possible to successfully probe for Fli-I on Western immunoblots. Both IPs yielded a band in the 145kDa region which corresponds to the Mr of Fli-I (Figure 3A). Although α CaMK-II was used in the MS/MS screen it is not normally expressed in NIH 3T3 cells, so it was not possible to test this endogenous interaction.

Conversely, Fli-I was immunoprecipitated from untreated 3T3 lysates. The immunoprecipitate was transferred and probed with CaM to screen for CaM binding proteins. For this purpose this approach is more effective than a traditional immunoblot. Many CaMK-II isoymes have a similar Mr as IgG heavy chain and so they migrate at similar rates during SDS-PAGE experiments. It is not possible to discern whether the bands that are detected because the secondary antibody is bound to the IgG heavy chain or to CaMK-II. Using CaM conjugated to biotin makes it possible to eliminate off-target interactions of the secondary antibody and to screen only for CaM binding proteins. As was expected the CaM overlay experiment revealed bands in the 50-60 kDa region, which corresponds to CaMK-II isozyme migration patterns (Figure 2B).

Additionally we used Fli-I IPs to screen for associated CaMK-II with CaMK-II enzymatic activity assays. Using autocamtide-2, an artificial CaMK-II substrate it was
possible to detect CaMK-II enzymatic activity by measuring the incorporation of $^{32}\text{P}$ from Fli-I IPs into the peptide. Kinase reactions run with this IP showed that almost 20% of the CaMK-II activity detected in whole cell lysates could be recovered in the IP. This is in comparison to immunoprecipitation with an anti-integrin antibody, which is a protein known to not bind CaMK-II, in which only 1% of the total cellular activity could be recovered. This experiment confirmed the interaction enzymatically (Figure 3C).

**CaMK-II Activity Dependent Interaction with Fli-I.**

The next question I raised explored the nature of the CaMK-II-Fli-I interaction in relation to CaMK-II activity. For this, NIH 3T3 cells were transfected with FLAG/mGδE constructs and the level of CaMK-II activity was varied. Several approaches were utilized to vary CaMK-II activity; one was to use a constitutively active CaMK-II mutant (FLAG/mGδECon), which had an Asp$^{287}$ point mutant at Thr$^{287}$, the autophosphorylation site. The other cells were transfected with wild type constructs (FLAG/mGδEWT). To inhibit CaMK-II we used two different drugs, KN-93 a reversible CaM antagonist which prevents CaM binding and hence CaMK-II activation and a myristoylated autoinhibitory peptide (mAIP), a membrane permeant peptide which mimics the autoinhibitory domain of CaMK-II, inactivating CaMK-II. The lysates of the transfected cells were immunoprecipitated with an antibody against the GFP tag and then probed with Fli-I antibodies. The constitutively active CaMK-II co-precipitated a much greater amount of Fli-I than did the untreated wild-type CaMK-II suggesting that the increased level of activity resulted in a greater degree of association. Conversely, the KN-93 and mAIP inhibited lysates immunoprecipitated less Fli-I than the wild type sample and
considerably less than the constitutively active sample (figure 4A). Interestingly, when the samples were immunoblotted for GFP as a control, the FLAG/mGδeCon was expressed at a lower level that then other samples (not shown). Additionally, when the total CaMK-II activity in the samples was measured as a means of determining the relative concentration of CaMK-II it was seen that the inhibited CaMK-II samples were expressed at 5 times the levels of the wild type and twenty times the level of the constitutively active sample (figure 4B). This is consistent with what is normally seen; the constitutively active CaMK-II is expressed at lower levels, although the exact cause of this phenomenon is unknown. The percent of CaMK-II autonomy in the samples was determined enzymatically. It revealed that over 50% of the CaMK-II in the constitutively active sample was autonomous as compared to less than 20% in the untreated sample and no more than 2% in the inhibited samples (figure 4C). These three results create an interesting picture in which the CaMK-II in the constitutively active sample, although expressed at a lower level bound more Fli-I than did the inhibited samples, which were expressed at greater levels. The activation state of CaMK-II dictates whether it is capable of binding Fli-I.

