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The MEF2 family of transcription factors is regulated by class II histone deacetylaces in the nucleus. MEF2-dependent gene expression in cardiomyocytes is augmented by the 14-3-3 chaperone family which binds and sequesters class II HDACs in the cytoplasm upon the activation of CaM kinase I & IV. A 14-3-3β mutant was made by conservatively substituting aspartate for serine 60 and serine 65. In MEF2 enhancer-reporter transfection assays, expression of the 14-3-3β double mutant silenced transcription mediated by CaM KI & IV in both cardiomyocytes and vascular smooth muscle cells. Co-expression of the 14-3-3β double mutant was also able to suppress MEF2 enhancer activation by phenylephrine in cardiomyocytes and vascular smooth muscle cells. Mammalian two-hybrid cloning of the 14-3-3β wild-type and double mutant genes will allow analysis of the protein-protein interaction between the different 14-3-3β monomers. These data suggest that 14-3-3β plays a critical role in the silencing of MEF2 mediated hypertrophy-sensitive gene transcription.

THE ROLE OF 14-3-3 IN THE SIGNALING OF CARDIAC HYPERTROPHY

Joel J. Ellis, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Chair, Department of Biomedical Sciences

Dean, Graduate School of Biomedical Sciences

THE ROLE OF 14-3-3 IN THE SIGNALING OF CARDIAC HYPERTROPHY

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Joel J. Ellis, B.S.

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CHAPTER 1

INTRODUCTION

Cardiac hypertrophy is the result of an up-regulation in the expression of a battery of cardiac growth related and contractile protein genes caused by various forms of stress (i.e. volume overload, chronic hypertension, myocardial stress from disease states, coronary occlusion, myocardial infarction, or severe mechanical overload). One of the first effects in the on-set of hypertrophy is the re-emergence of embryonic genes that are normally present only in early neonatal development of a fetus. This includes a switch from the expression of α-myosin heavy chain to β-myosin heavy chain, the presence of α-cardiac actin, and the re-emergence of α-skeletal actin [1, 2]. In addition, atrial naturetic factor (ANF), also known as atrial naturetic peptide (ANP), and b-type naturetic peptide (BNP) are up-regulated in response to increased vascular tension [3, 4]. Since the fetal genetic program constitutes a discreet switch between the expression of fetal isoforms form the adult counterparts, fetal genes have been instituted as genetic markers for hypertrophy induction [5]. Induction of these markers usually leads to increased ventricular wall thickening, cardiomyocyte mass gains, and ultimately reduced cardiac output [6, 7].

Increased intracellular calcium concentration has been identified as one of the major signals for cardiac hypertrophy [8]. Recent studies have shown that activation of

the calcium/calmodulin (CaM)- dependent protein kinase signaling cascade results in the activation of hypertrophy sensitive gene expression [9]. Calcinurin, a CaM-dependent phoshpatase, activates the transcription factor NFAT3 in cardiomyocytes [10-13]. CaM kinase I and IV have been shown to activate the myocyte enhancer factor-2 (MEF-2) family of transcription factors [14]. The active forms of MEF-2 transcription factors are a result of the activation of the calcium/calmodulin-dependent protein kinase cascade resulting in increased MEF-2 dependent transcription of hypertrophy sensitive genes [15-18].

Previous work by us and others has shown that another member of the CaM kinase family, CaM kinase II (CaM KII) is capable of silencing the hypertrophic induction of calcinurin, CaM kinase I or CaM kinase IV [19]. CaM KII is a multifunctional serine/threonine kinase that has been implicated in the regulation of hypertrophy sensitive genes [20]. CaM kinase II δB (nuclear isoform) has been shown to modestly induce ANF in cardiomyocytes [21]. Recently, we showed that a constitutively over expressed cytoplasmic CaM kinase II isoform (δC) could effectively silence hypertrophy induction in rat neonate cardiomyocytes. It was shown that CaM kinase II-mediated silencing could be halted with an inducible expression system. This resulted in the re-expression of hypertrophy sensitive genes in the presence of either calcinurin, CaM kinase I or CaM kinase IV [19]. These data suggested that we begin to explore the possible mechanisms by which a cytoplasmic isoform of CaM kinase II would be able to silence hypertrophy.

Transcriptional events are regulated in part by the acetylation/deacetylation of histones resulting in chromatin remodeling. When core histones are acetylated by histone acetyl transferases (HATs), chromatin is relaxed and the DNA is exposed allowing molecular events (transcription, replication, etc.) to occur. Histone deacetylases (HDACs) repress transcriptional events by associating with co-repressor complexes that can deacetylate core histones as well as transcription factors [22-26]. It has been shown that class II HDACs can partner with transcription factors. Specifically, class II histone deacetylases (HDAC4 and 5) associate with the transcription factor MEF2 to repress MADS-box dependent gene promoters [27]. HDACs 4 and 5 are expressed predominantly in cardiac, skeletal and smooth muscle as is MEF2 [28-30]. Under normal conditions class II HDAC repression is predominant in cardiomyocytes preventing the expression of hypertrophy sensitive genes. This is accomplished by the formation of HDAC/MEF-2 complexes in the nucleus preventing MADS box dependent transcription. The interaction of MEF-2 with class II HDACs is disrupted by CaM kinase I and IV mediated phosphorylation of HDAC. Once phosphorylated, HDAC is translocated to the cytoplasm via the chaperone 14-3-3 and MEF-2 is then able to drive transcription of MADS box-dependent promoters [14].

The 14-3-3-chaperone protein family is ubiquitous and highly conserved throughout most mammalian species and has been found to be present on almost all tissue types [31]. There are over fifty signaling proteins that have been demonstrated to bind with 14-3-3 solidifying its role as a key regulatory molecule involved in transduction pathways, cell cycle regulation, apoptosis, and cellular growth [32, 33]. The binding of

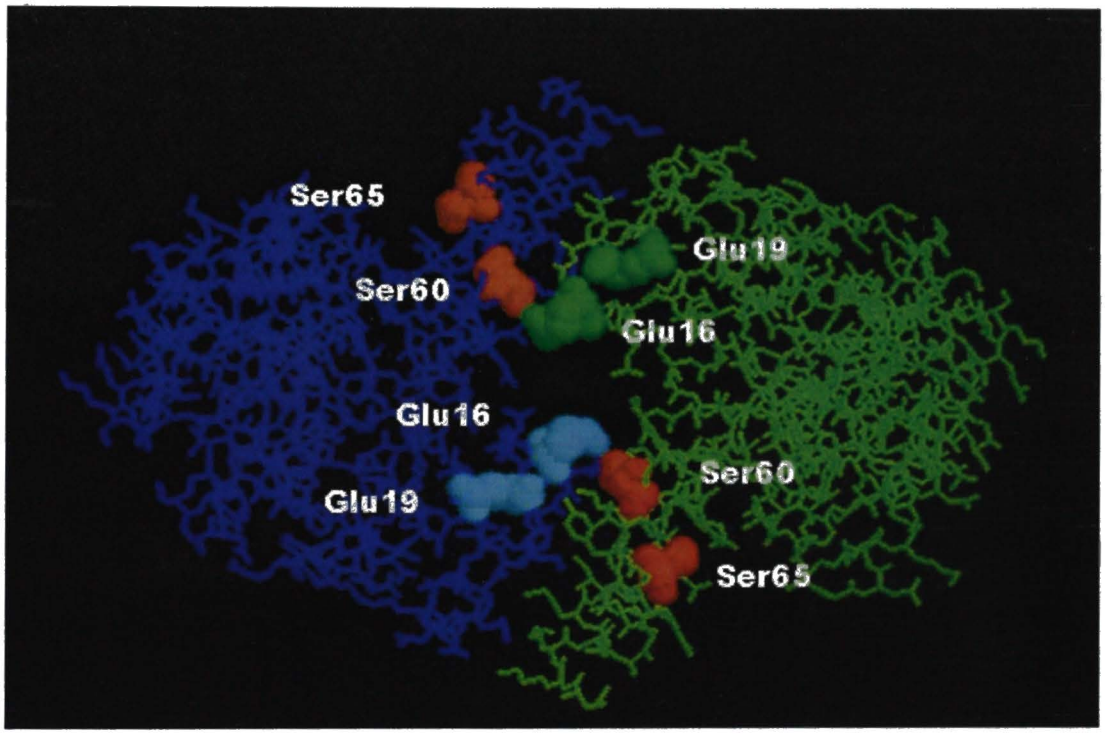
14-3-3 to other cellular proteins by recognition of phospho-protein motifs, results in the translocation of these partner proteins from one sub-cellular compartment to another. Ligands of 14-3-3 serve a broad range of functions within cells such as phosphatases, kinases, transcription factors, and oncoproteins [34]. The ability to act as a chaperone protein, mediated by Crm I, to export molecules from the nucleus to the cytoplasm adds to the evidence of 14-3-3 as a regulatory protein in cardiac hypertrophy signaling [35-38]. Seven isoforms of mammalian 14-3-3 have been found (β, ε, γ, η, σ, τ, ζ). Of these 14-3-3 isoforms beta, gamma and epsilon are predominantly expressed in heart.

Structurally, 14-3-3 proteins exist as homo or heterodimers and crystallographic data have been collected revealing many insights into the structure and function of 14-3-3. The structure of 14-3-3 is made-up of a bundle of nine helices ordered in an antiparallel fashion. There is a C-terminal amphipathic groove that is thought to be the interaction domain of most of its target proteins due to the highly conserved nature of this region [39, 40]. The N-terminal of 14-3-3 is involved in the formation of dimers, and its ability to bind two ligands simultaneously suggest that 14-3-3 dimers exist as both homo and heterodimers [41-43]. Interestingly, in the 14-3-3β region of dimerization, there are two tandem consensus CaM kinase II phosphorylation motifs (Serines 60 and 65). Immediately adjacent to the phosphorylation sites on the partnering subunit lie two glutamate residues (Figure 1). It is our hypothesis that phosphorylation of the CaM kinase II sites will disrupt homodimerization of 14-3-3 due to charge repulsion and steric hindrance resulting in the inability to sequester class II HDACs in the cytoplasm even in the presence of CaM kinase I or IV signaling. We believe that point mutations of 14-3-

3β changing the serines 60 and 65 to aspartates will mimic phosphorylation of CaM kinase II and silence MEF-2 mediated hypertrophy sensitive gene induction.

The ability to prevent or reverse a hypertrophy state in cardiac cells could be a key step toward our ability to treat heart disease and the debilitating loss of pump function that hypertrophy causes. Several pathways such as NFAT activation and the CaM kinase signaling cascade have been shown to drive hypertrophy-sensitive gene transcription. I suggest that a return to a repressed state, such as our proposed 14-3-3 point mutation model, could give insight into possible applicable mechanisms to treat heart disease. The work for this Internship Practicum is based on the hypothesis that global silencing of hypertrophy sensitive genes by CaM kinase II does, in fact, work though direct phosphorylation of 14-3-3 within the HDAC/14-3-3 complex in the cytoplasm.

Figure 1. A crystallographic model of the 14-3-3β homodimer shows serine residues 60 and 65 colored red and the complimentary glutamate residues 16 and 19 colored green. Using a commercial protocol (QuickChange™, Stratagene) I will point mutate the conserved serine 60 and 65 residues to aspartate creating a 14-3-3 double mutant.


Ser65
Ser60
Glu16
Glu19
Glu19
Glu16
Ser60
Ser65

CHAPTER 2

POINT MUTATIONS OF 14-3-3β

Introduction

Examination of the primary amino acid sequence of 14-3-3β has revealed two CaM KII phosphorylation sites. When these sites are phosphorylated, the 14-3-3β homodimer will be destabilized. I have designed DNA primers for in vitro site directed mutagenesis of the two R-X-X-S (Arginine-X-X-Serine) CaM KII phosphorylation sites (serine 60 and serine 65) changing them to aspartates. The original amino acid sequence is :NH_2-R-R-S-**S**-W-R-V-I-**S**-S-I-E-$COOH^-$ and the double mutated sequence is: NH_2-R-R-S-**D**-W-R-V-I-**D**-S-I-E-$COOH^-$. Two mutated versions of 14-3-3β will be produced, one with only serine 60 mutated and the other with both serine 60 and 65 mutated. Using a commercial protocol (QuickChange™, Stratagene) I generated expression plasmids containing both mutated versions of 14-3-3β. Expression plasmids were grown in bacteria for selection of mutation per the QuickChange™ protocol and positive colonies were selected, amplified, and plasmid DNA was harvested. The resulting DNA was sequenced to confirm mutations.

The principle behind this mutagenesis kit is to incorporate the desired point mutation into the gene without having to cleave, mutate and re-ligate DNA. Forward and

reverse primers were designed in the same way that PCR primers are designed. Incorporated into the primers is the changed sequence that will result in the point mutation. The reaction runs just like a PCR reaction with denaturing, annealing and elongation steps. The reaction is cycled from 12-20 cycles depending on your construct. After amplification of the point mutated plasmid, the reaction is digested with Dpn I restriction enzyme to chop up the original un-mutated DNA template. Dpn I recognizes methylated DNA. Since the newly amplified mutant DNA is not methylated, the original template DNA is degraded and only the mutated DNA remains. This DNA is then used to transform bacterial cells for quantitative DNA analysis.

Methods

Point Mutation of Serine 60 to Aspartate

Using a commercially available point mutation kit (QuickChange™, StrataGene) I set-up reactions to change serine 60 to aspartate creating a conservative point mutation within the 14-3-3β gene. The mutation reaction was set-up as follows:

Control	14-3-3β
5μl 10X reaction buffer	5μl 10X reaction buffer
2μl pWhiteScript 4.5 control plasmid	2μl of (5, 10, 20, 50) ng of dsDNA template
1.25μl of primer #1 (100μg/μl)	1.25μl of Ser 60 primer #1 (100μg/μl)
1.25μl of primer #2 (100μg/μl)	1.25μl of Ser 60 primer #2 (100μg/μl)
1μl of dNTP mix	1μl of dNTP mix
38.5μl ddH_2O	38.5μl ddH_2O
50μl total reaction (plus polymerase)	50μl total reaction (plus polymerase)

The sequence of the SER60ASP(A) primer was 5'-ggcgcccgctctgactggcgtgtcatctc -3' and the SER60ASP(S) primer was 5'-gagatgacacgccagtcagagcggcgggcgccc-3'. To each point mutation reaction 1μl of PfuTurbo polymerase was added and the reactions were stored on ice. The cycle reaction for the point mutation was set-up as follows:

1. 95°C for 30 seconds
2. 95°C for 30 seconds*
3. 55°C for 1minute*
4. 68°C for 15 minutes*
5. Hold at 4°C for ≥ 2 minutes

* Steps 2-4 were repeated for 17 cycles. This reaction was performed in an Eppendorf Mastercycler® Gradient thermal cycler. After the reaction cycle was completed all five reactions were digested with 1µl of Dpn I restriction enzyme (10U/µl) at 37°C for 1 hour.

