Journal of Molecular and Cellular Cardiology 54 (2013) 35–44

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Cross-talk between glycogen synthase kinase 3β (GSK3β) and p38MAPK regulates myocyte enhancer factor 2 (MEF2) activity in skeletal and cardiac muscle

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ARTICLE INFO

Article history:
Received 3 October 2012
Received in revised form 26 October 2012
Accepted 28 October 2012
Available online 5 November 2012

Keywords:
p38MAPK
Glycogen synthase kinase 3-beta
Myocyte enhancer factor 2

ABSTRACT

Characterizing the signaling network that controls MEF2 transcription factors is crucial for understanding skeletal and cardiac muscle gene expression. Glycogen synthase kinase 3β (GSK3β) regulates MEF2 activity indirectly through reciprocal regulation of p38MAPK. Cross-talk between GSK3β and p38MAPK regulates MEF2 activity in skeletal and cardiac muscle. Understanding cross-talk in the signaling network converging at MEF2 control has therapeutic implications in cardiac and skeletal muscle pathology. Glycogen synthase kinase 3β (GSK3β) is a known regulator of striated muscle gene expression suppressing both myogenesis and cardiomyocyte hypertrophy. Since myocyte enhancer factor 2 (MEF2) proteins are key transcriptional regulators in both systems, we assessed whether MEF2 is a target for GSK3β. Pharmacological inhibition of GSK3β resulted in enhanced MEF2A/D expression and transcriptional activity in skeletal myoblasts and cardiac myocytes. Even though *in silico* analysis revealed GSK3β consensus (S/T)XXX(S/T) sites on MEF2A, a subsequent *in vitro* kinase assay revealed that MEF2A is only a weak substrate. However, we did observe a post-translational modification in MEF2A in skeletal myoblasts treated with a GSK3β inhibitor which coincided with increased p38MAPK phosphorylation, a potent MEF2A activator, indicating that GSK3β inhibition may de-repress p38MAPK. Heart specific excision of GSK3β in mice also resulted in up-regulation of p38MAPK activity. Interestingly, upon pharmacological p38MAPK inhibition (SB203580), GSK3β inhibition loses its effect on MEF2 transcriptional activity suggesting potent cross-talk between the two pathways. Thus we have documented that cross-talk between p38MAPK and GSK3β signaling converges on MEF2 activity having potential consequences for therapeutic modulation of cardiac and skeletal muscle gene expression.

1. Introduction

Mitogen activated protein kinase (MAPK) signaling pathways are prominently involved in many cellular processes including cell proliferation and growth [1], development, migration [2] and differentiation [3,4]. Deregulation of MAPK signaling almost invariably leads to developmental defects and diseases including cardiac hypertrophy, muscular atrophy and cancer [5]. Proper regulation of this pathway in the context of elaborate and highly complex signaling networks within the cell is strongly dependent on communication with other signaling molecules, resulting in either synergistic or antagonistic relationships that produce a spectrum of biological outcomes. Understanding the nature of cross-talk between signaling pathways is indeed a major hurdle to understanding the molecular basis of all cellular processes. In the studies described here, we take advantage of the convergence of several signaling pathways on the MEF2 family of transcriptional regulators in order to gain insight into how cross-talk between GSK3β and p38MAPK signaling influence a single effector molecule which functions as a signaling conduit for the control of cardiac and skeletal muscle gene expression.

MEF2 proteins belong to the MADS (MCM1, agamous, deficiens, serum responsive factor) superfamily of transcription factors. There are four isoforms of MEF2 in vertebrates, MEF2A–D, that contain a highly conserved 57aa MADS-box domain at their amino-termini immediately adjacent to their 29aa MEF2 domain. Collectively these two domains are involved with DNA-binding, dimerization and interaction with co-factors. MEF2 factors regulate transcription as homo- or heterodimers by binding to the consensus DNA sequence (C/T)TA(A/T)$_4$TA(G/A) found in the regulatory regions of most cardiac and muscle specific genes [9,10]. Less conserved amongst the MEF2 isoforms are the C-termini which are subject to alternative splicing [11,12] and a variety of posttranslational modifications such as acetylation [13–15], sumoylation [16,17] and phosphorylation, many of which have proved important in regulating MEF2.