Localization of Fli-I in NIH 3T3 Cells.

The confirmation of the interaction between CaMK-II and Fli-I in untransfected cells and the dose dependent interaction in transfected cells raised questions about the patterns of Fli-I localization in vivo. This was addressed by immunohistochemistry using Fli-I antibodies. NIH 3T3 cells were grown on glass coverslips then fixed in methanol.
Methanol rather than formaldehyde was the fixative used because the latter caused a loss of Fli-I antigenicity.

We observed that in untreated cells there are several distinct localization patterns. Fli-I is capable of nuclear translocation under certain conditions and was found in many cells (Figure 5A), but in many of the cells in the same sample it localized outside of the nucleus. This is consistent with published results on Fli-I localization patterns. It is believed that when Fli-I is located in the cytosol it is associating with actin based structures, such as those found at the leading edge of motile cells and in perinuclear regions (Figures 4B and C). The patterns observed in the experiments are consistent with the current models of Fli-I behavior. Fli-I is known to act as a transcriptional co-activator and nuclear receptor binding protein, both of which may be coupled to its nuclear translocation, but it is believed that this translocation occurs primarily when cells are cell-cycle arrested or non-motile. Although it was observed that Fli-I could assume one of several localization patterns, groups of neighboring cells seemed to generally exhibit similar patterns, which is likely the result of the cells responding to cues in their microenvironment which influence their behavior.

Fli-I Localizes to the Nucleus in Arrested Cells.

Since within a single culture Fli-I could assume one of several localization patterns it was a challenge to create a consistent trend throughout each experiment. Based on published findings that Fli-I is sequestered in the nucleus of arrested cells, it seemed logical that arresting the cells via serum deprivation would synchronize the entire population of cells in each experiment. With this treatment it was possible to induce
arrest throughout the culture, which caused the cells to display only nuclear localization patterns (Figure 6B and C). This induced arrest allowed more direct control of the cells’ progression through the cell cycle and their migratory state. It was also realized that greater regulation could be established if the cells were plated on glass coverslips that had been pre-incubated with fibronectin (FN). This allowed the cells to attach at a faster rate so it was possible to more directly regulate the cell density. The density of the culture was important because the cells are inhibited by contact and will readily arrest when they touch neighboring cell and cause Fli-I to translocate to the nucleus (Figure 6D).

**Fli-I Does Not Localize to the Nucleus in Serum Stimulated Cells.**

It was necessary to accurately characterize the behavior of Fli-I under various conditions before being able to assess the effects of CaMK-II activity on its localization. Serum starving cells overnight after they had been plated on FN coated coverslips was adopted as the standard practice for all of the Fli-I localization experiments. Once the cells had been arrested overnight, they were then released by stimulation with 10% FBS containing medium. Cells that were fixed at various time points post-stimulation showed an increasing return of Fli-I to the leading edges of the cells. Six hours after the cells were stimulated, Fli-I could be seen associating with actin arches throughout the cells (Figure 7C). It seemed as although by 15-16 hours the majority of the cell’s Fli-I is extra-nuclear, with some being localized in the perinuclear region and much of it localized to ruffles at the cell’s edge (Figure 7D). Although it is possible to induce exportation of Fli-I from the nucleus of arrested cells by stimulating, we still observed that once the cells begin to contact one another it induced Fli-I’s return to the nucleus,
meaning that the contact induced arrest is able to overcome the effects caused by the serum stimulation.

