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Regulation of scleraxis transcriptional activity by serine phosphorylation

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ABSTRACT

Cardiac fibroblasts are the major extracellular matrix producing cells in the heart. Our laboratory was the first to demonstrate that the transcription factor scleraxis induces collagen 1α2 expression in both cardiac fibroblasts and myofibroblasts. Here we identify a novel post-translational mechanism by which scleraxis activity is regulated and determine its effect on transcription of genes targeted by scleraxis. Putative serine phosphorylation sites on scleraxis were revealed by in silico analysis using motif prediction software. Mutation of key serine residues to alanine, which cannot be phosphorylated, significantly attenuated the expression of fibrillar type I collagen and myofibroblast marker genes that are normally induced by scleraxis. Down-regulation of collagen 1α2 expression was due to reduced binding of the non-phosphorylated scleraxis mutant to specific E-box DNA-binding sites within the promoter as determined by chromatin immunoprecipitation in human cardiac myofibroblast cells and by electrophoretic mobility shift assay. This is the first evidence suggesting that scleraxis is phosphorylated under basal conditions. The phosphorylation sequence matched that targeted by Casein Kinase 2, and inhibition of this kinase activity disrupted the ability of scleraxis to modulate the expression of its target genes while also attenuating TGFβ-induced expression of type I collagen and myofibroblast phenotype conversion marker genes. These results demonstrate a novel mechanism for regulation of scleraxis activity, which may prove to be tractable for pharmacologic manipulation.

1. Introduction

Cardiac fibrosis remains a complex clinical dilemma for millions of patients worldwide, yet continues to elude any therapeutic remedies. The basis for this pathology arises in part from an induction of collagen production by cardiac myofibroblasts following either acute injury to the heart such as myocardial infarction, or following long-term stress such as the increased afterload induced by hypertension [1–3]. Cardiac fibroblasts synthesize numerous extracellular matrix (ECM) proteins that are critical for maintaining the structural integrity of the heart in normal physiological conditions, with the chief among these proteins being type I fibrillar collagen [1,2]. With pathological stress, increased expression and/or release of cytokines and growth factors, such as Transforming Growth Factor β (TGFβ), promotes the phenotypic conversion of fibroblasts into hyper-synthetic myofibroblasts that produce ECM at a rate that exceeds basal turnover, resulting in fibrosis [1–4]. Although this process is beneficial for initial wound stabilization and scar formation after myocardial infarction, over time fibrosis gradually stiffens the myocardium and interferes with the heart's ability to maintain cardiac output, frequently leading to heart failure and death [2,3,5]. To date, no therapies directed specifically at reducing or blocking fibrosis have been introduced due in part to poorly understood mechanisms underlying fibrosis. In particular, the transcriptional regulators of fibrotic genes, and the regulatory mechanisms underlying their function, remain poorly defined.

Scleraxis is a basic helix-loop-helix (bHLH) transcription factor that has been found to play a critical role in the embryological development of tendons and heart valves [6–8]. Our lab previously demonstrated that scleraxis was able to induce type I fibrillar collagen gene expression in cardiac fibroblasts and myofibroblasts via direct transcriptional regulation, and that TGFβ can induce scleraxis expression via a Smad-dependent pathway [9–11]. In addition, scleraxis was found to be up-regulated in the infarct scar following myocardial injury, suggesting a possible role for scleraxis in cardiac pathogenesis [10].

Protein phosphorylation is an important post-translational modification process that plays a critical role in the regulation of protein function. Phosphorylation is involved in the regulation of virtually every biological process, including enzymatic function, metabolic pathways, and gene expression [12]. To date, however, the potential for protein phosphorylation to regulate scleraxis activity has not been examined.

Abbreviations: αSMA, α-smooth muscle actin; bHLH, basic helix-loop-helix; CK2, Casein Kinase 2; ECM, extracellular matrix; TBB, 4,5,6,7-tetrabromo-2-azabenzimidazole; TGFβ, Transforming Growth Factor β.

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Based on an in silico analysis of the scleraxis peptide sequence, we hypothesized that scleraxis is phosphorylated by Casein Kinase 2 (CK2). Here we provide evidence that scleraxis activity requires phosphorylation at several highly-conserved serine residues, and that interfering with this phosphorylation by either mutating key residues or blocking CK2 activity dramatically attenuates the ability of scleraxis to regulate downstream target genes involved in fibrosis, including collagen I and α-smooth muscle actin (αSMA). Together, these findings support our contention that modulation of scleraxis phosphorylation status and thereby its effect on fibrotic gene expression may be an effective strategy for the development of anti-fibrotic strategies.

2. Materials and methods

2.1. Site-directed mutagenesis

The expression vector encoding mouse scleraxis (pECE-HA-FLAG-Scx) was used as the parent construct for generating point mutations [10]. Serine residues in the SESS motif were mutated to either alanine or aspartic acid to block or mimic serine phosphorylation, respectively. PCR-based reactions using specific mutagenesis primers (Supplementary Table 1) were performed using the Quikchange XL-II Site-Directed Mutagenesis Kit (Agilent Technologies) per manufacturer's instructions. Mutations were confirmed by sequencing.