Transformation of Bacteria with Point Mutated Plasmid DNA

Epicurian coli XL1-Blue super competent cells were thawed on ice for 15 minutes (started before Dpn I digestion was complete). Five sterile Falcon 2059 (10ml) polypropylene tubes were pre-chilled on ice. 50µl of XL1-Blue cells were placed into chilled tubes with 1µl of Dpn I digested mutation reaction DNA and incubated on ice for 30 minutes. The cells were then heat pulsed for 45 seconds in a 42°C water bath then placed immediately on ice for 2 minutes. Each tube received 0.5ml of NZY+ broth and was shaken and incubated at 225 rpm at 37°C for 1 hour.

An IPTG and X-gal solution was made up in 100µl of NZY+ broth. 20µl of 10% X-gal and 20µl of 100mM IPTG was added to 100µl of NZY+ broth. The solution was spread onto control agar/ampicillin plate and allowed to dry. Agar culture plates were made with 25g/L Luria Broth Base (LB), 15 g/L of agar and 1ml/L of 100mg/ml of ampicillin. After incubating for 1hour, 125µl of culture was placed and spread onto 4 plates for each mutation reaction. The control reaction was plated with 250µl on one culture plate. The plates were allowed to dry (~30 minutes) and then incubated at 37°C overnight.

Colony Selection and DNA Characterization

After overnight incubation, 5 positive colonies were selected from the culture plates and were named 1A, 2B, 3C, 4D, and 5E. The picked colonies were cultured in 2 10ml sterile culture tubes with 3ml of LB + ampicillin each (10 total culture tubes). The cultures were incubated in a shaker at 37°C and 225 rpm overnight. After overnight incubation, freeze down samples were made for storage in -80°C freezer (freeze downs were made by adding the culture cells to 60% glycerol in a 4:1 (Cells to glycerol) dilution). The remaining culture volume was placed into 1.5ml microfuge tubes and the cells were pelleted at 10,000 x g for 5 minutes. The supernatant was discarded and plasmid DNA was purified using Quiagen mini-prep spin column commercial protocol. Final elution of DNA was with 50µl of filter-sterilized ddH_2O and the samples were stored in -20°C freezer. Using a Beckman DU-68 UV spectrophotometer, the DNA concentration of each sample was determined at A = 260λ (Absorbance at 260 x 50 x 75). The DNA concentration of the samples were calculated as follows:

1A – 248ng

2B – 293ng

3C – 387ng

4D – 319ng

5E – 495ng

The Serine 60 mutation DNA for all five samples was sent to Iowa State University sequencing facility for confirmation of the point mutation (figure 2).

Point Mutation of Serine 65 to Aspartate

After the point mutation of serine 60 was confirmed by sequencing, the mutation of serine 65 to aspartate was set-up using the serine 60 mutated DNA as the reaction template. The serine 65 mutation primers SER65ASP(S) 5'-gtcttctgctcaatgctgtcgatgacac gccagtcag-3' and SER65ASP(A) 5'-ctgactggcgtgtcatcgacagcatt gagcagaagac-3'were used. The same QuickChange™ commercial protocol was followed as previously described for the serine 60 conservative point mutation. The serine 65 mutagenesis reaction was set-up as follows:

Control	**14-3-3β**
5μl 10X reaction buffer	5μl 10X reaction buffer
2μl pWhiteScript 4.5 control plasmid	2μl of (5, 10, 20, 50)ng of dsDNA template
1.25μl of primer #1 (100μg/μl)	1.25μl of Ser 65 primer #1 (100μg/μl)
1.25μl of primer #2 (100μg/μl)	1.25μl of Ser 65 primer #2 (100μg/μl)
1μl of dNTP mix	1μl of dNTP mix
38.5μl ddH_2O	38.5μl ddH_2O
50μl total reaction (plus polymerase)	50μl total reaction (plus polymerase)

The mutated DNA was processed and bacterial cells were transformed as previously described. Colonies were selected and the plasmid DNA was purified following the Quiagen mini-prep spin column commercial protocol. Using a Beckman

DU-68 UV spectrophotometer, the plasmid DNA concentration of each sample was determined at A = 260λ. The concentration of the samples were calculated as follows:

1A2 = 134ng/μl

2B2 = 151ng/μl

3C2 = 155ng/μl

4D2 = 317ng/μl

5E2 = 190ng/μl

The Serine 65 double mutation DNA for all five samples was sent to Iowa State University Sequencing Facility for confirmation of the point mutation (figure 3).

Maxi-prep Plasmid Purification of Mutated 14-3-3β DNA

After conformation of the double point mutation by sequencing, starter cultures were made by placing 15μl of sample 1A – mutant #1(serine 60) and 15μl of sample 1A2 – mutant #2 (serine 60 and 65) into separate 10 ml sterile culture tubes containing 1ml S.O.C. nutrient rich culture media. The cultures were incubated in a shaker for 1hour at 225 rpm and 37°C. After 1 hour, 100μl of the starter S.O.C. culture was placed into 250ml of 25g/L LB and 1ml/L of 10mg/ml ampicillin culture flask. The two cultures were incubated at 37°C in a shaker at 225 rpm overnight. The culture solution was placed into sterile 40ml Oakridge tubes and pelleted at 6,000 x g for 10 minutes in a Sorvol RC-5B refrigerated ultracentrifuge. The supernatant was discarded and the plasmid DNA was purified following Quiagen Maxi-prep commercial protocol. Final elution of the DNA was with 350μl of 10mM Tris-Cl, pH 8.0. The 14-3-3β mutant 1 and

mutant 2 DNA was stored at -20°C. Using a Beckman DU-68 UV spectrophotometer, the DNA concentration of each sample was determined at A = 260λ. The concentration of the samples was determined as follows:

Mutant 1 – 1.959μg/μl

Mutant 2 – 2.322μg/μl

Restriction Digestion of Mutated 14-3-3β DNA

DNA of both single and double 14-3-3β mutations were digested with Not I and EcoR I enzymes to verify the correct DNA purification. With this enzyme digestion we expected to see a 750 base pair fragment. The digestion reactions were set-up as follows:

14-3-3β mutant 1	**14-3-3β mutant 2**
1μl mutant 1 DNA (1μg)	1μl mutant 2 DNA (1μg)
1μl 10X React 3 Buffer (Gibco BRL)	1μl 10X React 3 Buffer (Gibco BRL)
7.5μl ddH_2O	7.5μl ddH_2O
0.5μl Not I enzyme	0.5μl Not I enzyme
10μl total reaction	10μl total reaction

After 1.5 hours of incubation at 37°C, 1.1μl of Multi-Core 10X buffer (Promega) and 0.5μl of EcoR I enzyme was added to each digestion reaction and incubated another 1.5 hours at 37°C. 1μl of 6X DNA loading dye was added to the digestions and they were run on a 0.8% agarose gel at 35mA and 75 volts for 1 hour. The agarose gel was then

stained with ethidium bromide (EtBr) for 10 minutes and the DNA was viewed by UV trans-luminator and documented with AlphaImager software and camera (figure 4).

Results

Sequence Verification

After making the 14-3-3β point mutation using the QuickChange™ mutagenesis kit, the point mutated plasmid DNA was sequenced and the point mutations were verified by sequence alignment with the original wild type sequence. Four DNA samples that were sequenced had the single point mutation incorporated into the construct sequence. By using the single mutation DNA as a template the second point mutation was made and the DNA was also verified by sequence analysis. Four mutated DNA samples all contained the double mutation of 14-3-3β. The resulting constructs are a 14-3-3β single mutation and a 14-3-3β double mutation.

Figure 2. The nucleotide sequence for four of the selected transformed colonies containing the serine 60 point mutation to aspartate. Colonies 1A, 3C, 4D, and 5E all show the point mutation for aspartate at codon 60 (red box) of the 14-3-3β sequence. The 14-3-3β wild-type sequence shows the original codon 60 as serine.

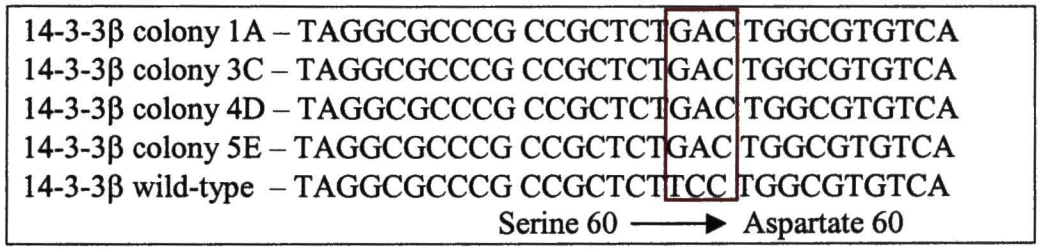

14-3-3β colony 1A – TAGGCGCCCG CCGCTCTGAC TGGCGTGTCA
14-3-3β colony 3C – TAGGCGCCCG CCGCTCTGAC TGGCGTGTCA
14-3-3β colony 4D – TAGGCGCCCG CCGCTCTGAC TGGCGTGTCA
14-3-3β colony 5E – TAGGCGCCCG CCGCTCTGAC TGGCGTGTCA
14-3-3β wild-type – TAGGCGCCCG CCGCTCTTCC TGGCGTGTCA
Serine 60 ⟶ Aspartate 60

Figure 3. These are the nucleotide sequences for the second point mutation, serine 65 to aspartate. In addition to the first serine 60 to aspartic acid mutation (red box), the DNA from transformed colonies 1A2, 3C2, 4D2, and 5E2 also have the serine to aspartic acid mutation at codon 65 (blue box). The 14-3-3β wild-type sequence at the bottom shows the original serine codons for both 60 and 65 residues of the peptide.

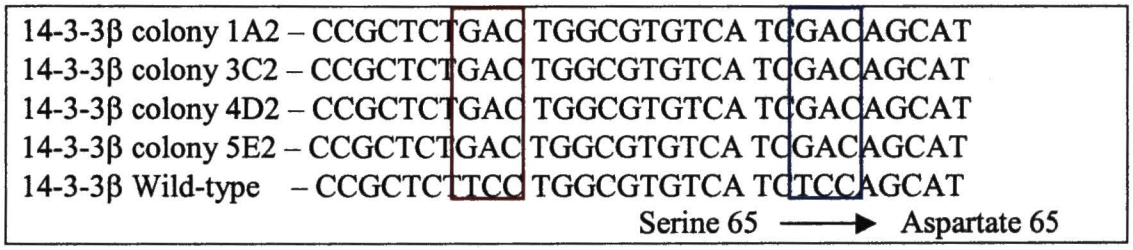
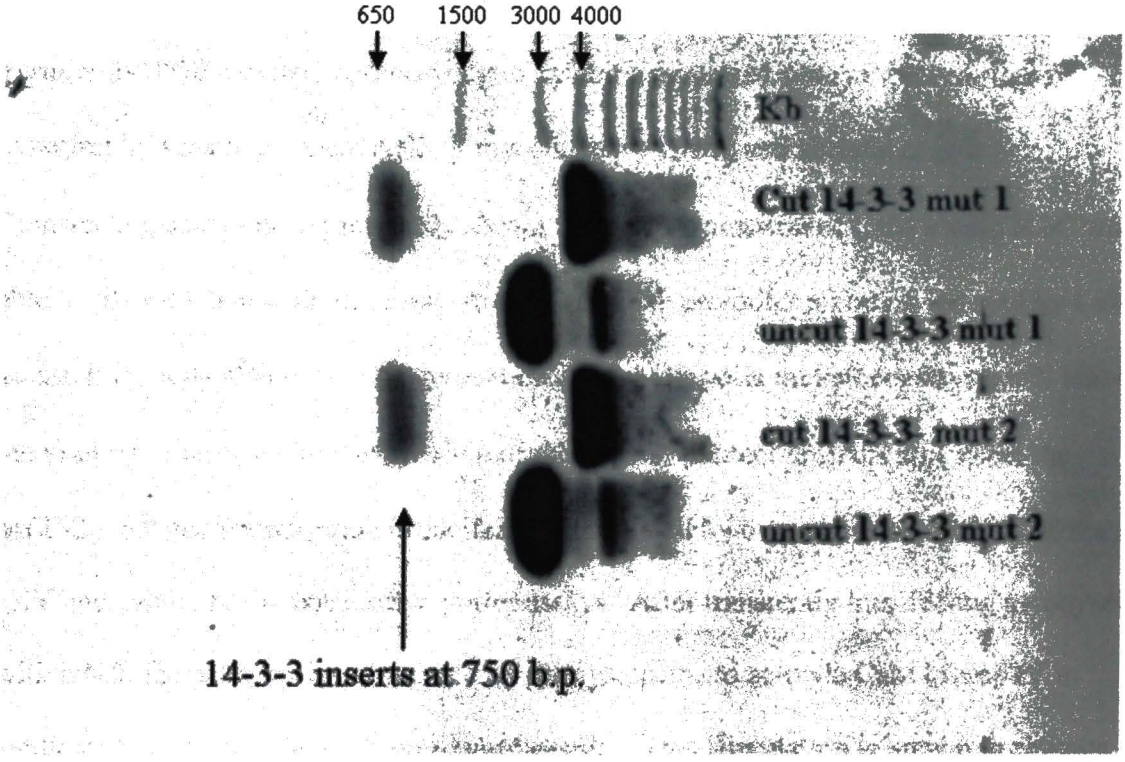

14-3-3β colony 1A2 – CCGCTCTGAC TGGCGTGTCA TCGACAGCAT
14-3-3β colony 3C2 – CCGCTCTGAC TGGCGTGTCA TCGACAGCAT
14-3-3β colony 4D2 – CCGCTCTGAC TGGCGTGTCA TCGACAGCAT
14-3-3β colony 5E2 – CCGCTCTGAC TGGCGTGTCA TCGACAGCAT
14-3-3β Wild-type – CCGCTCTTCC TGGCGTGTCA TCTCCAGCAT
Serine 65 ⟶ Aspartate 65

Figure 4. This is a 0.8% agarose gel with 14-3-3β mutant 1 and mutant 2 DNA digested with Not I and EcoR I. Description of the gel lanes is as follows: 1. Kb ladder DNA, 2. Digested 14-3-3β mutant 1 DNA, 3. Un-digested 14-3-3β mutant 1 DNA, 4. Digested 14-3-3β mutant 2 DNA, 5. Un-digested 14-3-3β mutant 2 DNA. This electrophoresis DNA assay verifies that the correct plasmid DNA was purified and that the 14-3-3β mutant genes are within the purified plasmids.