**CaMK-II Inhibition Also Targets Fli-I to the Nucleus.**

It was apparent from the initial localization experiments that Fli-I is a very dynamic molecule and that its localization is closely coupled to the activity state of the cell. Once protocols had been established for regulating Fli-I’s behavior as consistently as possible, it was feasible to determine the effects of CaMK-II activity on its localization. The first goal was to limit CaMK-II activity and assess the effects on Fli-I localization. This was accomplished with the CaMK-II inhibitor KN-93. The cells were plated in the same fashion as they were in the serum dependence experiments, but 10 μM KN-93 was added to the media at the same time point that serum free medium has previously been added. In a very similar manner to serum starvation, KN-93 caused Fli-I to shuttle into the nucleus. This effect was observed in a time dependent fashion with the effect being seen within four hours (figure 8B), continuing to translocate in at 12 hours (figure 8C), and being all in by 18 hours (figure 8D). A similar experiment was performed using 10 μM mAIP as the CaMK-II inhibiting agent. This treatment resulted in an effect very similar to the KN-93 dependent inhibition. The mAIP caused Fli-I to exit the cytoplasm of the cells and migrate to the nucleus within 90 minutes (Figure 9B) and being non-cytosolic by three hours (figure 9C). The mAIP had maximal effect within three hours, which was much faster than what was seen with the KN-93 treatment, but this is likely due to the differences in the two drugs’ specific modes of action. It was very interesting to be able to induce Fli-I localization patterns similar to those seen in
arrested cells by inhibiting CaMK-II. This complemented the Fli-I Western blot of the various CaMK-II activity treatments in an *in vivo* model.

**Fli-I Leaves the Nucleus in Artificially Activated CaMK-II Cells.**

Once it was clear that Fli-I could be sequestered to the nucleus by restricting CaMK-II activity, I wondered if I could reverse this localization by artificially activating CaMK-II. The first thought was to transfected cells with a constitutively active CaMK-II and localize Fli-I, which was the most straight forward approach to forcing Fli-I to return to the cytosol. Although we were able to successfully transfected the cells, there was no noticeable effect on Fli-I localization. It was observed however, that cells that had been transfected with the constitutively active construct had arrested, which resulted in Fli-I assuming nuclear localization. It was unclear if this effect was the result of the CaMK-II activity or another pathway, but it was apparent that it would not be an effective effective for demonstrating the effect of CaMK-II activity of Fli-I localization. The next method employed was to artificially activate endogenous CaMK-II by treating cells with the Ca$^{2+}$ ionophore ionomycin. Ionomycin causes Ca$^{2+}$ influx into cells, which results in CaMK-II activation. The cells were maintained in serum free media, which we had previously shown to cause Fli-I to transport to the nucleus. Although the culture was maintained in the absence of serum, treatment with ionomycin caused Fli-I to exit the nucleus and associate with stress fibers and in the perinuclear region of the cell. The transport of Fli-I out of the nucleus occurred in a time dependent fashion, with the effects becoming noticeable between 3 to 6 hours post-treatment (figure 10B) and seemingly complete by 16 hours (figure 10C) after the drug was added. When compared to the effects seen when
cells are treated with serum free media, the translocation of Fli-I in response to Ca\(^{2+}\)
establishes a clear link between it and cellular Ca\(^{2+}\) and when combined with the results
of CaMK-II inhibition it confirms that CaMK-II serves as a regulator of Fli-I.
Discussion

The role that CaMK-II plays in essential cellular functions is often underappreciated. Its high level of evolutionary conservation, broad patterns of expression, and unique regulation exemplify the essential role of CaMK-II in cellular function (Tombes et al., 2003). To identify new functions of the protein it is necessary to use open minded approaches. An unbiased process for achieving this goal is to discover new binding partners in the hope that they could be correlated to unknown functions. The MS/MS used in study was ideal for this type of screen.

The MS/MS screen is an excellent tool for the identification of binding partners, although the artificial nature of the system made it susceptible to erroneous results. Although false positive results were a possibility the identification of both CaM and CaMK-II in the screen validated the methodology. Although the data appeared genuine it was necessary to confirm the endogenous interaction. In particular Fli-I was a fascinating result and it had a commercially available high quality antibody against it, so it was the logical choice for study. Several additional targets were considered including tropomodulin-3 and cofilin, but the antibodies purchased against these proteins could not be confirmed as efficient in endogenous systems so they were not pursued further (data not shown).

