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Identification of novel regulatory NFAT and TFII-I binding elements in the calbindin-D28k promoter in response to serum deprivation*

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Abstract

Calbindin-D28k, a key regulator of calcium homeostasis plays a cytoprotective role in various tissues. We used serum free (SFM) and charcoal stripped serum (csFBS) culture media as models of cellular stress to modulate calbindin D28k expression and identify regulatory cis-elements and trans-acting factors in kidney and beta cells. The murine calbindin-D28k promoter activity was significantly upregulated under SFM or csFBS condition. Promoter analysis revealed evolutionary conserved regulatory cis-elements and deletion of 23nt from +117/+139 as critical for basal transcription. Bioinformatics analysis of the promoter revealed conserved NFAT and TFII regulators elements. Forced expression of NFAT stimulated promoter activity. Inhibition of NFAT transcriptional activity by FK506 attenuated calbindin-D28k expression. TFII-I was shown to be necessary for basal promoter activity and to act cooperatively with NFAT. Using chromatin immunoprecipitation (ChIP) assays, NFAT was shown to bind to both proximal and distal promoter regions. ChIP assays also revealed recruitment of TFII to the -36/+139 region. Knockdown of TFII-I decreased promoter activity. In summary, calbindin-D28k expression during serum deprivation is partly regulated by NFAT and TF-II. This regulation may be important *in vivo* during ischemia and growth factor withdrawal to regulate cellular function and maintenance.

Keywords

apoptosis; calbindin-D28k; calcium binding proteins; NFAT transcription factors; serum deprivation; TFII-I

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Introduction

Calbindin D28k (D28k) was originally identified as a vitamin D-dependent protein expressed in chick intestinal mucosa [1]. It is a cytosolic calcium binding protein highly expressed in the distal tubule, intestines, central nervous system, primary murine osteoblast cells [2] and in several other organs [3,4,5]. D28k plays an important role in the intracellular calcium homeostasis, its strong buffering capacity prevents cytotoxic effect of high concentration of free calcium in kidney, brain, pancreas, and intestine [3,5]. In pancreatic β cells, D28k attenuates intracellular calcium influx induced by KCl depolarization as well as apoptosis induced by pro-inflammatory cytokines [6,7]. Moreover there was a significant increase in insulin release in islets isolated from the KO mice indicating a role for calbindin in the modulation of depolarization-stimulated insulin release.

Extracellular calcium chelators reduce beta cell death induced by serum withdrawal and intracellular influx [8]. In various mammalian cells, calbindin-D28k plays an important cytoprotective role (reviewed in [9]). In neurons, the pro-apoptotic effects of a mutant presenilin are blocked by calbindin-D28k overexpression [10]. Calbindin-D28k prevented glucocorticoid induced osteocytes and osteoblast cell death through inhibition of endogenous caspase-3 activity [2,11]. Calbindin D28k expression is induced in retinal neurons under ischemic stress [12,13]. Despite its role in apoptosis and calcium homeostasis transcriptional regulation of calbindin-D28k in response to pro-apoptotic conditions, such as ischemic stress, is not well understood.

Calcium influx regulates the activity of the nuclear factor of activated T-cells (NFAT) family of transcription factors. They consists of 5 members: NFATc1, NFATc2, NFATc3 NFATc4 and NFAT5 [14]. NFATc1 through NFATc4 activity are calcium dependent and tightly regulated by the phosphatase calcineurin complex (CN). Activated CN rapidly dephosphorylates NFAT proteins, resulting in conformational changes that trigger their nuclear translocation. NFAT regulates multiple signaling pathways that plays a critical role in response to elevated extracellular calcium signaling [15]. Targeting of NFATc2 promotes caspase-dependent apoptosis of neoplastic cells [16,17].