650
1500
3000
4000
Kb
Cut 14-3-3 mut 1
uncut 14-3-3 mut 1
cut 14-3-3- mut 2
uncut 14-3-3 mut 2
14-3-3 inserts at 750 b.p.

CHAPTER 3

14-3-3β TRANSFECTION ASSAYS

Introduction

MEF-2 has been shown to be an integral player in the induction of hypertrophy. Terminally differentiated cardiomyocytes exhibit a repressed state of transcription of hypertrophy sensitive genes (ANF, β myosin heavy chain, and skeletal α actin). Transfection assays have previously shown that over expression of CaM kinase II δC was able to silence the activation of hypertrophy sensitive promoter reporters induced by CaM kinase I, IV and calcinurin. The generation of 14-3-3β point mutant constructs will allow me to identify how the over expression of the 14-3-3β mutant proteins will affect the MEF-2 mediated transcription of luciferase, stimulated by over expression of CaM kinase I, IV and calcinurin, in enhancer-reporter assays. After transiently transfecitng myocytes with DNA, the cells can be stimulated by phenylephrine to activate CaM kinase IV in cardiomyocytes and PAC-1 smooth muscle cells . This stimulation is known to activate MEF-2 mediated transcription.

Transfection of Cardiomyocytes

Primary cardiomyocyte cultures were prepared from 2–4 day old Sprague-Dawley rats as described by Argentin et al. [44]. Ventrticular heart tissue was disected and stored in ice cold 199 media (4°C) until all tissue was collected. The ventricular tissue was washed two times with new 199 media then minced with tissue scissors until the tissue was pureed. The minced tissue was then placed into 40 mL of pancreatic enzyme solution and stired and incubated for 18 minutes at 37°C. The digested supernatant was then pipetted out and placed in a sterile pre chilled 50 mL tube and placed on ice. The digestion is performed a total of 4 times. The cell suspensions are centrifuged at 3,000 rpm for 15 minutes. The supernatant is discarded and the cells are resuspended in 199 media supplemented with 10% fetal bovine serum. Cells were cultured at 37°C in 5% CO_2 using Falcon 12-well tissue culture plates. Cultures were maintained in 199 media supplemented with 10% fetal bovine serum (FBS). To avoid changes in intracellular Ca^{2+} that the Ca_2PO_4 transfection method might cause, we chose LipofectAMINE Reagent (Life Technologies, Grand Island, NY) for transfection assays. Transfections were performed 24-48 hours post plating. Cell lysates were assayed for relative luciferase activity using a Turner Designs luminometer (Model TD-20e).

Treatment groups were set-up utilizing a luciferase based MEF-2 enhancer-reporter to study the effects that the 14-3-3β mutant 2 would have on luciferase transcription due to CaM KI and IV. An equal amount of DNA (140ng) was transfected

to ensure that DNA concentration did not cause differing results. The six treatment groups were designed as follows:

Treatment 1. 100ng MEF-2, 5ng CaM KI, 35ng pCMV5
Treatment 2. 100ng MEF-2, 20ng CaM KIV, 20ng pCMV5
Treatment 3. 100ng MEF-2, 5ng CaM KI, 20ng 14-3-3β mutant 2, 15ng pCMV5
Treatment 4. 100ng MEF-2, 20ng CaM KIV, 20ng 14-3-3β mutant 2
Treatment 5. 100ng MEF-2, 40ng pCMV5 (Control)
Treatment 6. 100ng MEF-2, 20ng 14-3-3β mutant2, 20ng pCMV5

Transfection solutions consist of 50μl 199 media for each treatment well and DNA incubated with LipofectAMINE™ for 45 minutes. Cardiomyocytes were transfected following Life Technologies LipofectAMINE™ protocol.

After 16 hours incubation at 37°C, transfected cultures were washed with 0.5ml serum free 199 media twice. The cells were then given 1ml of 0.2% serum in 199 media and incubated for 48 hours at 37°C. Transfected cardiomyocytes were harvested by washing twice with 0.5ml 1X PBS. Cells were then lysed by adding 25μl 1X lysis buffer (Tropix) for 10 minutes. Using a rubber policeman, the lysis solution was scraped vigorously in the culture well and transferred into a luminometer cuvette. 100μl of luciferase substrate (Promega) was added to each cuvette and vortexed vigorously. Luciferase readings were taken using a luminometer.

Transfection of Phenylephrine Stimulated Cardiomyocytes

Phenylephrine is an α-1 receptor agonist that activates MEF-2 through the Ca^{2+}/calmodulin-dependent protein kinase cascade, specifically CaM KIV in the nucleus. Cardiomyocytes were cultured as previously described and transfected following Life Technologies LipofectAMINE™ protocol. An equal amount of DNA (140ng) was used in the transfection solutions for each treatment. The treatments were set-up as follows:

Treatment 1. 100ng MEF-2, 5ng CaM KI, 35ng pCMV5

Treatment 2. 100ng MEF-2, 20ng CaM KIV, 20ng pCMV5

Treatment 3. 100ng MEF-2, 5ng CaM KI, 20ng 14-3-3β mutant 2, 15ng pCMV5

Treatment 4. 100ng MEF-2, 20ng CaM KIV, 20ng 14-3-3β mutant 2

Treatment 5. 100ng MEF-2, 40ng pCMV5 (Control)

Treatment 6. 100ng MEF-2, 20ng 14-3-3β mutant 2, 20ng pCMV5

Treatment 7. P.E.- 100ng MEF-2, 20ng 14-3-3β mutant 2, 20ng pCMV5

Treatment 8. P.E.- 100ng MEF-2, 40ng pCMV5

Treatment 9. P.E.- 100ng MEF-2, 20ng 14-3-3 w/t, 20ng pCMV5

Treatment 10. P.E.- 100ng MEF-2, 20ng of 14-3-3β mutant 2, 20ng 14-3-3β w/t

After 16 hours of transfection, cardiomyocytes were washed twice with serum free 199 media and re-fed with 199 media supplemented with 0.2% serum. The treatments that required Phenylephrine were stimulated at a 10μM concentration. Cells

were then incubated for 24 hours at 37°C. The cardiomyocytes were then lysed and assayed for luciferase activity as previously described.

Transfection of PAC-1 Cells

Pulmonary arterial smooth muscle cells (PAC-1) were plated and cultured in 3-12 well plates. The cells were washed with 0.5ml of serum free 199 media per well two times. The cells were transfected as previously described using the LipofectAMINE™ protocol. The treatments were set-up as follows:

Treatment #1. 200ng MEF-2, 40ng pCMV5
Treatment #2. 200ng MEF-2, 5ng CaM KI, 35ng pCMV5
Treatment #3. 200ng MEF-2, 20ng CaM KIV, 20ng pCMV5
Treatment #4. 200ng MEF-2, 5ng CaM KI, 20ng 14-3-3β mutant 2, 15ng pCMV5
Treatment #5. 200ng MEF-2, 20ng CaM KIV, 20ng 14-3-3β mutant 1
Treatment #6. 200ng MEF-2, 20ng CaM KIV, 20ng 14-3-3β mutant 1

After 16 hours of transfection, cells were washed twice with 0.5ml/well serum free 199 media and re-fed with 1ml/well 199 media supplemented with 0.2% serum. PAC-1 cells were then incubated for 24 hours at 37°C. The cells were then lysed and assayed for luciferase activity as previously described.

Transfection of Phenylephrine Stimulated PAC-1 cells

PAC-1 cells were cultured as previously described and transfected following Life Technologies LipofectAMINE™ protocol. An equal amount of DNA (240ng) was used in the transfection solutions for each treatment. The treatments were set-up as follows:

Treatment #1. 200ng MEF-2, 40ng pCMV5
Treatment #2. P.E., 200ng MEF-2, 40ng pCMV5
Treatment #3. P.E., 200ng MEF-2, 20ng14-3-3β mutant 2, 20ng pCMV5
Treatment #4. P.E., 200ng MEF-2, 20ng 14-3-3β mutant 1, 20ng pCMV5
Treatment #5. P.E., 200ng MEF-2, 20ng 14-3-3β w/t, 20ng pCMV5
Treatment #6. P.E., 200ng MEF-2, 10ng 14-3-3β mutant 2, 30ng pCMV5

After 16 hours of transfection, the PAC-1 cells were washed twice with serum free 199 media and re-fed with 199 media supplemented with 0.2% serum. The treatments that required Phenylephrine were stimulated at a 10μM concentration. Cells were then incubated for 24 hours at 37°C. The PAC-1 cells were then lysed and assayed for luciferase activity as previously described.

Results

Cardiomyocyte Transfection

These results suggest the 14-3-3β mutant 2 silences CaM KI and IV induction of the MEF-2 enhancer-reporter in cardiomyocytes in the same manner as CaM KII (Figures 5 and 6). This supports the hypothesis that CaM KII works through the phosphorylation and dimer disruption of 14-3-3β in the cardiomyocyte to repress hypertrophy-sensitive gene induction.

The stimulation of cardiomyocytes by phenylephrine is an α-1 agonist response that activates CaM KIV resulting in MEF-2 activation and transcription of hypertrophy-sensitive genes. This activation relies on the ability of 14-3-3β to sequester HDAC4/5 in the cytoplasm. Figure 7 shows the 14-3-3β mutant 2 recombinant protein is unable to sequester HDAC4/5, preventing the P.E. induction of MEF-2 dependent transcription.

Figure 5. Transient transfection of rat neonate cardiomyocytes with CaM KI shows induction of the MEF-2-luciferase enhancer-reporter above empty pCMV5 vector control levels. Co-transfection of 14-3-3β mutant 2 with CaM KI shows a silencing of MEF-2 enhancer-reporter activity similar to the control level.

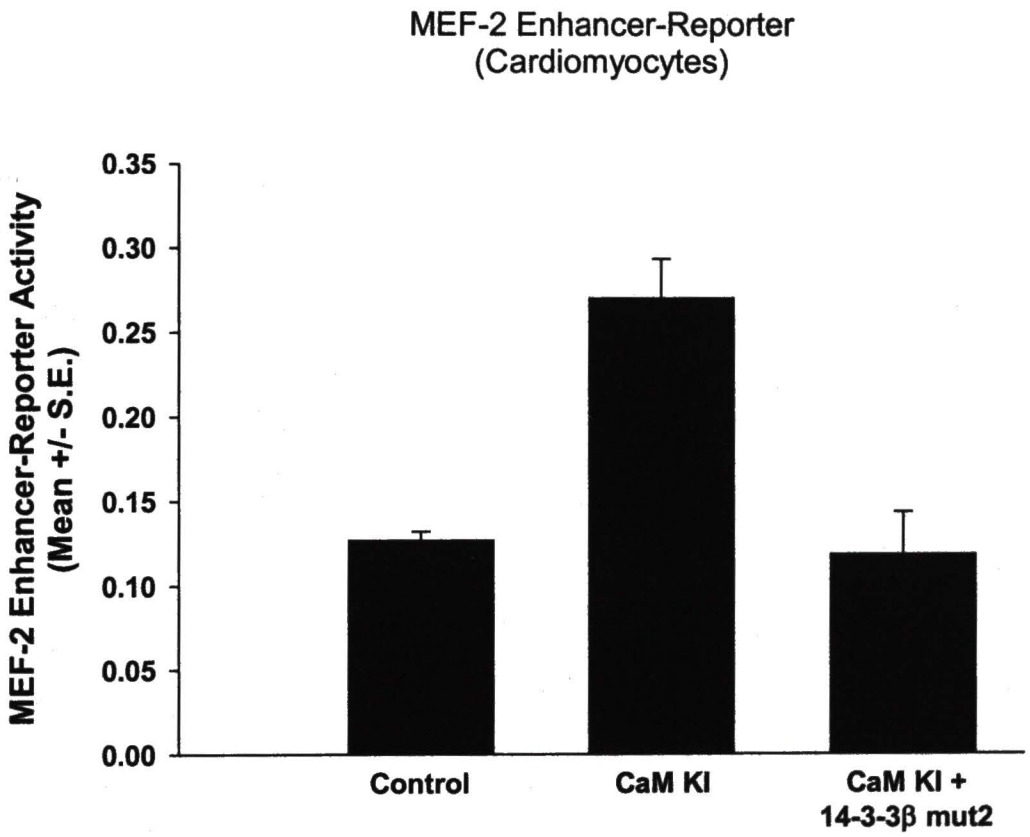

MEF-2 Enhancer-Reporter
(Cardiomyocytes)
MEF-2 Enhancer-Reporter Activity
(Mean +/- S.E.)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
CaM KI
CaM KI +
14-3-3β mut2

Figure 6. Transient transfection of cardiomyocytes with CaM KIV shows induction of the MEF-2-luciferase enhancer-reporter above control levels, similar to that of CaM KI. Co-transfection of 14-3-3β mutant 2 with CaM KIV also shows complete silencing of CaM KIV induction of the MEF-2 enhancer-reporter.

