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PKG-1α mediates GATA4 transcriptional activity

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ABSTRACT

GATA4, a zinc-finger transcription factor, is central for cardiac development and diseases. Here we show that GATA4 transcriptional activity is mediated by cell signaling via cGMP dependent PKG-1α activity. Protein kinase G (PKG), a serine/tyrosine specific kinase is the major effector of cGMP signaling. We observed enhanced transcriptional activity elicited by co-expressed GATA4 and PKG-1α. Phosphorylation of GATA4 by PKG-1α was detected on serine 261 (S261), while the C-terminal activation domain of GATA4 associated with PKG-1α. GATA4's DNA binding activity was enhanced by PKG-1α via by both phosphorylation and physical association. More importantly, a number of human disease-linked GATA4 mutants exhibited impaired S261 phosphorylation, pointing to defective S261 phosphorylation in the elaboration of human heart diseases. We showed S261 phosphorylation was favored by PKG-1α but not by PKA, and several other kinase signaling pathways such as MAPK and PKC. Our observations demonstrate that cGMP-PKG signaling mediates transcriptional activity of GATA4 and links defective GATA4 and PKG-1α mutations to the development of human heart disease.

1. Introduction

GATA binding protein 4 (GATA4), is a zinc-finger transcription factor essential for early embryonic cardiac development and for maintenance of the adult heart [1,2]. GATA4 null embryos exhibited defective heart tube formation [3,4]. Haploid insufficient mice and or mice with a conditional cardiomyocyte-specific ablation of GATA4 elicit decreased cardiac function and heightened disease phenotypes when challenged with pressure overload [5,6]. However, even cardiac overexpression of GATA4 may also induce cardiac hypertrophy [7]; thus, demonstrating the importance of the optimal "balanced" transcriptional activity of GATA4 in the normal heart development and in the adult. So, either increased or decreased GATA4 levels may be potentially detrimental to cardiac health. As a cardiac transcription factor, GATA4 activates several important downstream targets including NPPA, NPPB, and MHC, which are also involved in the maintenance of normal heart function [8]. Targeted activation occurs directly via binding to the GATA4 cognate DNA sequences in the promoter regions of target genes and indirectly through protein–protein interactions with its cofactors [9,10]. A number of naturally occurring human GATA4 missense mutants such as G303E and G296S, in which the respective glycine (G) is converted to glutamate (E) or serine (S), were etiologically linked to human congenital cardiac diseases [11–13]. Further, these mutations negatively affected GATA4's physical interaction with co-factor SMAD4, and consequently impaired gene activity [14]. Thus, deciphering the upstream regulatory network that mediates GATA4's activity is fundamental to further understand its role in vivo.

One of the major mechanisms that regulate GATA4's activity is cellular signaling through posttranslational modifications. For instance, sumoylation and acetylation potentiated GATA4's capability to activate downstream target genes [15,16], while methylation of GATA4 by polycomb repressive complex 2 (PRC2) suppressed its activity [17]. Also, GATA4's transcriptional activity is regulated by phosphorylation of it's own sites by distinct protein kinases. For example, phosphorylation of S105 by ERK and p38 MAPK [18,19], and of S419/410 by protein kinase C (PKC) potentiated GATA4's function [20], whereas glycogen synthase kinase 3 beta (GSK 3b) phosphorylated the N-terminal domain of GATA4 (2–205 aa), which decreased its nuclear occupancy, and

Abbreviations: GATA4, GATA binding protein 4; NPPA, natriuretic peptide A; NPPB, natriuretic peptide B; MHC, myosin heavy chain; ERK, extracellular regulated protein kinases; p38 MAPK, p38 mitogen-activated protein kinases; PKC, protein kinase C; GSK 3β, glycogen synthase kinase 3 beta; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PKG, Protein kinase G; DTT, dithiothreitol; DMEM, Dulbecco's Modified Eagle's Medium; EMSA, Nuclear Extracts and Electrophoretic Mobility Shift Assay; AVSD, atrioventricular septal defects; SRF, serum response factor.

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subsequently repressed the GATA4-dependent gene activation program [21]. Of particular interest, neither S105 nor S419/420 is localized within GATA4's DNA binding domain; however, their phosphorylation impacted GATA4's DNA binding affinity [19,20]. In addition, in gonadal cells, the cAMP/protein kinase A (PKA) signaling pathway enhanced the physical association of GATA4 with its cofactor, CREB-binding protein, via phosphorylation of S261 on GATA4, thereby potentiating GATA4's capacity to activate downstream target genes [22]. Phosphorylation and or activation of GATA4 was blocked by a specific PKA inhibitor [22]. However, the cAMP/PKA-dependent mechanism of phosphorylation and activation of GATA4 remains to be examined in the context of cardiac target genes. The outcome of mediating GATA4's functional activity by posttranslational modification may be context-dependent, and as regulatory mechanisms may play an important role for modulating GATA4's activity under various physiological and pathological settings.