TFII-I, a basal transcription factor with multiple functions, stimulates transcription of various genes [18,19] and is involved in several cellular processes such as cell growth, cell-survival, and intracellular endoplasmic reticulum stress caused by depletion of intracellular calcium stores [18,20,21,22,23]. We identified conserved NFAT and TFII-I potential binding sites in the murine calbindin D28k promoter. Given their common functions in the regulation of intracellular calcium signaling and apoptosis, in this study for the first time we tested whether NFAT and TFII are involved in transcriptional regulation of calbindin-D28k during apoptotic signals. Serum deprivation was used as a model system of apoptosis induction and beta cell dysfunction. We show here the regulatory functions of NFAT and TFII in the expression of calbindin-D28k and identify previously unreported binding sites in the calbindin-D28k promoter.

MATERIALS AND METHODS

Cell Culture

Madin-Darby canine kidney (MDCK), HEK-293, and Hela cells (ATCC) were cultured in DMEM medium with high glucose (GIBCO-Invitrogen) supplemented with 10% or 5% FBS (Atlanta Biological) and 1x antibiotic/antimycotic (GIBCO-Invitrogen) except during transfection. The rodent insulinoma MIN6 cell was maintained in DMEM medium (Gibco-Invitrogen), as described previously [24]. The calcineurin inhibitor FK506 was used at a final concentration of 0.3 μ M for up to 48 hours as indicated.

Plasmid Constructs and Luciferase Assays

The 3.2kb mouse calbindin-D28k promoter reporter, kind gifts from Dr. Harry T. Orr (University of Minnesota) [25] as well as the promoter deletion constructs were cloned in the pGL3 basic vector (Promega). Bioinformatics analysis of murine 3.2kb calbindin-D28k promoter was performed using the public domain Transcription Element Search System (TESS) software (<http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME>). NFAT expression vectors were kindly provided by Chi-Wing Chow (Albert Einstein College of Medicine). All transient transfection experiments were done in 96 well plates using Fugen-6 (Roche) or lipofectamine 2000 (Invitrogen) following manufacturer's recommendation. The pSV- β -galactosidase expression vector (Invitrogen) was a normalization control. Luciferase activity was measured 24 hours post transfection, using the manufacturer's protocol in the FLUOstar Omega luminometer (BMG LABTECH).

Apoptosis assay

The annexin V-FITC Apoptosis Detection Kit (BioVision) was used to assess cell apoptosis according to the manufacturer's instructions. In brief, HEK-293 and MDCK cells were incubated in 10% FBS or SFM medium for 24hours and assayed according to manufacturer's recommendations. Each sample was analyzed immediately using the FACSCalibur flow cytometer (Becton Dickinson). Data analysis was done with FlowJo (TreeStar Inc.).

ChIP assays and PCR analysis of Calbindin-D28k Promoter Regulation

Twenty-four hours after serum deprivation treatments ChIP assays were performed as previously reported for MIN6 cells [24]. Briefly, cells were cross-linked with 4% paraformaldehyde and the chromatin was fragmented by sonication using the Bioruptor 200 (Diagenode) in an ice-cold water bath. Antibodies against NFATc1 (H-110), NFATc2 (G1-D10) or TFII-I (Santa Cruz Biotechnology) were immobilized on protein A-Sepharose beads and used to precipitate protein:DNA complexes. The bound DNA fragments were quantified by qPCR using SYBR Green Master Mix (Applied Biosystem) with primer sets to amplify various promoter fragments (−3036/−2826, −2775/−2432, −2245/−1855, −309/−91, and −36/+139). All primer sequences can be provided upon request.

Calbindin-D28k Expression Analysis

Total RNA was extracted from MIN6, grown for up to 24 hrs in DMEM containing 10%FBS or SFM using TRI reagent (Sigma). Generated cDNA (iScript Bio-Rad) was used to measure the expression of mouse calbindin-D28k relative to cyclophilin B using Power SYBR Green (Applied Biosystems).

TFII-I siRNA knockdown

Hela cells were grown in phenol-free DMEM supplemented with 5%FBS in 48-well plate. siRNA oligos targeting human TFII-I gene (Ambion) were delivered using lipofectamine RNAimax (Invitrogen) according to manufacturer's instructions. Five hours post-transfection, the medium was replaced with fresh medium containing 10FBS for 48hrs and transfected with pGL3-Basic vector or 3.2kbp promoter with or without FK506 for 24hrs. TFII-I knockdown efficiency was determined by western blotting.