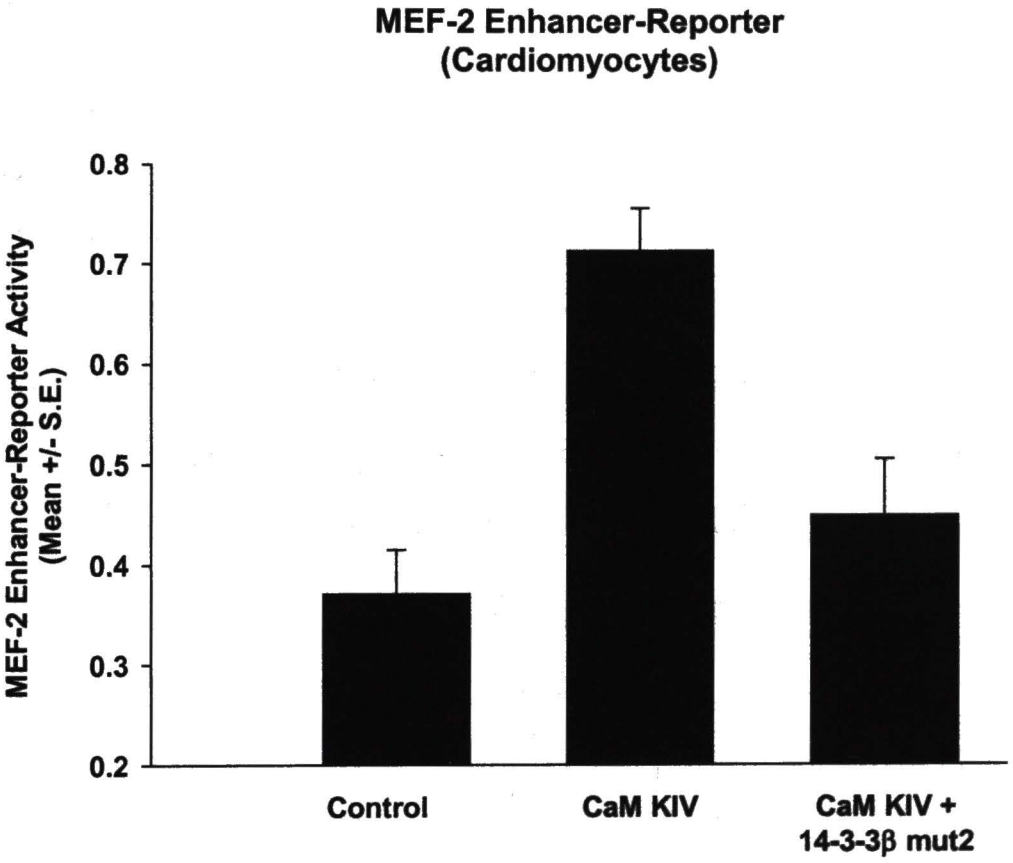
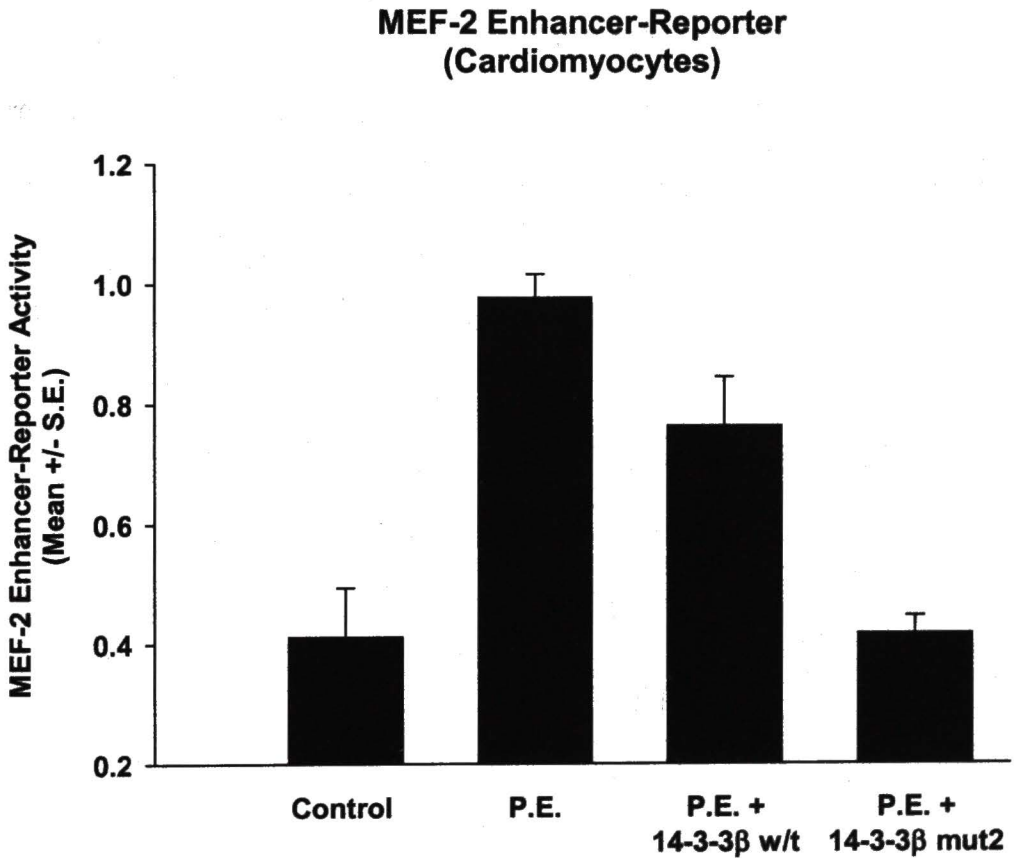

MEF-2 Enhancer-Reporter
(Cardiomyocytes)
MEF-2 Enhancer-Reporter Activity
(Mean +/- S.E.)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Control
CaM KIV
CaM KIV +
14-3-3β mut2

Figure 7. Rat neonate cardiomyocyte cell lysates were analyzed for luciferase activity caused by the induction of the MEF-2 enhancer-reporter. Cardiomyocytes that were stimulated with phenylephrine (P.E.) show a dramatic induction of MEF-2 enhancer-reporter activity over pCMV5 empty vector control. Transient transfection of cardiomyocytes with wild-type 14-3-3β before P.E. stimulation still shows activation over control levels. Transfection with the 14-3-3β mutant 2 gene results in the silencing of P.E. activation of MEF-2 enhancer-reporter activity.


MEF-2 Enhancer-Reporter
(Cardiomyocytes)
MEF-2 Enhancer-Reporter Activity
(Mean +/- S.E.)
1.2
1.0
0.8
0.6
0.4
0.2
Control
P.E.
P.E. +
14-3-3β w/t
P.E. +
14-3-3β mut2

PAC-1 cell Transfection

In the same way that 14-3-3β mutant 2 silences CaM KI and IV MEF-2 induction in cardiomyocytes, 14-3-3β mutant 2 also silences this induction in PAC-1 cells (Figures 7 & 8). The 14-3-3β mutant 2 silences activation of MEF-2 dependent transcription by P.E.. with the same efficiency in smooth muscle as it does in cardiomyocytes (Figure 9). 14-3-3β mutant 2 is working in a dose dependent manner. The 10ng/well transfection did not silence MEF-2 activity as strongly as the 20ng/well treatment. Further more detailed dose response assays are needed to verify this result. The 14-3-3β mutant 1 does not silence like the double mutation. It appears that both serine 60 and 65 are required for transcriptional silencing.

Figure 8. Transient transfection of pulmonary arterial smooth muscle cells (PAC-1) with CaM KI shows induction of the MEF-2-luciferase enhancer-reporter above empty pCMV5 vector control levels. Co-transfection of 14-3-3β mutant 2 with CaM KI shows a silencing of MEF-2 enhancer-reporter activity similar to the control levels.

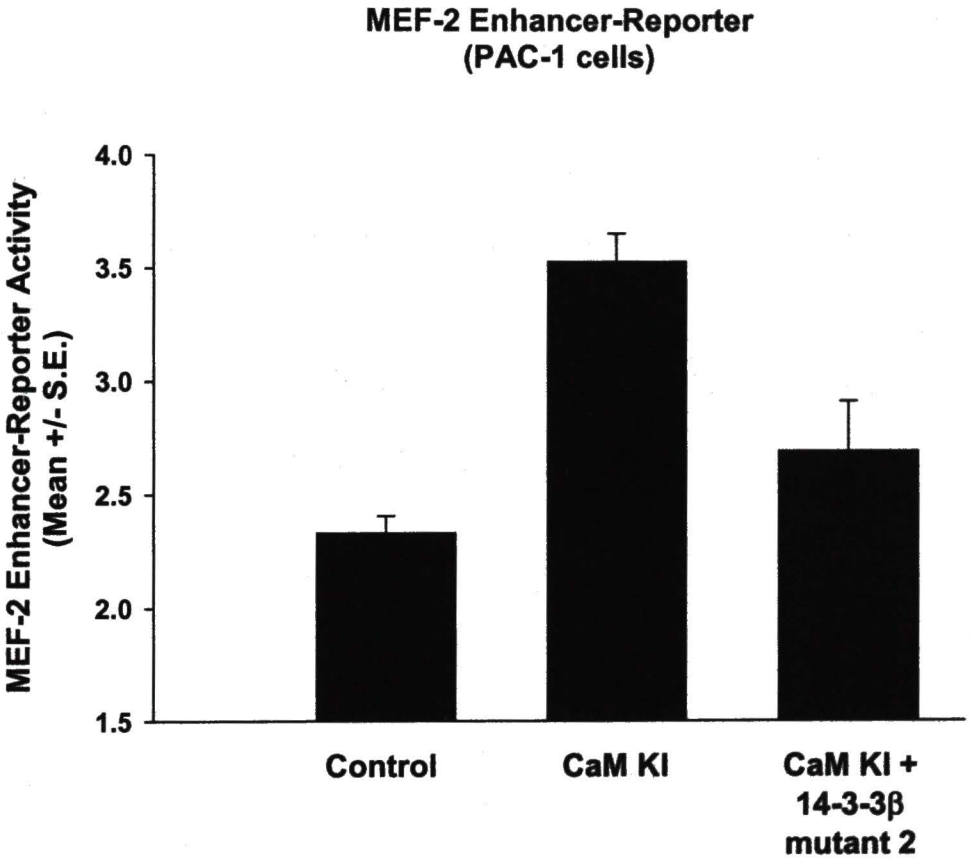

MEF-2 Enhancer-Reporter
(PAC-1 cells)
MEF-2 Enhancer-Reporter Activity
(Mean +/- S.E.)
4.0
3.5
3.0
2.5
2.0
1.5
Control
CaM KI
CaM KI +
14-3-3β
mutant 2

Figure 9. Transient transfection of PAC-1 cells with CaM KIV shows induction of the MEF-2-luciferase enhancer-reporter above control levels, similar to that of CaM KI. Co-transfection of 14-3-3β mutant 2 with CaM KIV also shows complete silencing of CaM KIV induction of the MEF-2 enhancer-reporter.

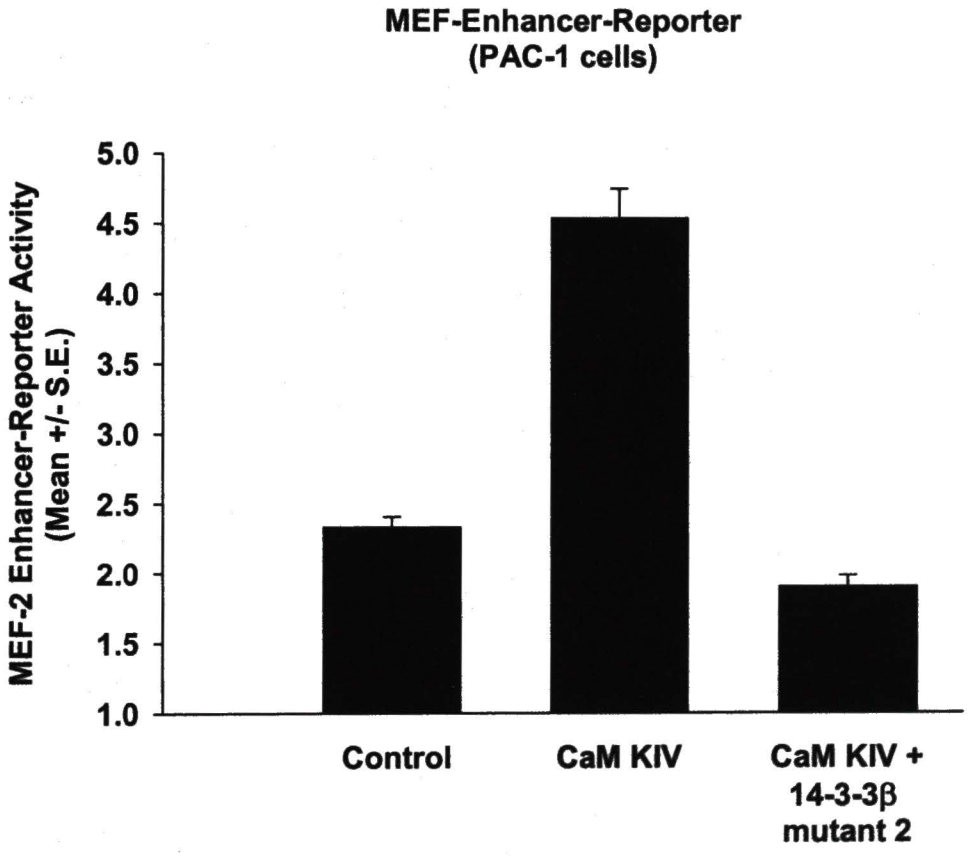
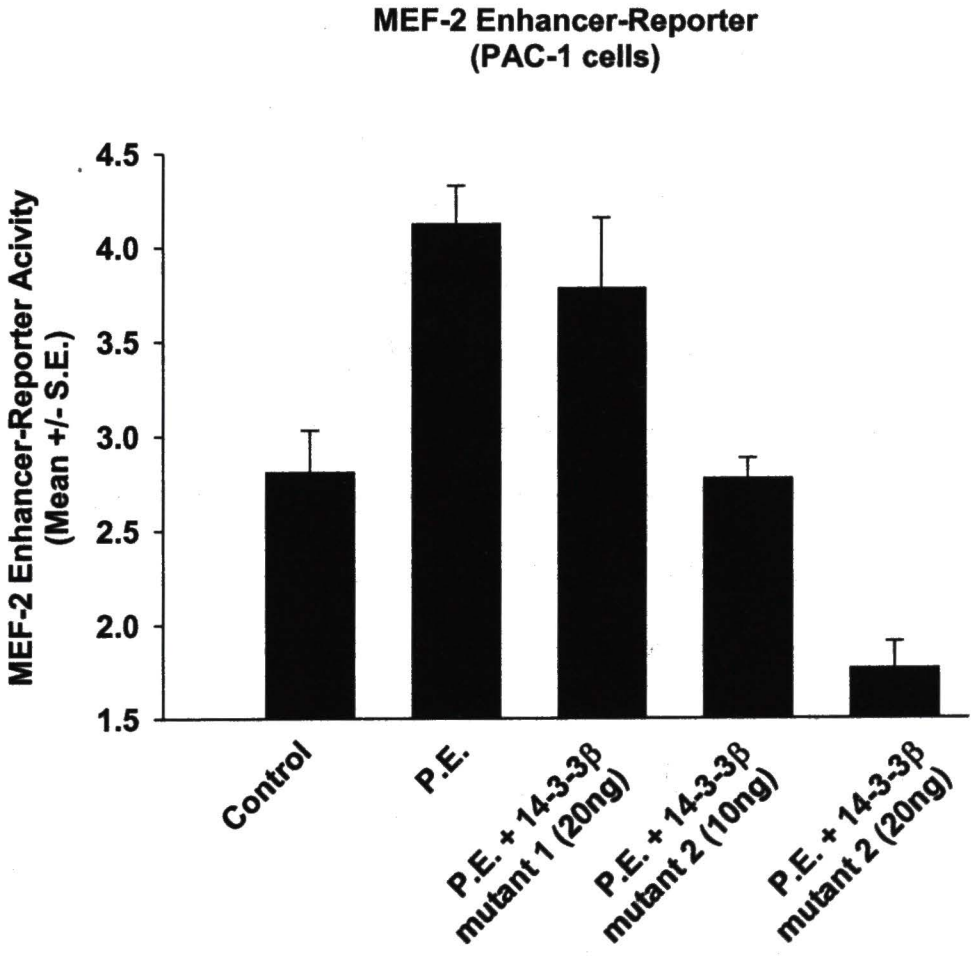

MEF-Enhancer-Reporter
(PAC-1 cells)
MEF-2 Enhancer-Reporter Activity
(Mean +/- S.E.)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Control
CaM KIV
CaM KIV +
14-3-3β
mutant 2

Figure 10. PAC-1 cell lysates were analyzed for luciferase activity caused by the induction of the MEF-2 enhancer-reporter. PAC-1 cells that were stimulated with P.E. show a dramatic induction of MEF-2 enhancer-reporter activity over pCMV5 empty vector control. Transient transfection of PAC-1 cells with 14-3-3β mutant 1 gene before P.E. stimulation still shows activation over control levels. Transfection with the 14-3-3β mutant 2 gene (10ng/well) results in the silencing of P.E. activation of MEF-2 enhancer-reporter activity level similar to the control cells. Transfection with the 14-3-3β mutant 2 gene (20ng/well) shows the silencing of P.E. activation well below the control levels.