Fli-I is a member of the Gelsolin Related Actin Binding Partner (GRABP) family. It is a 145 kDa protein that is highly conserved and expressed in all metazoans. The C-terminal side of Fli-I is a Gelsolin-like domain which gives Fli-I its actin binding and capping ability and the N-terminal half is a Leucine Rich Repeat (LRR), a conserved
domain known to be involved in protein protein interactions (Archer et al., 2005; Campbell et al., 2000). The gene was identified in *Drosophila melanogaster* mutants that did not develop the ability to fly. It was discovered that they were heterozygous mutants of the gene located on the 19F region of the X-chromosome and that the loss of flight was the result of a failure to successfully cross bridge the actin filaments of in-direct flight muscles. Homozygous Fli-I null mutants are embryonic lethal, which is a more severe phenotype than villin or double Cap-G and Gelsolin knockouts, both GRABPs family members. The lethality of the knockouts and the high degree of genetic conservation are illustrative of the importance of this molecule (Lee et al., 2004; Straub et al., 1996; Campbell et al., 2002).

Fli-I possesses a variety of functions. It is known to have actin binding function and to associate with actin based structures during development and cell motility (Davy et al., 2000; Davy et al., 2001). Additionally, Fli-I functions as a transcriptional co-activator through its interactions with nuclear receptor based complexes as identified during yeast-two hybrid screens (Archer et al., 2004; Lee et al., 2004; Liu and Yin, 1998; Archer et al., 2005). This broad range of functions is apparent by the intra- and extranuclear localization patterns it exhibits.

Fli-I lacks a domain that would allow it to respond to Ca$^{2+}$, which is something that the other members of the GRABP family possess (Fong and de Couet, 1999). Ca$^{2+}$ based signaling is fundamental to many cellular processes including regulation of cytoskeletal dynamics. Fli-I is a critical component of this process and although it is not directly regulated by Ca$^{2+}$ transients it could be controlled by them through its interaction with CaMK-II. It has been speculated that Fli-I could serve as an intermediate between
Ca\textsuperscript{2+} based signal transduction pathways and the regulation of cytoskeletal dynamics (Claudianos and Campbell, 1995; Davy et al., 2000; Davy et al., 2001).

The Fli-I antibody was specific in immunoblots of endogenous 3T3 and P19 lysates. This made it possible to probe for its presence in CaMK-II IPs and also to screen for CaM-II in Fli-I IPs. The CaMK-II IPs contained Fli-I on Immunoblots, which verified the interaction and was supported by the detection of CaMK-II in Fli-I IPs, both immunologically and enzymatically. The complementary nature of these experiments which were performed on native proteins corrected for the synthetic nature of the initial screens. The reciprocal verification of the interaction confirmed the legitimacy of the MS/MS result.

The authenticity of the interaction was apparent and seemed to have potential as molecularly significant, but nothing was known of the nature of the interaction. All of the MS/MS experiments contained Fli-I so it was not possible to discern any information from the variations in the screens. Since CaM-II function is closely coupled to its activation state, it was logical to wonder if that had an effect on the association.

Transfection of NIH 3T3 cells with GFP-tagged constructs offered several advantages in charactering the CaMK-II-Fli-I association. It made it possible to use a constitutively active variant and to immunoprecipitate against the GFP-tag which is more specific than antibodies against CaMK-II. The inhibited samples were also transfected to maintain consistency between the samples. These experiments illustrated the activity dependence of the interaction. The increased quantity of Fli-I bound to constitutively active CaMK-II and decreased association as a result of lowered activity suggest that the nature of the interaction makes it possible to determine the activation state of CaMK-II.
Little is currently known about the domains that are facilitating the interaction of these proteins, but it is clear that the activation state of CaMK-II somehow regulates the interaction. A likely possibility is that upon CaMK-II activation a Fli-I binding site is either created or exposed. Recent work on the crystal structure of CaMK-II suggests that activation causes the core domain of the CaMK-II holoenzyme to expand, which could expose a Fli-I binding site (Gaertner et al., 2004; Rosenberg et al., 2005). Fli-I possesses a traditional nuclear localization signal (PKKKRKV). It is possible that when Fli-I is bound to CaMK-II the sequence is obstructed, preventing the formation of a nuclear import complex. Although CaMK-II activation occludes Fli-I from the nucleus, the isozymes of CaMK-II examined in this study are non-nuclear, so they can not enter the nucleus to retrieve Fli-I. An explanation is that CaMK-II is not responsible for Fli-I’s export from the nucleus, but maintains its presence in the cytosol when it is active.