Previous studies have identified several kinases that regulate MEF2 transactivation properties. Casein kinase II (CK2) phosphorylates MEF2C at serine 59 enhancing its DNA-binding capacity and

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http://dx.doi.org/10.1016/j.yjmcc.2012.10.013

hence transcriptional activity [18]. ERK5 interacts with the N-termini of MEF2A/C/D [19] and can phosphorylate serine 387 in the transactivation domain [20]. Of the kinases that target MEF2, p38MAPK has been most extensively studied and deemed to be a key requisite for skeletal and cardiac muscle differentiation. Defects in this pathway have also been associated with muscle related diseases, such as embryonal rhabdomyosarcoma (ERMS) [21]. MEF2A has multiple p38MAPK phosphoacceptor sites as indicated by mass spectrometric analysis [8] and all four isoforms have been repeatedly demonstrated to be activated by this kinase. During embryogenesis, p38MAPK activation of MEF2 is necessary for proper heart development [23] and is also involved in cardiac hypertrophy in adult heart tissue, both *in vivo* and *in vitro* [22,23]. Similarly, as well as being a key regulator of skeletal myogenesis *in vitro*, p38MAPK critically interacts with and activates MEF2 in the somite myotome during development [24]. Thus MEF2 is a key convergence point for several cellular signaling pathways in the control of striated muscle gene expression.

Two kinases that actively repress skeletal and cardiac muscle differentiation are PKA and GSK3β. Whilst the effect of PKA has been shown to be mediated through repression of MEF2 transactivation properties [25], the effect of GSK3β on this process is less clear. GSK3β is involved in multiple cellular processes including glycogen metabolism, embryonic development, cell proliferation and apoptosis [26,27]. Several unique features distinguish GSK3β from other protein kinases; it is constitutively active in unstimulated cells and paradoxically, it is inhibited in response to cellular signals such as growth factors [28]. More importantly phosphorylation of its substrates often leads to their subsequent ubiquitylation followed by proteasomal degradation [26]. GSK3β usually targets proteins that have already been phosphorylated by another kinase at a serine or threonine residue located four amino acids C-terminal to a consensus (S/T)XXX(S/T)-PO_4 motif [26,27]. In addition to this canonical consensus recognition sequence, GSK3β has been shown to phosphorylate KSP motifs in neurofilament proteins [29] and microtubule associated proteins [30] leading to their inactivation.

GSK3β has been studied extensively in insulin and Wnt signaling. Upon insulin binding to its receptor, activation of the phosphatidylinositol-3-kinase (PI3K) pathway occurs, leading to phosphorylation and hence inactivation of serine 9 on GSK3β via protein kinase B (PKB) [31] as well as C-terminal phosphorylation on serine 389 by p38MAPK [38]. In a myogenic context, PI3K activation has been shown to lead to cardiac and skeletal muscle hypertrophy [6,7,26,28,32,33] as well as being an activator of p38MAPK [34] and a co-requisite for p38MAPK induced chromatin remodeling [35,36]. Defects in the PI3K pathway lead to activation of GSK3β and consequently repression of myogenic differentiation [35]. In kidney cells induction of GSK3β results in the repression of JNK and p38MAPK through the inhibition of their upstream mitogen activated protein kinase kinase kinase (MAPKKK), MEKK4 [37]. Thus there is considerable circumstantial evidence suggesting an intersection between GSK3β and p38MAPK signaling pathways.

In this report, based initially on informatics analysis, we hypothesized that GSK3β is involved in the regulation of cardiac and skeletal muscle gene expression either by directly phosphorylating and hence destabilizing MEF2, or indirectly abrogating MEF2 activity through inhibition of p38MAPK. Whilst we report that MEF2A is a weak substrate of GSK3β *in vitro* we document that GSK3β activity represses MEF2 transactivation properties in both skeletal and cardiac myocytes both *in vitro* and *in vivo*. Pharmacological inhibition of GSK3β resulted in (i) increased MEF2 activity and (ii) de-repression of p38MAPK. Heart specific excision of GSK3β also resulted in up-regulation of p38MAPK activity. Gain of function assays using constitutively active GSK3β (S9A) repressed MEF2 activity which can be counteracted by exogenous activation of p38MAPK. Based on these data we propose integration of GSK3β and p38MAPK into the signaling network converging on the MEF2 transcription factors regulating both skeletal and cardiac gene expression.