MEF-2 Enhancer-Reporter
(PAC-1 cells)
MEF-2 Enhancer-Reporter Acivity
(Mean +/- S.E.)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
Control
P.E.
P.E. + 14-3-3β
mutant 1 (20ng)
P.E. + 14-3-3β
mutant 2 (10ng)
P.E. + 14-3-3β
mutant 2 (20ng)

CHAPTER 4

MAMMALIAN TWO-HYBRID SYSTEM

Introduction

Most of the 14-3-3 regulation and chaperone activity occurs while a homo or heterodimer complex of 14-3-3 proteins exists. In order to study the effectiveness of the 14-3-3β double mutation to form a dimer, a mammalian two-hybrid system construct will be implemented. This system has a Gal-4 driven luciferase-based reporter gene that results in luciferase transcription when the Gal-4 DNA binding domain is in close proximity to the VP-16 transactivating domain. The constructs are created by cloning the genes of interest, in this case 14-3-3β wild-type and 14-3-3β mutant 2, into the pACT (VP-16 activation domain) and the pBIND (Gal-4 binding domain) cloning vectors. These clones will result in a fusion protein with the respected activation or binding domains. When the resulting cloned DNA is transfected with the Gal-4 luciferase reporter DNA and the proteins of interest interact, luciferase activity will be induced. If the proteins of interest do not interact then there will be diminished luciferase activity (Figure 11).

Due to the (A) nucleotides added into the BamH I recognition sites of the pACT and pBIND cloning vectors there were three approaches taken to generate the 14-3-3β

mammalian two-hybrid system constructs; 1) Blunt end ligation, 2) TOPO TA cloning and 3) Designed cloning sites in PCR products.

Methods

Blunt-end Ligation of 14-3-3β mutant 2 and pACT + 1

This method of cloning involves using a blunt end cutting restriction enzyme that does not leave any sticky end overhangs on either the 5' or 3' ends. The insert and vector DNA is ligated using T4 DNA ligase. After the ligation the sequence need to be analyzed for directional orientation of the insert. Using the blunt cutting restriction enzyme EcoR V a digestion reaction was set-up to cut the ends of the pACT+1 cloning vector. EcoR V recognizes sites on both the 5' and 3' ends of the multi-cloning region of pACT+1 creating a linearized vector. The endonuclease digestion was set-up as follows:

100.25µl of pACT+1 DNA (10µg)
12µl 10X React 2 buffer
2µl EcoR V enzyme
6µl ddH_2O
120µl total reaction

The digestion reaction was incubated for three hours at 37°C. After 3 hours, 1U of Calf Intestinal Alkaline Phosphatase (CIAP) was added to the digestion reaction and incubated for 5 minutes at 50°C. The reaction was heat inactivated at 65°C for 15

minutes and stored at 4°C overnight. The blunt end ligation reactions were set-up in insert: vector ratios as follows:

Ligation #1 (15:45)	Ligation #2 (30:90)
1.15µl pACT+1 DNA	2.3µl pACT+1 DNA
1.0µl 14-3-3β mutant 2 DNA	1.91µl 14-3-3β mutant 2 DNA
4.0µl 5X ligase buffer	4.0µl ligase buffer
1.0µl T4 DNA ligase	1.0µl T4 DNA ligase
12.85µl ddH_2O	10.79µl ddH_2O
20µl total reaction	20µl total reaction

Ligation #3 (45:135)	Ligation #4 (60:180)
3.44µl pACT+1 DNA	4.5µl pACT+1 DNA
2.87µl 14-3-3β mutant 2 DNA	3.83µl 14-3-3β mutant 2 DNA
4.0µl 5X ligase buffer	4.0µl ligase buffer
1.0µl T4 DNA ligase	1.0µl T4 DNA ligase
8.69µl ddH_2O	6.67µl ddH_2O
20µl total reaction	20µl total reaction

Ligation reactions were incubated at 14°C for 20 hours. DH5α competent cells were transformed as previously described and cultured on LB + ampicillin agar plates overnight at 37°C.

Vector Purification of pACT and pBIND

The TOPO TA protocol utilizes the (A) nucleotide overhangs resulting from PCR reactions using Taq polymerase. Topoisomerase molecules are covalently bound to the cloning pCR2.1 vector so that no enzyme is required for insert and vector ligation. In order to use this TOPO-TA commercial protocol, (Invitrogen) BamH I and Not I cloning sites were needed on the pACT and pBIND vectors at the 5' and 3' ends respectively. We used the control vectors from the CheckMate™ system and used Bam HI and Not I to cut out the control inserts ID and MyoD. The restriction digest reactions were set-up as follows:

Reaction #1	Reaction #2
8µl pACT+1 DNA (800ng)	3µl pBIND+1 DNA (800ng)
2µl 10X React 3 Buffer	1µl 10X React 3 Buffer
1µl BamH I enzyme	1µl BamH I enzyme
9µl ddH_2O	5µl ddH_2O
20µl total	10µl total

Reaction #3	Reaction #4
10µl pACT MyoD DNA (800ng)	1.1µl pBIND ID DNA (800ng)
2µl 10X React 3 Buffer	1µl 10X React 3 Buffer
1µl BamH I enzyme	1µl BamH I enzyme

7µl ddH_2O	6.9µl ddH_2O
20µl total	10µl total

After digestion reactions were incubated for 1.5 hours at 37°C, 1µl Not I enzyme was added to all four reactions and incubated for another 1.5 hours at 37°C. At the end of the digestion, the samples were placed at 4°C until loaded onto an agarose gel. 1µl of 6X loading dye was added to the 10µl reactions and 2µl 6X loading dye was added to the 20µl reaction. The reactions were then loaded onto a 0.6% agarose gel and run at 40mA and 40 volts for 1 hour. The gel was documented using AlphaImager software and digital camera.

After confirmation of ID and MyoD inserts cutting out using BamH I and Not I restriction enzymes, reactions were set-up to digest and gel purify a larger quantity of vector for ligation of 14-3-3β wild-type and mutant 2 after TOPO cloning. The reactions were designed as follows:

pACT-MyoD Reaction	pBIND-ID Reaction
134.7µl pACT-MyoD DNA (10µg)	14.1µl pBIND-ID DNA (10µg)
16µl 10X React 3 Buffer	2µl 10X React 3 Buffer
2µl BamH I enzyme	2µl BamH I enzyme
7.3µl ddH_2O	1.9µl ddH_2O
160µl total	20µl total

After 1.5 hours of incubation at 37°C 1µl of Not I enzyme was added to the reaction and digested for another 1.5 hours at 37°C. The reactions were then placed at 4°C until loaded onto agarose gel. Loading dye was added to each reaction and the entire volume was loaded onto a 0.6% LMP agarose gel and run at 40mA and 40 volts for 1 hour, keeping the temperature cool with ice so the gel did not melt. The linearized vectors were cut out of the gel using a trans-luminator and the gel blocks were placed into 1.5ml microfuge tubes and stored at -20°C.

The LMP agarose blocks with the linearized vectors was melted at 65°C for 15 minutes. Following the Life Technologies protocol for β-Agarase digestion of LMP agarose, the β-Agarase buffer and enzyme were added to the LMP agarose solution as follows:

pACT	pBIND
150µl 10X β-Agarase buffer	110µl 10X β-Agarase buffer
4µl β-Agarase I enzyme	3µl β-Agarase I enzyme

The 10X buffer was added first and equilibrated at 40°C for 30 minutes. The temperature was then increased to 45°C and the β-Agarase I enzyme was then added. The solutions were incubated overnight at 40°C. The reactions were then placed at 4°C.

The pACT and pBIND vectors were then phenol extracted following the phenol extraction protocol (appendix). Final elution was in 50µl of 10mM Tris-Cl, pH 8.0. DNA was stored at -20°C until ligation.

TOPO TA Cloning

Using the TA TOPO cloning kit from Invitrogen, the following ligation reactions were set-up to clone 14-3-3β wild-type and mutant 2 into the pCR2.1 cloning vector. The reactions were set-up as follows:

14-3-3β wild-type #1	**14-3-3β wild-type #2**
2μl 14-3-3β w/t PCR product	4μl 14-3-3β w/t PCR product
1μl salt solution (kit)	1μ salt solution (kit)
1μl TOPO vector (kit)	1μl TOPO vector (kit)
2μl ddH_2O	0.0μl ddH_2O
6μl total	6μl total

14-3-3β mutant 2 #1	**14-3-3β mutant 2 #2**
2μl 14-3-3β mutant 2 PCR product	4μl 14-3-3β mutant 2 PCR product
1μl salt solution (kit)	1μl salt solution (kit)
1μl TOPO vector (kit)	1μl TOPO vector (kit)
2μl ddH_2O	0.0μl ddH_2O
6μl total	6μl total

Reactions were incubated for 15 minutes at room temperature then placed on ice (4°C). Transformation of OneShot competent cells (Invitrogen) was done by adding 2μl

of each TOPO reaction to a vial of cells. The vials were incubated on ice for 20 minutes then heat shocked at 42°C for 30 seconds and then placed on ice for 2 minutes. 250µl of room temperature S.O.C. culture media was added to each vial of cells and the vials were placed in a shaking incubator at 37°C and 225 rpm for 1 hour. The agar + kanamycin plates were prepared with 40µl of 40mg/ml X-gal and allowed to dry. For each transformation 30 and 50µl of the cell suspension was spread onto the agar plates and incubated overnight at 37°C. White colonies were selected according to the TOPO TA cloning protocol. Five colonies were selected for each of the cloned 14-3-3β and wild-type genes. Colonies were selected and grown overnight in 3ml of LB + kanamycin overnight at 37°C and 225 rpm. The picked colonies were labeled as Wild-type A-J and Mutant A-J.

Characterization of TOPO TA Cloned 14-3-3β

Mini-prep plasmid DNA isolation was done for each colony culture (Quiagen). Final elution was with 40µl of 10mM Tris-Cl, pH 8.0. To verify the orientation of the 14-3-3β inserts Pst I restriction enzyme was used to digest the plasmid DNA. The Pst I enzyme digestion will result in the following fragments for correct and incorrect orientation:

Correct	Incorrect
201 base pairs	84 base pairs
552 base pairs	201 base pairs
1168 base pairs	1168 base pairs
2794 base pairs	3189 base pairs

The Pst I digestion reactions were set up as follows:

25µl DNA
3µl 10X React 2 buffer
1µl Pst I enzyme
1µl ddH_2O
30µl total reaction volume

All digestion reactions were incubated for 3 hours at 37°C. After the digestion was complete 1.5µl 6X loading dye was added to each reaction and then the entire volume was loaded onto a 1% agarose gel. The gel was run at 40mA and 90 volts for 1.5 hours. The gel was stained with ethidium bromide and visualized using UV light.

Primer Design for Two-Hybrid Cloning

As a result of the inability of being able to get the correct orientation of the 14-3-3β inserts in the TOPO TA cloning system, primers were designed to incorporate the

correct cloning sites into a PCR product for cloning directly into the pACT and pBIND vectors. Using the New England Biolabs reference book, BamH I and Not I sites were designed at the 5' and 3' ends, respectively, of HDAC4,5 and the 14-3-3β genes. The primer sequences are as follows:

14-3-3β forward and reverse primers

forward: 5' – cgggatcccgatgacaatggataaaagtgag – 3'

reverse: 5' – atagtttagcggccgcattcttatttagttctctccctccccag – 3'

HDAC4 forward and reverse primers

forward: 5' – cgggatcccgatgagctcccaaagccatcc – 3'

reverse: 5' – atagtttagcggccgcattcttatctacaggggcggctcctcttc – 3'

HDAC5 forward and reverse primers

forward: 5' – cgggatcccgatgaactctcccaacgagtc – 3'

reverse: 5' – atagtttagcggccgcattcttatgaatgtgtgacttttttgttttt – 3'

Primer sequences were sent to Integrated DNA Technologies to be synthesized.

HDAC 4, 5 & 14-3-3β wild-type, mutant PCR

PCR reactions were set-up to optimize the annealing temperature and verify that the primers will work for PCR amplification of 14-3-3β wild-type, 14-3-3β mutant, HDAC4, and HDAC5 genes. Using a Promega PCR master mix, which includes Taq polymerase, PCR buffer, and salts, PCR reactions were set-up as follows:

HDAC 4 flag
6.25µl Promega Master Mix
1.0µl HDAC4 forward primer (0.20mM)
1.0µl HDAC4 reverse primer (0.20mM)
1.6µl HDAC4 template (250ng)
2.65µl ddH_2O
12.5µl total volume

HDAC 5 flag
6.25µl Promega Master Mix
1.0µl HDAC5 forward primer (0.20mM)
1.0µl HDAC5 reverse primer (0.20mM)
2.0µl HDAC5 template (250ng)
2.25µl ddH_2O
12.5µl total volume

The PCR annealing temperature was determined using a temperature gradient across the heating block of the Eppendorf Mastercycler® Gradient thermal cycler. The annealing temperature gradient was 10°C and went from 60°C to 70°C. The PCR cycle sequence was set-up as follows:

1. 95°C for 2 minutes
2. 95°C for 30 seconds *
3. 60°-70°C for 30 seconds *
4. 73°C for 3 minutes *
5. 73°C for 5 minutes
6. Hold at 4°C

*Steps 2-4 were repeated for 30 cycles.