Fli-I is very active in its localization. Since it becomes sequestered to the nucleus of arrested cells, it made sense that the translocation could be correlated to its association with CaMK-II. Localizing Fli-I in control cultures proved to be a challenging task. To accomplish this it was necessary to synchronize the progression of the cell’s cycles and to closely control their density. This was achieved by arresting and releasing them and culturing them on FN. Much information about the nature of their binding became apparent when the localization patterns of Fli-I were characterized during various cell activity states and comparing them to multiple levels of CaMK-II activity. In arrested cells Fli-I is found primarily in the nucleus and although some can be detected outside the nucleus, it is non-specifically present. The location of Fli-I is similar in arrested cells and
CaMK-II inhibited cells suggesting that under naturally occurring conditions their interaction may be somehow correlated to the cell cycle and to cytoskeletal dynamics.

It was asked if Fli-I was not only a CaMK-II binding partner, but also a substrate. Phosphorylation could represent an additional level of regulation in addition to the activity dependent binding between the two proteins. There are five potential CaMK-II consensus sequences in Fli-I, but the structure of the protein is unknown so they may not actually be phosphorylatable. This was tested by partially purifying CaMK-II and combining it with Fli-I IPs in the presence of [γ-32P-ATP]. The assay was crude in nature, but the initial autoradiographs indicate that Fli-I is not a CaMK-II substrate (data not shown). Although the reactants were not completely pure and the results can not be considered definitive, they are still convincing. Binding that is dependent on autophosphorylation is a seen throughout the cell, such as receptor tyrosine kinases, which autophosphorylate when they bind ligands to mediate their signal transduction by only binding signaling proteins when they are phosphorylated. It is possible that CaMK-II binds Fli-I through a similar mechanism.

A clue to the function of the interaction is another binding partner identified in the screen, the protein known as leucine rich repeat region of Fli-I interacting protein II (LRRFIP2). Little had been known of the function of LRRFIP2 other than its association with Fli-I, until recently when it was shown to be a positive regulator of the Wnt signaling pathway in yeast two-hybrid screens (Liu et al., 2005; Liu et al., 1998). It was shown that LRRFIP2 is a positive effector of the canonical Wnt pathway through an interaction with Disheveled (Dvl). When a Wnt ligand binds to its receptor, Frizzled
(Fz), Dvl is activated and inhibits GSK-3β, releasing β-catenin, which then translocates to the nucleus and interacts with TCF to function as a transcription factor.

If Fli-I is localized in the nucleus, LRRFIP2 can not interact with Dvl to up regulate β-catenin mediated transcription. Since Fli-I is extra-nuclear in a manner proportional to CaMK-II activity, Wnt signal transduction would be elevated at times of high activity. This model suggests that extracellular signaling has regulators inside of the cell, which would mean that the pathway has increased sensitivity to a variety of stimuli. In transformed cells CaMK-II expression is down regulated (Tombes et al., 1999), meaning that extracellular signals which are transduced by the canonical Wnt pathway are not efficiently relayed, which could contribute to the carcinogenic characteristics exhibited by these cells. This could be a previously undescribed regulatory pathway that needs to be further characterized.