2.2. Cell culture and treatments

NIH-3T3 fibroblasts (ATCC) cultured in Dulbecco's Minimal Essential Medium (HyClone) supplemented with 10% FBS (Gibco) and 1% antibiotics (HyClone) were plated 24 h prior to transfections or treatments and used at ~70% to 80% confluency. NIH-3T3 cells express endogenous scleraxis as we have previously demonstrated [9,10]. Cells were transfected with DNA vectors using Lipofectamine 3000 (Life Technologies) and OptiMEM reduced serum media (Life Technologies). For CK2 inhibition experiments, cells were incubated with 30 μM 4,5,6,7-tetrabromo-2-azabenzimidazole (TBB; Calbiochem) or vehicle (DMSO) in media with 1% FBS (Gibco). Human cardiac myofibroblasts were obtained commercially (Cell Applications Inc., USA) and used for chromatin immunoprecipitation assays.

2.3. Luciferase gene reporter assay

NIH-3T3 cells were plated in 6-well plates and co-transfected with the human collagen Iα2 proximal reporter vector (500 ng) and one of the following expression vectors (500 ng): empty vector control (pECE), scleraxis (Scx), phospho-blocker (triple mutant ScxS/A, double mutants S31/33A or S31/34A or S33/34A, or individual mutants S31A or S33A or S34A), phospho-mimetic (ScxS/D), or an inactive scleraxis mutant lacking both the DNA-binding and protein interaction domains (ScxΔΔ) [9]. In another set of experiments, NIH-3T3 fibroblasts were co-transfected with a scleraxis proximal promoter reporter construct (1.6 kb sequence spanning −1525 to +78 bp relative to the human scleraxis transcriptional start site in pGL4.10) plus expression vectors for scleraxis (Scx) or control empty vector (pECE). Cells were then treated post-transfection for an additional 24 h with TBB or vehicle (DMSO). Cell lysates were processed and analyzed using a Dual-Luciferase Reporter Assay System (Promega) on a Glomax multimode plate reader (Promega). All values were normalized to co-transfected *Renilla* luciferase (pRL).

2.4. Gene expression analysis

NIH-3T3 cells were plated in 6-well plates and transfected (500 ng) with empty vector pECE, Scx, ScxS/A, ScxS/D or ScxΔΔ. For CK2 inhibition assays, cells were transfected with pECE, Scx or ScxS/A mutant for 24 h followed by treatment with 30 μM TBB (Calbiochem) for another 24 h. For experiments with the CK2α dominant negative mutant, cells were co-transfected with expression vectors for the mutant (CK2dn; OriGene Technologies) [13] and scleraxis (Scx) or control empty vector (pECE) for 24 h, followed by recombinant TGFβ$_1$ (10 ng/ml; Peprotech) or vehicle (water) treatment of the cells for an additional 24 h. Total RNA was isolated from all cells and purified using a GeneJET RNA Purification Kit (Thermo Fisher Scientific) and subjected to quantitative real-time PCR analysis using specific primers (Supplementary Table 2) [9]. GAPDH was used as a housekeeping gene. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Chromatin immunoprecipitation (ChIP) assay

Human cardiac myofibroblasts were transfected with mammalian expression vectors (2 μg) encoding either wild type Scx or the phosphorylation-deficient scleraxis triple mutant (ScxS/A) using Lipofectamine 3000. Cell lysates collected 72 h post-transfection were cross-linked, sonicated, pre-cleared and soluble chromatin was incubated with 10 μg anti-scleraxis antibody or pre-immune serum (negative control) [9]. The DNA-protein immune complexes were precipitated using protein A/G agarose resin (Thermo Fisher Scientific), eluted, reverse cross-linked and purified by phenol/chloroform extraction. The purified DNA fragments were subjected to qPCR using specific primers for the scleraxis-binding E-box sites (E1/E2 and E3) in the human COL1α2 gene promoter (Supplementary Table 3) [9]. Genomic DNA was used as positive (input) control.

2.6. Electrophoretic mobility shift assay (EMSA)

Gel mobility shift assays were performed as described previously [9]. COS7 cells were transfected with Scx or ScxS/A mutant for 48 h, and nuclear extracts isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, USA) per manufacturer's instructions. The nuclear fractions were incubated with biotin-labeled oligonucleotide probes corresponding to either E-box 1 or 3 of the human collagen Iα2 gene promoter [9]. Binding reactions were incubated at room temperature for 30 min in a buffer containing 50 ng poly (dI·dC), 5% glycerol, 0.05% NP-40, 0.35 mM $MgCl_2$, 100 mM KCl, 0.5 mM EDTA, and 20 fmol biotin end-labeled probe. A 500-fold molar excess of unlabeled oligonucleotide was used to compete with the biotin-labeled Scx and ScxS/A DNA complexes. DNA-protein complexes were separated on a 6% polyacrylamide gel. Chemiluminescent identification of the complexes was performed using a Lightshift Chemiluminescent EMSA Kit (Pierce Biotechnology, USA), and complexes detected using ECL on CL-Xposure blue X-ray film (Thermo Scientific).

2.7. Western blotting

Total cell protein was isolated using radioimmunoprecipitation buffer (RIPA) as described previously [9,10]. Proteins were separated on SDS-PAGE gels and transferred to PVDF membranes (Millipore). Primary antibodies used for western blotting were mouse anti-FLAG (F3165, Sigma Aldrich), rabbit anti-collagen type I (CL50141AP-1; Cedarlane) and mouse anti-α-tubulin (12G10; Developmental Studies Hybridoma Bank). Antibodies were detected using enhanced chemiluminescence reagents (Santa Cruz Biotechnology) and CL-Xposure blue X-ray film (Thermo Fisher Scientific). Quantity One software (Bio-Rad) was used to measure relative band intensity. The intensity of the collagen bands was normalized to that of α-tubulin. Nuclear lysates used in EMSA experiments were subjected to western blotting using antibodies for detection of the FLAG epitope tag and Lamin A/C (nuclear loading control; 05-714(CH), Millipore).