14-3-3 wild-type
6.25µl Promega Master Mix
1.0µl 14-3-3β forward primer (0.20mM)
1.0µl 14-3-3B reverse primer (0.20mM)
1.0µl 14-3-3β wild-type template (250ng)
3.25µl ddH_2O
12.5µl total volume

14-3-3β mutant 2
6.25μl Promega Master Mix
1.0μl 14-3-3β forward primer (0.20mM)
1.0μl 14-3-3β reverse primer (0.20mM)
1.0μl 14-3-3β mutant 2 template (250ng)
3.25μl ddH_2O
12.5μl total volume

The PCR annealing temperature was determined using a temperature gradient across the heating block of the Eppendorf Mastercycler®. The annealing temperature gradient was 10°C and went from 60°C to 70°C. The PCR cycle sequence was set-up as follows:

7. 95°C for 2 minutes
8. 95°C for 30 seconds *
9. 60°-70°C for 30 seconds *
10. 73°C for 1 minute *
11. 73°C for 5 minutes
12. Hold at 4°C

*Steps 2-4 were repeated for 30 cycles.

Larger PCR reactions (final volume of 50µl) were set-up for HDAC4 and 14-3-3β wild-type and mutant 2 using the optimal annealing temperatures in order to produce product for the cloning into the mammalian two-hybrid system. After the PCR cycles were completed, the samples were cleaned using the Millipore Microcon PCR cleanup spin filter. Final elution was with 20µl of ddH_2O and samples were placed at 4°C until restriction digestion with BamH I and Not I.

Restriction Digestion of PCR Products with BamH I and Not I

The Not I digestion was done first due to the lower buffer salt concentration. This digestion will be incubated at 37°C for 20 hours in order to assure >90% enzyme digestion of the PCR products. The endonuclease reactions were set-up as follows:

14-3-3β w/t & mutant 2	**HDAC 4**
10µl PCR product	10µl PCR product
2.5µl 10X React 3 buffer	2.5µl 10X React 3 buffer
1.0µl Not I enzyme	1.5µl Not I enzyme
11.5µl ddH_2O	11µl ddH_2O
25.0µl total reaction volume	25.0µl total reaction volume

After 20 hours of incubation at 37°C the following amounts of reagents were added to each digestion reaction:

3.0µl 10X buffer E, 0.3µl BSA, and 1µl of BamH I enzyme.

The reactions were incubated at 37°C for three hours then heat inactivated at 65°C for 10 minutes. Using Millipore's Micropure – EZ spin filters the digestion reactions were cleared of enzymes and the resulting digested DNA was stored at -20°C. The samples were analyzed for DNA concentration using a UV spectrophotometer. The following DNA concentrations were determined:

HDAC 4 – 52ng/μl

14-3-3β wild-type – 28ng/μl

14-3-3β mutant 2 – 37ng/μl

The femtomolar concentration was determined for all of the digested PCR samples and the following ligation reactions (insert: vector femtomolar ratio) were set-up as follows:

HDAC 4

Ligation #1 (3:9)	Ligation #2 (15:45)	Ligation #3 (30:90)
0.3μl pACT DNA	1.38μl pACT DNA	2.75μl pACT DNA
1.0μl HDAC4 insert	0.62μl HDAC4 insert	1.2μl HDAC4 insert
4.0μl 5X ligase buffer	4.0μl ligase buffer	4.0μl ligase buffer
1.0μl T4 DNA ligase	1.0μl T4 DNA ligase	1.0μl T4 DNA ligase
13.7μl ddH_2O	13.0μl ddH_2O	11.05μl ddH_2O
20μl total reaction	20μl total reaction	20μl total reaction

14-3-3β wild-type

Ligation #1 (3:9)	Ligation #2 (15:45)	Ligation #3 (30:90)
1.0μl pBIND DNA	1.3μl pBIND DNA	2.7μl pBIND DNA
1.0μl 14-3-3β w/t insert	1.0μl 14-3-3β w/t insert	1.0μl 14-3-3β w/t insert
4.0μl 5X ligase buffer	4.0μl ligase buffer	4.0μl ligase buffer
1.0μl T4 DNA ligase	1.0μl T4 DNA ligase	1.0μl T4 DNA ligase
13.0μl ddH_2O	12.7μl ddH_2O	11.3μl ddH_2O
20μl total reaction	20μl total reaction	20μl total reaction

14-3-3β mutant 2

Ligation #1 (3:9)	Ligation #2 (15:45)	Ligation #3 (30:90)
0.3μl pACT DNA	1.38μl pACT DNA	2.75μl pACT DNA
1.0μl 14-3-3β mut 2 insert	1.0μl 14-3-3β mut 2 insert	1.0μl 14-3-3β mut 2 insert
4.0μl 5X ligase buffer	4.0μl ligase buffer	4.0μl ligase buffer
1.0μl T4 DNA ligase	1.0μl T4 DNA ligase	1.0μl T4 DNA ligase
13.7μl ddH_2O	12.6μl ddH_2O	11.25μl ddH_2O
20μl total reaction	20μl total reaction	20μl total reaction

All ligation reactions were incubated at room temperature for 15 minutes then stored at -20°C overnight. Chemically competent DH5α cells were transformed as

previously described. Cells were transformed with 10μl of the ligation reactions and also with 10μl of a 1:10 dilution of the ligation reaction. Culture solutions were grown on ampicillin selective LB agar culture plates and incubated overnight at 37°C. Ten colonies were selected for each ligation and plasmid DNA was purified following the Quiagen mini-spin commercial protocol. Final elution was with 50μl of Tris-Cl, pH 8.0 buffer.

Characterization of Cloned 14-3-3β Wild-type, Mutant 2 and HDAC4

DNA samples for all three cloned genes were digested with BamH I and Not I to verify cloned inserts. Loading dye was added and the samples were run on 1.0% agarose gels at 40mA and 40 volts for 1hour. DNA was visualized and documented using a UV trans-luminator and ethidium bromide staining.

pACT - HDAC4 was characterized using Xho I endonuclease that only cuts once within the HDAC4 sequence and does not cut within the pACT vector. Kpn I was also used to cut the 3' end of the 14-3-3β genes in case the Not I site was altered during the cloning. For the pBIND – 14-3-3β wild-type clone, Bcl I was used to cut within the 14-3-3β insert but not the pBIND vector DNA. All enzymes were incubated at 37°C for ≥1.5 hours except for Bcl I, which was incubated at 50°C. DNA was run on 1.0% agarose gels at 40mA and 40 volts for 1 hour. The DNA was stained with ethidium bromide and visualized using an UV trans-luminator.

Blunt End Ligation

Figure 11 shows the characterization of the pACT+1, pBIND+1 DNA, pACT/MyoD, and pBIND/Id vectors. I was able to use BamH I and Not I enzymes to cut out the MyoD and Id inserts out of the cloning vectors. The MyoD fragment is visible in lane 6 and the Id fragment is visible in lane 7. The presence of the Id and MyoD fragments after digestion with BamH I and Not I confirms that the pACT and pBIND vectors can be purified for cloning. The blunt end ligation reaction produced no transformed bacteria after the overnight culture on LB agar culture plates with selection for ampicillin resistance. The blunt end ligation reaction was repeated and resulted again in no colonies. I proceeded with the TOPO TA cloning.

TOPO TA Cloning

These agarose gels show Pst I digested TOPO TA cloned 14-3-3β wild-type and mutant 2 DNA (Figures 11 & 12). The fragments at 84 base pairs signifies the incorrect orientation of both 14-3-3β wild-type and 14-3-3β mutant 2 genes within the pCR2.1 vector. The consistent backwards orientation of the 14-3-3β inserts suggests the possibility that this may be the most thermodynamically efficient way for the 14-3-3β inserts to be cloned into the pCR2.1 vector.

Figure 11. CheckMate™ mammalian two-hybrid system diagram shows how protein-protein interaction results in luciferase activity induction (A). When the two proteins of interest do not interact then there is no luciferase transcription (B).

CheckMate™ mammalian two-hybrid system

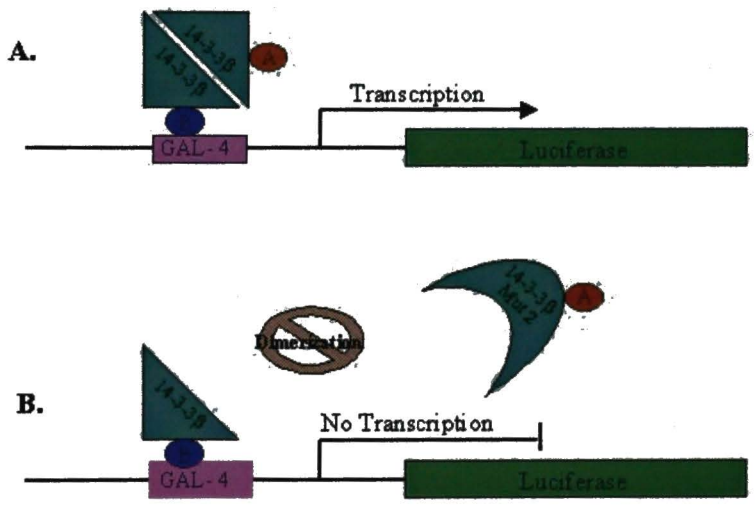


Figure 12. The BamH I and Not I restriction digested DNA was run on a 0.6% agarose gel. The lane descriptions are as follows: 1. Kb plus DNA ladder, 2. pACT+1 undigested, 3.pACT+1 digested, 4. pBIND+1 digested, 5. pACT-MyoD undigested, 6. pACT-MyoD digested, 7. pBIND-Id undigested, 8. pBIND-Id digested. Lanes 6 and 8 show the cut out MyoD and Id inserts.

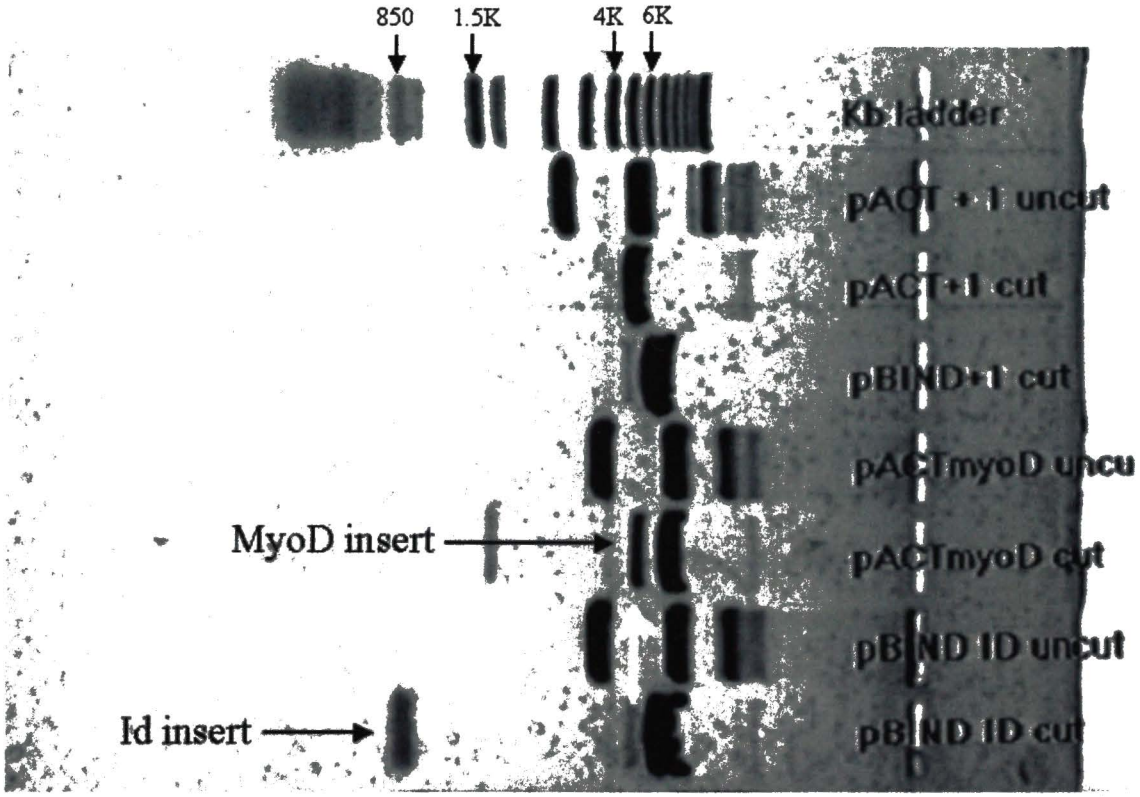

850
1.5K
4K
6K
Kb ladder
pACT + I uncut
pACT +I cut
pBIND+I cut
pACTmyoD uncu
pACTmyoD cut
pBIND ID uncut
pBIND ID cut
MyoD insert
Id insert

Figure 13. Pst I digested TOPO cloned 14-3-3β wild-type (A-E) and mutant 2 (A-E) DNA was run on a 1.0% agarose gel and stained with ethidium bromide. The lane descriptions are as follows: 1. Kb plus DNA ladder, 2. Undigested pCR2.1 vector, 3. Digested 14-3-3β w/t A clone, 4. Digested 14-3-3β w/t B clone, 5. Digested 14-3-3β w/t C clone, 6. Digested 14-3-3β w/t D clone, 7. Digested 14-3-3β w/t E clone, 8. Digested 14-3-3β mutant A clone, 9. Digested 14-3-3β mutant B clone, 10. Digested 14-3-3β mutant C clone, 11. Digested 14-3-3β mutant D clone, 12. Digested 14-3-3β mutant E clone.