A number of additional experiments are necessary to more specifically define the nature of the interaction. They include:

- Adding the GFP tag to Fli-I
  
  Fluorescently tagging Fli-I would make it possible to characterize its movement in living cells and to analyze its kinetics into and out of the nucleus under different conditions

- Colocalizing CaMK-II and Fli-I
  
  The localization of Fli-I can be complimented by showing the position of CaMK-II and Fli-I at the same time under the various experimental conditions that Fli-I alone was localized during. This could be done by imaging each protein in different fluorescent channels.
• **Domain Mapping of the CaMK-II-Fli-I interaction**

To further understand the properties of the interaction it is essential to know where they binding is occurring. This can be done by using deletion constructs, which create truncated proteins and determine which domains are essential for binding. This could be followed up by using point mutants to define the required amino acid residues to facilitate binding.

• **In vitro translation and direct binding assay**

Using reticulolysate it is possible to translate proteins *in vitro* and test for their interaction. mRNA made from CaMK-II and Fli-I could be translated and the interaction could be confirmed. This would eliminate any question of whether or not the association of Fli-I and CaMK-II is direct or indirect. There are commercially available kits for this type of assay which makes this a practical and worthwhile experiment.

• **Biochemical Characterization of the CaMK-II-Fli-I interaction**

Currently nothing is known of the biochemical aspects of the interaction and will need to be defined. This will include studying the kinetics of the Fli-I translocation, its rate of turnover, the binding affinity of the two proteins, and identifying which growth factors are essential to keep Fli-I in the cytosol. These experiments will reveal in greater detail the already confirmed interaction and offer insight to its intracellular functions.

• **Gene expression effected by the CaMK-II-Fli-I interaction**

Using CaMK-II inhibitors to minimize its activity and RNAi to lower Fli-I expression gene expression patterns can be characterized using DNA
microarray analysis. Genes that are down regulated when both CaMK-II is inhibited and Fli-I expression is minimized could potentially be dependent on the interaction.

- The effects of CaMK-II on Wnt pathway components

Since it is possible that through its interaction with Fli-I and LRRFIP2 that CaMK-II could be effecting Wnt based transcription it will be necessary to examine what is happening to components in the pathway. This can be done immunologically to look at the phosphorylation state of GSK-3β or for the presence of β-catenin, for example.

- Whole organism studies

It is important to also characterize the effects of this interaction in a whole organism. Since both proteins are developmentally essential a good system for studying their roles during development would be the zebra fish, *Danio rerio*.