Protein kinase G (PKG) is a serine/tyrosine specific kinase and the major effector of cGMP [23]. PKG-1 has two isoforms, PKG-1α and PKG-1β, products of alternative splicing from the same gene [24]. PKG-1 harbors three major functional domains (cGMP binding domain, C-terminal catalytic domain, and N-terminal domain [24], and is the major mediator elicited by the activation of the NO/cGMP signaling pathway [25,26]. In the absence of cGMP, the N-terminal domain of PKG-1 auto-inhibits the kinase activity; however, binding of cGMP to PKG-1α causes a conformational change, subsequently reversing this inhibition and enabling PKG-1 to phosphorylate its substrates [27]. Previous findings suggested that these two isozymes may play differential roles in the cellular events at baseline than in the progression and or development of pathological conditions. First, the activation of PKG-1α required a much lower level of cGMP compared with that of PKG-1β [28,29]. Secondly, PKG-1α and 1β have distinct substrates [30]. A clue leading to our interest in PKG-1α was its impact on the cardiovascular system and its abundant expression in rabbit cardiomyocytes and in human atrial myocytes [31,32]. Also, PKG-1α played an important role in protecting cells from apoptosis associated with pathophysiological conditions in cardiomyocytes [33,34], may be through phosphorylation of its targets such as CREB [35]. The protective effects of PKG-1 on cardiomyocytes were further corroborated by the observations that cardiac-specific deletion of PKG-1 exacerbated the cardiac phenotypes in response to various stressors in vivo [36]. Finally, potential crosstalk shared between PKG-1α and other signaling pathways such as ERK, GSK3β I, and p38 MAPK may be cytoprotective [33,37].

Here, we report that the GATA4 DNA binding was regulated by PKG-1α by means of phosphorylation and protein–protein interaction. We further tested a number of human GATA4 mutants that exhibited decreased phosphorylation by PKG-1α and DNA binding activity. Thus, our study demonstrates that cGMP-PKG signaling, as a unique regulatory pathway, for mediating the transcriptional activity of GATA4.

2. Materials and methods

2.1. Expression vectors and reporter constructs

The NPPA promoter-fused luciferase reporter construct (NPPA-Luc), GATA4 wild type and mutant expression vectors were previously described [20,38]. PKA expression vectors were provided by Dr. Dinakar Iyer (University of Houston). Full length GATA4 cDNA and its deletion mutants 41–207 aa, 208–328 aa, 329–441 aa and 208–328 aa were PCR-amplified and subcloned into pGEX-4T vector via BamHI and EcoRI sites, correspondingly generating GST tagged bacterial expression vectors. The full length PKG-1α cDNA was PCR-amplified on the template pBS-PKG-1α, and subcloned into PCGN vector via XbaI and BamHI sites. All point mutations were created using site-directed mutagenesis (QuikChange® Site-Directed Mutagenesis Kit, Stratagene), and were confirmed by sequencing.

2.2. Cell cultures and transient transfections

CV1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone). Transient transfections were performed using lipotectamine 2000 (Invitrogen) on CV1 cells based on the manufacturers' protocol. For luciferase assay, reporter construct was transfected with GATA4 wild type or one of its mutants, respectively, in the absence or presence of PKG-1α. When applicable, cGMP (100 μmol/L), KT5823 (a PKG-1α inhibitor, 1 μmol/L) or H89 (a PKA inhibitor, 10 μmol/L) was added 24 h after transfection. Cells were harvested 48 h posttransfection, and luciferase activity was determined using luminometer (BD Monolight 3010). The amount of reporter construct was kept at 0.2 μg per well for a 24 well/plate. The data shown for transfections are represented as means ± SEM obtained from at least three independent assays.

2.3. In vitro phosphorylation assays

Recombinant GST, GST-GATA4 wild type or its point/deletion proteins were prepared as previously described [39]. 1 μg recombinant protein, GST–GATA4 full length, GST–GATA 41–207, GST–GATA4 208–328, GST–GATA4 329–441 or GST–GATA4 208–328 with S261A (conversion of serine 261 to arginine) was incubated with 100 units of PKG-1α (Promega) for 30 min at 30 °C in the buffer containing 40 mM Tris–HCl (pH 7.5), 20 mM magnesium acetate, 0.2 mM ATP, 2.86 μM cGMP and 30,000 cpm/μL γ-^{32}P ATP. The proteins were separated by SDS-PAGE gel, dried and exposed to X-ray film. Total protein was visualized by Coomassie Blue staining of the gel.

2.4. In vivo phosphorylation assay

CV1 cells with expression vectors encoding GATA4 alone, or together with PKG-1α, were homogenized in the lysis buffer containing of 20 mM Tris–Cl [pH 7.5], 0.5% NP-40, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol [DTT] and 0.5% proteinase inhibitor. 80 μg of cell lysates were loaded for Western blots, and the membranes were blotted with antibodies against GATA4, phosphor-S261-GATA4, or myc, respectively (Santa cruz Biotechnology), and revealed by ELC (GE Health).

2.5. GST-pull down and coimmunoprecipitations (Co-IP) assays

GST-pull down assay was performed as detailed previously [39]. Co-IP was performed on whole cell lysis containing overexpressed myc-tagged GATA4 wild type or its mutants in the presence of HA-tagged PKG-1αusing IgG (as a control) or anti-myc antibody for pull down. The precipitated pellets were subjected to 10% NuPage gels, transferred to PVDF membrane, blotted with anti-HA antibody (Santa cruz Biotechnology). Protein bands were visualized by ECL.

2.6. Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins extracted from CV1 cells were overexpressed various GATA4 or PKG-1α proteins as indicated in each figure legend. Nuclear extract preparation and EMSA were carried out following the procedure described previously [20]. Briefly, a double-stranded oligonucleotide containing GATA binding motif on the NPPA promoter, 5′ CCAGGAAGATAACCAAGGAC3′, was labeled with [α-^{32}P]dCTP. 1 or 5 μg nuclear proteins were incubated with 50,000 cpm of ^{32}P-labeled NPPA probe at room temperature for 20 min in the buffer containing 500 ng poly(dI-dC), 120 mM KCl, 25 mM $MgCl_2$, 20 mM Tris–Cl [pH 7.9], 2 mM DTT and 2 mM EDTA. In super shift experiments, 2 μg anti-GATA4 antibody was added to the reaction tubes for additional 20 min incubation. A non-denaturing 5% polyacrylamide gel was used to separate the protein-DNA complexes. The gels were dried and exposed to X-ray films.