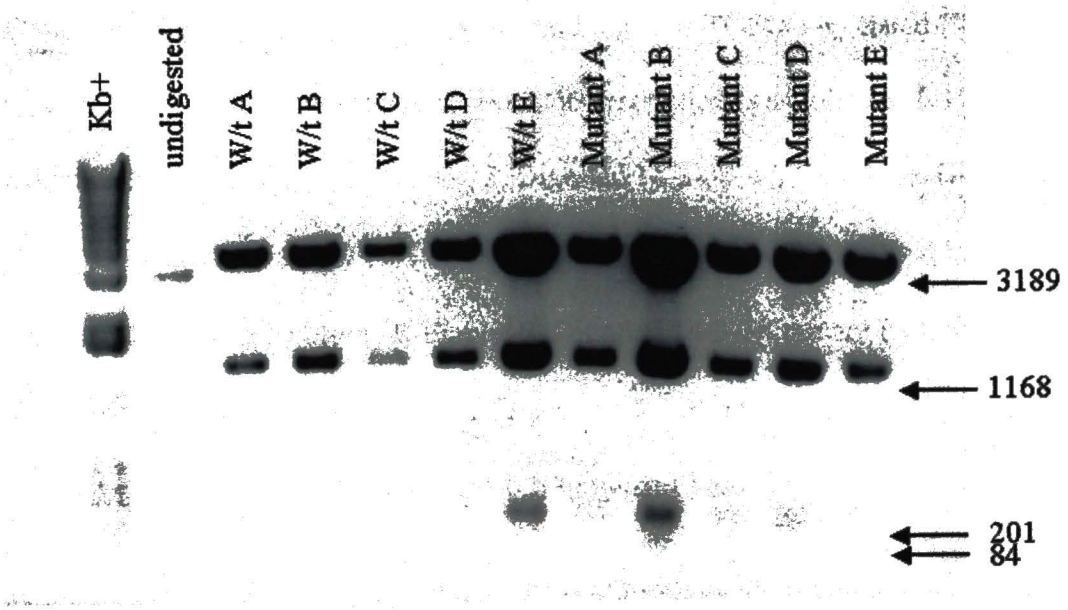
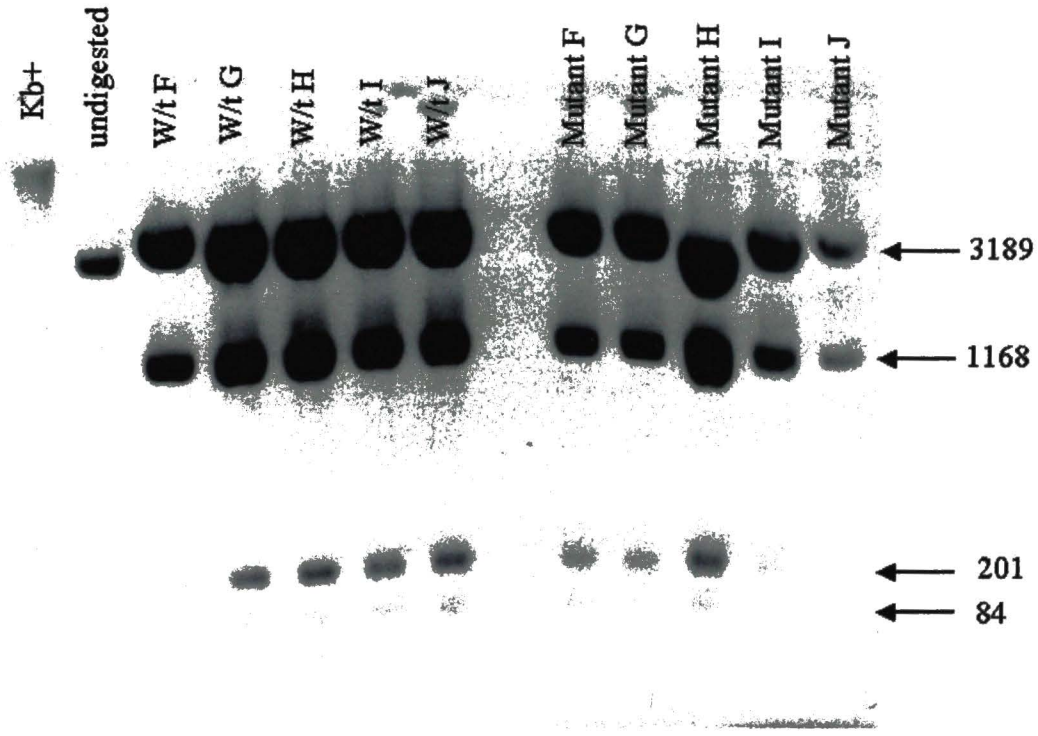

Kb+
undigested
W/t A
W/t B
W/t C
W/t D
W/t E
Mutant A
Mutant B
Mutant C
Mutant D
Mutant E
3189
1168
201
84

Figure 14. This 1.2% agarose gel shows Pst I digested TOPO TA cloned 14-3-3β wild-type (F-J) and mutant 2 (F-J) DNA. The lane description is as follows: 1. Kb plus DNA ladder, 2. Undigested pCR2.1 vector, 3. Digested 14-3-3β w/t F clone, 4. Digested 14-3-3β w/t G clone, 5. Digested 14-3-3β w/t H clone, 6. Digested 14-3-3β w/t I clone, 7. Digested 14-3-3β w/t J clone, 8. Empty, 9. Digested 14-3-3β mutant F clone, 10. Digested 14-3-3β mutant G clone, 11. Digested 14-3-3β mutant H clone, 12. Digested 14-3-3β mutant I clone, 13. Digested 14-3-3β mutant J clone.


Kb+
undigested
W/t F
W/t G
W/t H
W/t I
W/t J
Mutant F
Mutant G
Mutant H
Mutant I
Mutant J
3189
1168
201
84

PCR Products for HDAC 4, 5 and 14-3-3β Wild-type, Mutant 2

These PCR reactions were done to verify that the designed primers with 5' BamH I and 3' Not I cloning sites produced PCR products. All of the PCR reactions, except for HDAC 5, produced PCR products. Figure 15 shows 11 HDAC 4 PCR reactions run across an annealing gradient. This gel shows that 64°C is the optimal annealing temperature for HDAC 4. Figure 16 shows 11 HDAC 5 PCR reactions across an annealing gradient. There were no PCR product produced by these primers. Figures 17 and 18 show 11 14-3-3β wild-type PCR and mutant 2 reactions across an annealing gradient respectively. These gels identify 65°C as the optimal annealing temperature for both 14-3-3β wild-type and mutant PCR reactions.

Figure 15. HDAC 4 PCR product at 3259 base pairs is shown on a 0.8% agarose gel for 11 different PCR reactions, each with a different annealing temperature ranging from 60° to 70°C (left to right).

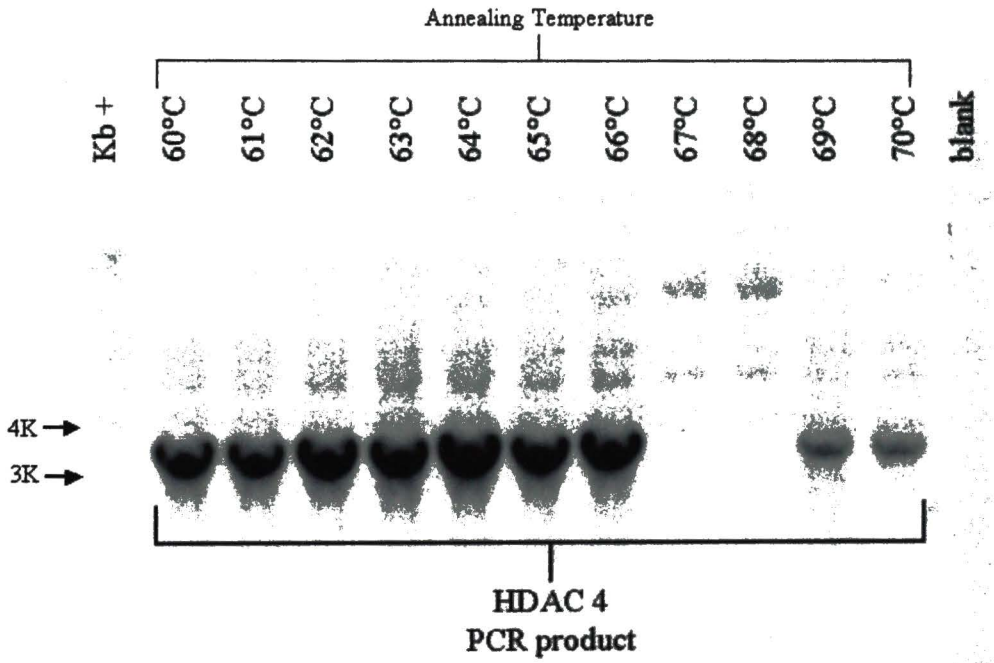

Annealing Temperature
Kb +
60°C
61°C
62°C
63°C
64°C
65°C
66°C
67°C
68°C
69°C
70°C
blank
4K
3K
HDAC 4
PCR product

Figure 16. HDAC 5 PCR results are shown for 11 different PCR reactions, each with a different annealing temperature ranging from 60° to 70°C (left to right). HDAC 5 PCR product is either not present or at a very low concentration and hard to distinguish. There is no evident optimal annealing temperature for these HDAC 5 cloning primers.

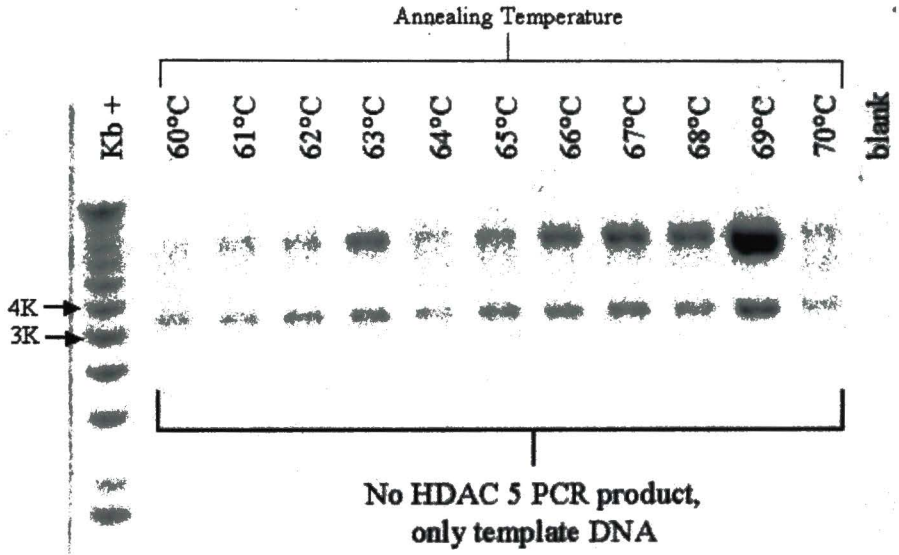

Annealing Temperature
Kb +
60°C
61°C
62°C
63°C
64°C
65°C
66°C
67°C
68°C
69°C
70°C
blank
4K
3K
No HDAC 5 PCR product,
only template DNA

Figure 17. 14-3-3β wild-type PCR product at 742 base pairs is shown for 11 different PCR reactions, each with a different annealing temperatures ranging from 60° to 70°C (left to right). The 14-3-3β wild-type PCR results are shown in a 1.0% agarose gel stained with ethidium bromide.

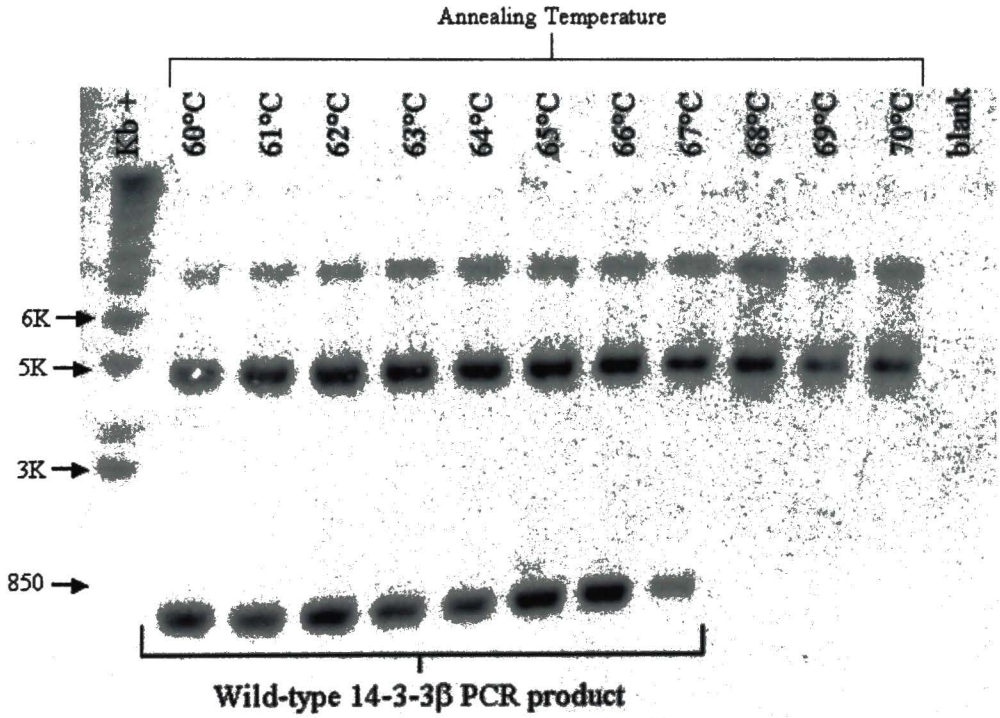
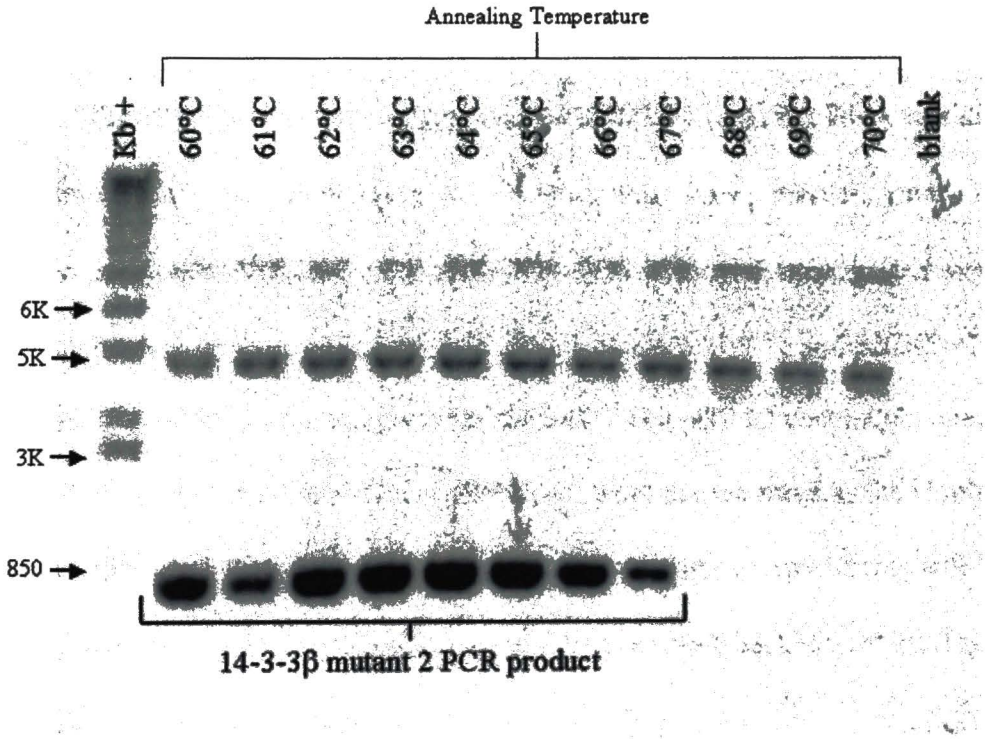

Annealing Temperature
Kb +
60°C
61°C
62°C
63°C
64°C
65°C
66°C
67°C
68°C
69°C
70°C
blank
6K
5K
3K
850
Wild-type 14-3-3β PCR product

Figure 18. 14-3-3β mutant 2 PCR product at 742 base pairs is shown for 11 different PCR reactions, each with a different annealing temperatures ranging from 60° to 70°C (left to right). 14-3-3β mutant 2 DNA is shown in a 1.0% agarose gel stained with ethidium bromide.