These experiments will make it possible to further describe the CaMK-II and Fli-I interaction. This work is the first description of what has the potential to be a new intracellular regulatory pathway. Both of these proteins are ubiquitously expressed and highly conserved throughout all metazoans. This is reflective of their cellular importance and means that their interaction could be quite ancient. The complexity with which each is regulated is profound, but is even more intricate when they cooperate. The identification of Fli-I and CaMK-II by MS/MS is exciting not only because of its cellular significance but because it is representative of a new class of biochemical approaches.
which will make it possible to characterize the cross talk that exists between well established cellular pathways.
Table 1. MS/MS Results Table. The Names, NCBI protein accession numbers, and total number of peptides detected of the proteins identified as potential CaMK-II binding partners using MS/MS. The total number of peptides is the number of peptides that were analyzed and analyzed by SEQUEST to determine the legitimacy of the result.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>NCBI Accession Number</th>
<th>Total Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMK-II</td>
<td>Q8K4W4, KCCB</td>
<td>42</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>CALM</td>
<td>14</td>
</tr>
<tr>
<td>Flightless-1</td>
<td>Q8V144</td>
<td>24</td>
</tr>
<tr>
<td>Myosin Heavy Chain</td>
<td>MYH9</td>
<td>35</td>
</tr>
<tr>
<td>β5-Tubulin</td>
<td>TBB5</td>
<td>14</td>
</tr>
<tr>
<td>γ-Actin</td>
<td>ACTG</td>
<td>12</td>
</tr>
<tr>
<td>Tubulin 1</td>
<td>TBA1</td>
<td>12</td>
</tr>
<tr>
<td>Tropomodulin-3</td>
<td>TMO3</td>
<td>30</td>
</tr>
<tr>
<td>β-Actin</td>
<td>ACTB</td>
<td>15</td>
</tr>
<tr>
<td>LRRFIP1</td>
<td>Q8BLV4</td>
<td>12</td>
</tr>
<tr>
<td>Vimentin</td>
<td>P08670</td>
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</tr>
<tr>
<td>β6-Tubulin</td>
<td>TBB6</td>
<td>6</td>
</tr>
<tr>
<td>LRRFIP2</td>
<td>Q9607</td>
<td>13</td>
</tr>
<tr>
<td>Myosin Light Chain</td>
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<tr>
<td>Cofilin</td>
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</table>
Figure 1. **Linear FLAG/GFP-CaMK-II map.** The FLAG-tag was fused to the N-terminal side of the GFP tag and is 8 residues long. The GFP-tag is 245 amino acids long and is located on the N-terminal side of CaMK-II. The catalytic domain of CaMK-II is just over 250 amino acids long and is adjacent to the regulatory domains, which is 30 residues in length. The variable domain differences in size between isoforms and is where alternative splicing occurs. The C-terminal most domain is the association domain.
Figure 2. The FLAG elution process eliminates non-specifically bound proteins. Panel A is a GFP western blot of the FLAG eluents. Lane 1 is the lysate of the transfected cells and lane 2 is the first elution and lane 3 is the second. Panel B is a SYPRO Ruby total protein stain of the FLAG eluents. The bands at Lane 1 is the lysate of the transfected cells, lane 2 is that first eluent and lane 3 is the second. In both images the bands at 25 and 50 kDa correspond to the IgG light and heavy chains and the band at 80 kDa is the Mₐ of FLAG/mGδₑWT. It is clear that the majority of the proteins are cleared from the lysate.
Figure 3. *Reciprocal IP of endogenous CaMK-II and Fli-I*. In panel A, CaMK-II antibodies were used against untreated lysates. Lane 1 is whole cell lysates, lane 2 is a β CaMK-II antibody and lane 3 is a δ CaMK-II antibody immunoprecipitation of whole cell lysate. All three were probed with an anti-Fli-I antibody and show a band in the 145 kDa region, corresponding to the Mr of Fli-I. Panel B shows a Fli-I IP that was probed with biotinylated CaM. Lane 1 is whole cell lysate and lane 2 is a Fli-I IP. There are multiple bands in the 54-60 kDa region, representative of the mass of various CaMK-II isozymes. Panel C shows a graph of the amount of CaMK-II activity that was recovered from a Fli-I IP as compared to an integrin IP, which is known to not bind CaMK-II.
Figure 4. CaMK-II activity dependent association with Fli-I. NIH 3T3 cells were transfected with FLAG/GFP-tagged δE CaMK-II. In panel A, lane 1 is non-immunoprecipitated untransfected lysate to illustrate input, lane 2 is FLAG/mGδEWT, lane 3 is FLAG/mGδECon, lane 4 is FLAG/mGδEWT with 10 μM KN-93, and lane 5 is FLAG/mGδEWT with 10 μM mAIP. Lanes 2-5 were immunoprecipitated using an anti-GFP antibody then western blotted for Fli-I. Lane 3 has more Fli-I associated than does the control, lane 2 and much more than the lanes 4 and 5, the CaMK-II inhibited samples. The relative amounts of CaMK-II expressed in the samples as determined enzymatically are represented in panel B. It is interesting to note that the KN-93 and mAIP treated samples are expressed at a much higher level, but had much less Fli-I and FLAG/mGδECon was the lowest and had the most Fli-I bound. Panel C shows the CaMK-II autonomy of the samples and illustrates that greater autonomy, which corresponds to greater quantity of Fli-I.