2. Materials and methods

2.1. Plasmids

MEF2 and MCK reporter constructs (pMEF2, pMCK, pMCKΔMEF2) in pGL3 and expression vectors for MEF2A in pMT2 were used in reporter gene assays. The Gal4-MEF2A fusions have been described previously [43]. HA tagged Pax3-fkhr was cloned into pcDNA3.1 and kindly donated by Dr. Malkin and Adam Durbin at MaRS, Toronto. HA tagged GSK3β(S9A) was cloned in pcDNA3 ORF 995–2305. p38 and MKK6(EE) expression vectors were previously described [8].

2.2. Antibodies

Anti-MEF2A rabbit polyclonal antibody was produced with the assistance of the York University Animal Care Facility; anti-MEF2D (1:1000; BD Biosciences); β-catenin, phospho-β-catenin, p38, phospho-p38, ATF2, phospho-ATF2 and GSK3β (1:1000; Cell Signaling); actin, α/β-tubulin (1:2000; SantaCruz) were used for immunoblotting experiments.

2.3. Cell culture and transfection

C2C12 and RH30 cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone), 1% L-glutamine and 1% penicillin–streptomycin. Cells were maintained in a humidified, 37 °C incubator with a 5% CO_2 atmosphere. For transfections, cells were seeded 1 day prior to transfection and transfected according to the standard calcium phosphate method previously described by Perry et al. A mixture of 50 μl 2.5 M $CaCl_2$ per 25 μg DNA with an equal volume of 2× HeBS (2.8 M NaCl, 15 mM Na_2HPO_4, 50 mM HEPES, pH = 7.15) was used and the cells were and incubated overnight followed by washing and addition of fresh media [54]. Neonatal cardiomyocytes were isolated from 2- to 5-day-old rats. Whole hearts were separated and minced in a buffer solution (calcium and bicarbonate free Hanks with Hepes) and then dissociated into single cells by trypsin enzyme (Gibco) during repeated digestion with slow stirring. 10% FBS (Sigma) DMEM F12 (w/1% penicillin/streptotocin, 50 mg/L gentamycin sulfate) (Invitrogen) was added to the suspended cells and centrifuge for 10 min in 1200 rpm. The pellet was resuspended in medium. The isolated cells were plated for 30–60 min at 37 °C, allowing differential attachment of non-myocardial cells. The cardiomyocyte cells were counted and transferred to pre-gelatin coated plates.

The HL1 cardiac cell line was cultured in Claycomb Medium (Sigma Aldrich) supplemented with 100 μM norepinephrine (Sigma Aldrich), 10% FBS and 4 mM L-glutamine (Invitrogen). Cells were maintained in a humidified 37 °C incubator with 5% CO_2. The HL-1 cell line was originally established from an AT-1 subcutaneous tumor excised from an adult female Jackson Laboratory inbred C57BLy6J mouse.

Transient transfections in neonatal cardiomyocytes and HL-1 cells were performed using lipofectamine 2000. A 1:2.5 mixture ratio of DNA to lipofectamine in 250 μl Opti-Medium (Gibco) was prepared for a 4 h incubation.

2.4. Protein extractions, immunoblotting and reporter gene assays

Cells were harvested using an NP-40 lysis buffer (0.5% NP-40, 50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA [pH 8.0], 0.1 M NaF) containing 10 μg/ml leupetin and aprotinin, 5 μg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM sodium orthovanadate. Protein concentrations were determined using the Bradford method (Bio-Rad) with bovine serum albumin (BSA) as a standard. Total protein extracts (20 μg) were used for immunoblotting, diluted in sample buffer containing 5% β-mercaptoethanol and boiled.