Annealing Temperature
Kb +
60°C
61°C
62°C
63°C
64°C
65°C
66°C
67°C
68°C
69°C
70°C
blank
6K
5K
3K
850
14-3-3β mutant 2 PCR product

Ligation of PCR Products with pACT and pBIND

Ligated pACT-HDAC4 clones were verified by digestion with Xho I restriction enzyme (Figure 19). The linearized DNA of lanes 5, 9, and 13 with Xho I enzyme indicate the presence of the HDAC4 gene in the pACT vector. Because Xho I does not recognize a site within pACT, the linearized pACT-HDAC4 suggests the presence of HDAC4 in the plasmid. The next step is to have this plasmid sequenced to ensure the correct reading frame of the cloned HDAC4.

The pBIND/14-3-3β wild-type and pact/14-3-3β mutant 2 DNA was digested with BamH I, Kpn I, and Bcl I (pact/14-3-3β mutant 2) restriction enzymes. In the case of both pact/14-3-3β mutant 2 and pBIND/14-3-3β wild-type, the digested plasmids ran at a smaller size on the agarose gel than did the empty pACT+1 and pBIND+1vectors (Figures 20 and 21). This suggests that the pACT and pBIND vectors obtained from the control plasmids, pACT-MyoD and pBIND-Id, were not the same as the cloning vectors of the CheckMate™ mammalian two-hybrid system. Future experiments will be done with un-tampered pACT and pBIND vectors from a new CheckMate™ kit (Promega) and the ligation procedures repeated.

Figure 19. Samples 1, 2, and 3 of pact/HDAC4 clone were digested with BamH I, Not I, and XhoI restriction enzymes and run on a 1.0% agarose gel and visualized with ethidium bromide. Lane descriptions are as follows: 1. Kb plus DNA ladder, 2. pACT/HDAC4 1 undigested, 3. pACT/HDAC4 1 digested with BamH I and Not I, 4. pact/HDAC4 1 digested with BamH I, Not I, and Xho I, 5. pACT/HDAC4 1 digested with Xho I, 6. pACT/HDAC4 2 undigested, 7. pACT/HDAC4 2 digested with BamH I and Not I, 8. pACT/HDAC4 2 digested with BamH I, Not I, and Xho I, 9. pACT/HDAC4 2 digested with Xho I, 10. pACT/HDAC4 3 undigested, 11. pACT/HDAC4 3 digested with BamH I and Not I, 12. pACT/HDAC4 3 digested with BamH I, Not I, and Xho I, 13. pACT/HDAC4 3 digested with Xho I.

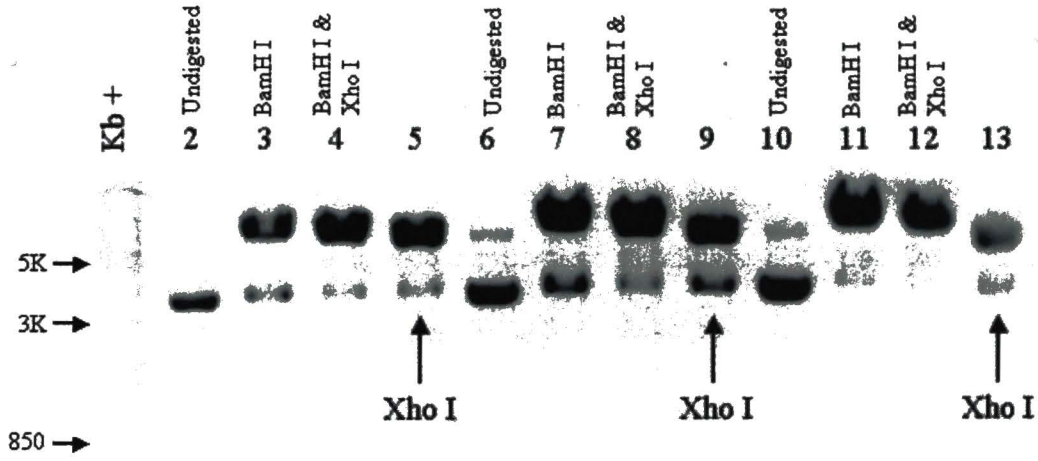

Kb +
2 Undigested
3 BamH I
4 BamH I & Xho I
5
6 Undigested
7 BamH I
8 BamH I & Xho I
9
10 Undigested
11 BamH I
12 BamH I & Xho I
13
5K
3K
850
Xho I
Xho I
Xho I

Figure 20. pBIND/14-3-3β wild-type was digested with BamH I and Kpn I and the empty vector pACT+1 was digested with Not I and run on a 1.0% agarose gel and stained with ethidium bromide. The lane descriptions are as follows: 1. Kb plus DNA ladder, 2. pBIND/14-3-3β wild-type undigested, 3. pBIND/14-3-3β wild-type digested with BamH I, 4. pBIND/14-3-3β wild-type digested with BamH I and Kpn I, 5. pBIND+1 digested with Not I, 6. pBIND+1 undigested. The digested pBIND/14-3-3βwild-type (lane 3) is a smaller DNA vector than the digested pBIND+1 (lane 5).

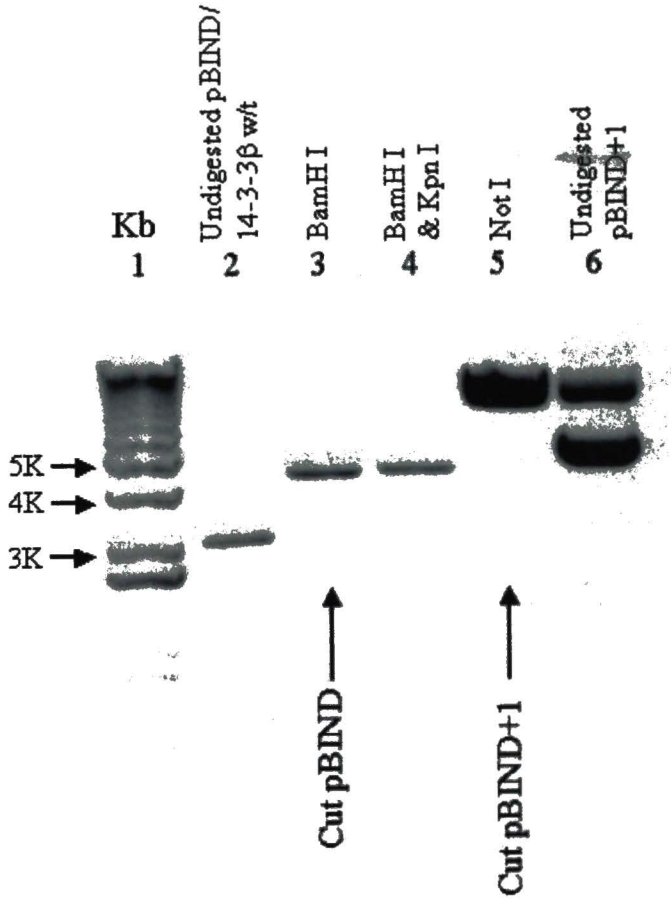

Kb
1
Undigested pBIND/ 14-3-3β w/t
2
BamH I
3
BamH I & Kpn I
4
Not I
5
Undigested pBIND+1
6
5K
4K
3K
Cut pBIND
Cut pBIND+1

Figure 21. pACT/14-3-3β mutant 2 was digested with BamH I, Bcl I and Kpn I and the pACT+1 empty vector was digested with Not I. The DNA was run on a 1.0% agarose gel and stained with ethidium bromide. The lane descriptions are as follows: 1. Kb plus DNA ladder, 2. pACT/14-3-3β mutant 2 undigested, 3. pACT/14-3-3β mutant 2 digested with BamH I, 4. pACT/14-3-3β mutant 2 digested with BamH I and Kpn I, 5. pACT/14-3-3β mutant 2 digested with Bcl I, 6. pACT/14-3-3β mutant 2 digested with BamH I and Bcl I, 7. pACT+1 digested with Not I, 8. pACT+1 undigested. Bcl I enzyme alone did not digest the pACT-14-3-3β mutant 2 DNA (lane 5). The digested pACT/14-3-3β mutant 2 (lane 3) is a smaller DNA vector than the digested pACT+1 (lane 7).

3K
4K
5K
Kb
1
Undigested pACT/
2 14-3-3β Mut2
Cut pACT
3 BamH I
BamH I
4 & Bcl I
5 Bcl I
BamH I
6 & Kpn I
Cut pACT+1
7 Not I
Undigested
8 pACT+1

DISCUSSION

The calcium/calmodulin-dependent protein kinase cascade activation has been shown to be a major contributor to the induction of MEF-2 mediated hypertrophy sensitive transcription. The repression of hypertrophy sensitive genes (β-myosin heavy chain, skeletal α-actin, and ANF) by class II HDACs (HDAC 4 & 5) in normal working cardiomyocytes is reversed by the activation of CaM kinase I and IV [45, 46]. Wild type-14-3-3 sequesters HDAC 4 and 5 in the cytoplasm and leaves MEF-2 free in the nucleus to activate hypertrophy sensitive transcription [14]. The work presented in this paper gives strong support for 14-3-3β/HDAC association being a point of regulation for this transcription induction. Conservative point mutations of 14-3-3β at serines 60 and 65 to aspartic acids has resulted in a protein unable to form homo or heterodimers due to steric hindrance and charge repulsion. When this mutated recombinant 14-3-3β protein is over expressed transiently in cardiomyocytes and smooth muscle PAC-1 cells, the induction of MEF-2 mediated activity by both CaM KI and CaM KIV is completely silenced (Figures 5,6,8 & 9). Phenylephrine stimulation of cardiomyocytes and PAC-1 cells was also completely silenced by the 14-3-3β double mutation (Figs 7 and 10). The single point mutation of 14-3-3β, serine 60, does not silence MEF-2 induction by phenylephrine in the same fashion as the double mutation (Fig 10). This suggests that a doublet phosphorylation of a 14-3-3β dimer on serines 60 and 65 or possibly a single

phosphorylation of serine 65 is required to disrupt the dimer stability and free any bound molecules.

While the attempts to generate a mammalian two-hybrid system have been unsuccessful, I believe that the information generated by this assay system will be of great importance. The mammalian two-hybrid constructs will allow me to study the effects that 14-3-3β mutant 2 will have on dimerization and other protein-protein interactions important in hypertrophy induction.

The role of 14-3-3 in cardiac hypertrophy is an important one that is demanding attention of researchers around the world. The regulation of class II HDACs by 14-3-3 is an important part of the hypertrophy induction caused by active CaM kinase I and IV. The repression of promoter reporter gene transcription by CaM kinase II reveals a possible way for reversing the hypertrophy effects seen in many cardiac disease states. Identification of the CaM kinase II recognition motif within the 14-3-3 amino acid sequence gives further evidence that the silencing activity of CaM kinase II is the result of a phosphorylation and subsequent dimer destabilization of 14-3-3 resulting in the release and re-entry of HDAC 4 or 5 into the nucleus.

APPENDIX

Phenol Extraction:

Materials

A) Phenol equilibrated with Tris.HCl pH 8.0 stored at 4°C

B) 24:1 chloroform:Isoamyl alcohol

Add 96ml chloroform: 4ml Isoamyl alcohol.

Stored at room temperature in a tight capped bottle.

Protocol

1) Add 400µl DNA in a microfuge tube. Add 400ml buffered saturated phenol taken from the bottom thick layer.
2) Vortex for 10 seconds.
3) Centrifuge 15 minutes at 14,000 rpm in a refrigerated centrifuge at 4°C.
4) Pipette out the upper layer (aqueous layer) into a fresh microfuge tube
5) Add 200µl phenol and 200µl chloroform: isoamyl alcohol.
6) Vortex for ten seconds and centrifuge at 14,000 rpm for 15 minutes.
7) Pipette upper aqueous layer into a fresh microfuge tube.
8) Add 400µl chloroform: Isoamyl alcohol.
9) Vortex for 10 seconds and centrifuge at 14,000 rpm for 15 minutes.
10) Pipette upper aqueous layer into a fresh microfuge tube.
11) Add 40µl of 3.3 M sodium acetate pH 5.2 and vortex well to facilitate precipitation.
12) Add 1ml of 100% ethanol.
13) Incubate at -80°C for 30 minutes.

14) Centrifuge for 20 minutes at 14,000 rpm (4°C).

15) Discard supernatant (careful not to disturb the pellet) and wash with 1ml 70% ethanol.

16) Centrifuge for 10 minutes at 14,000 rpm and discard supernatant.

17) Allow pellet to air dry for ~ 10 minutes.

18) Suspend the DNA pellet in appropriate amount of preferred buffer or ddH_2O.

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