Figure 5. Fli-I localization in NIH 3T3 cells. Fli-I can be specifically visualized in NIH 3T3 cells using Texas Red conjugated antibodies. Fli-I is a very dynamic molecule that localizes in a variety of patterns that are correlated to the cell’s activities. It can be entirely in the cytosol in cells that are motile and active (panel A). Fli-I can also assume a heterogeneous localization pattern, both in and out of the nucleus, in a single culture (panel B). It can also be primarily nuclear, which is suggestive of cell arrest (panel C). Scale bar represents 50 μm.
Figure 6. *Fli-I localizes to the nucleus in arrested cells.* During times of activity Fli-I is found outside of the nucleus and associating with actin based structures (panel A). When cells arrest is induced with serum starvation, Fli-I begins to translocate to nucleus and can be visualized there within 6 hours (panel B). Twelve hours after serum starvation, the majority of Fli-I is in the nucleus and that which remains in the nucleus is nonspecifically localized (panel C). If arrest is induced by contact, NIH 3T3 cells arrest. Cells at a high density have Fli-I localized to the nucleus and cytosolically in an undefined manner (panel D). Scale bar represents 50 µm.
Figure 7. Fli-I exits the nucleus in serum stimulated cells. Fli-I is sequestered in the nucleus of serum starved cells (panel A). If these cells are stimulated with serum containing medium, Fli-I can begin to be seen localizing along actin based structures in the cytosol within 6 hours (panel b). Twelve hours after stimulation, Fli-I can still be visualized in the nucleus, but at a lower intensity and more dynamically in the cytosol (panel C). Eighteen hours after serum has been added to the culture all of the Fli-I has translocated to the cytosol and is associated with actin structures and along the cell edges (panel D). Scale bar represents 50 μm.
Figure 8. **KN-93 causes Fli-I to translocate to the nucleus.** NIH 3T3 cells that are active exhibit cytosolic Fli-I localization patterns (panel A). If these cells are treated with 10 μM KN-93, Fli-I begins to be transported into the nucleus and can be seen there within 4 hours of treatment (panel B). Twelve hours after KN-93 is added to the culture the majority of Fli-I is localized to the nucleus, though some remains outside the nucleus, but is less specifically localized (panel C). Eighteen hours post-treatment Fli-I is sequestered to the nucleus (panel D). Scale bar represents 50 μm.
Figure 9. *Fli-I is sequestered to the nucleus by mAIP CaMK-II Inhibition.* In a manner very similar to treatment with KN-93, 10 μM mAIP causes Fli-I to translocate to the nucleus. It associates with various structures in the cytosol in active cells (panel A). 90 minutes after active cells are treated with mAIP much of the cell’s Fli-I has moved into the nucleus (panel B). Three hours after the mAIP treatment, Fli-I has become sequestered in the cell’s nucleus (panel C). Scale bar represents 50 μm.
Figure 10. *Fli-I is not sequestered to the nucleus in arrested cells when CaMK-II artificially activated.* In serum free conditions Fli-I is sequestered to the nucleus. It is possible to reverse this localization by treating cells with the Ca$^{2+}$ ionophore ionomycin which artificially activates CaMK-II. Prior to treatment with ionomycin, Fli-I is localized in the nucleus in serum free conditions (Panel A). When the culture is treated with ionomycin for 12 hours, a cytosolic presence of Fli-I can begin to be seen (Panel B). 18 hours after the treatment with ionomycin all of the cell’s Fli-I in the cytosol and is in the regions where it is found in the cell’s cytosol even though the cells are arrested (Panel C).
Figure 11. Potential Model of CaMK-II and Fli-I’s interaction. A potential graphical representation of the CaMK-II-Fli-I interaction. When Ca\(^{2+}\) rises in the cell, it becomes bound by the Ca\(^{2+}\) binding protein CaM. Ca\(^{2+}/CaM\) is bound by CaMK-II and becomes catalytically active. In its active state CaMK-II is capable of binding Fli-I, which is otherwise sequestered to the nucleus. When Fli-I exits the nucleus and CaMK-II is active they associate and travel to actin filaments throughout the cell and at its edges.
References


Tombes, R.M., E. Westin, S. Grant, and G. Krystal. 1995. G1 Cell Cycle Arrest and Apoptosis are Induced in NIH 3T3 Cells by KN-93, an Inhibitor of CaMK-II (the Multifunctional Ca²⁺/CaM Kinase). *Cell Growth and Differ.* 6:1063-1070.


Vita

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