Western Blotting and Antibodies

Protein samples from whole cell extracts were prepared for and separated by SDS-PAGE after Bradford quantification and transferred onto nitrocellulose membranes (Bio-Rad). The blots were incubated overnight at 4 °C with the antibodies against calbindin D28k (Santa Cruz Bio-technology, sc-7691, sc-365360;), TFII-1 (Santa Cruz Biotechnology sc-28716,) and NFATc1 (Pierce MA3-024, Santa Cruz Bio-technology); sc-7294. Blots were developed with chemiluminescent reagents (Pierce). Immunoblots were quantitated using the ImageJ software package (NIH) and knockdown efficiency was determined relative to β -tubulin or GAPDH loading controls.

Statistics

All experiments were performed least twice in triplicates for each condition. Data are reported as the mean $\pm$ sem. Data were analyzed by the *Student's t-test* for paired observations. When three or more means were compared ANOVA was applied followed by Dunnett's multiple comparison tests. All analysis was performed using the Prism 6.0 program (Graph Pad Software). A value of $p < 0.05$ was considered significant.

RESULTS

Serum deprivation stress increases apoptosis and activates the 3.2kbp calbindin-D28k promoter

MDCK, HEK-293 and Hela cells were placed under serum deprivation to induce apoptosis. Under SFM culture conditions, both cell lines showed increased percentage of apoptotic cells compared to the FBS condition. The percentage of apoptotic MDCK cells increased approximately 2.5 $\times$ (from 2.8% to 7%) while there was a $\sim$ 4 $\times$ increase of apoptotic HEK-293 cells (from 1% to 4.4%). Forced expression of D28k suppressed apoptosis in HEK-293 (4.4% to 2%).

The mouse -3036/+139 calbindin D28k promoter (3.2kbp) and several truncated promoter fragment reporters (Fig 1A) were transfected into MDCK, HEK-293 and Hela cells supplemented with various serum concentrations. Relative to FBS, serum deprivation

increased promoter activity in all cell lines. In MDCK cells the fold-increase ranged from 2.5× for 1%FBS to 10× in SFM (Fig 1B). In Hela cells grown in SFM there was approximately 60-fold activation. On the other hand promoter activity was decreased by deletion of the sequences upstream and downstream of the transcription start site. -3036/-1102, -3036/-916, and -3036/-141 truncated promoters showed progressive drops in fold induction (Fig 1C-D). Deletion of only a 23-nt fragment (+117/+139) upstream of the start site in the 3.2kb promoter reduced its activity to as low as only 6-fold over baseline (HEK-293 cells, Fig 1D) and deletion of the +2/+139 region further decreased the promoter activity essentially to the empty vector level. Therefore, important regulatory elements exist downstream of the transcriptional start site.

NFAT and TFII-I bind to the calbindin promoter and increase the promoter activity

Bioinformatics analysis of the 3.2kb promoter revealed multiple putative transcription factor binding sites (cis-elements) in the promoter. Considering their calcium dependent activities, we focused on the conserved NFAT and TFII-I sequences in the regulation of D28k promoter activity. Both NFATc2 and NFATc1 induced a two to three-fold increase in promoter activity in both Hela and HEK-293 cell lines (Figure 2A,C). The increased activity was abolished by treatment with FK506, a CN/NFAT inhibitor (Figure 2A,C, E). ChIP assays performed using anti-NFATc2 on HEK cell lysates co-transfected with the 3.2kb promoter and NFATc2 expression vector showed up to ~300 fold enriched NFATc2 binding at the proximal end (-140/+139, Figure 2D).

In Hela cells, TFII-I was knocked down nearly 100% (Figure 2B) resulting with a greater reduction of promoter activity compared to FK506 treatment. In Hela cells combined TFII-I knockdown with FK506 did not significantly decrease over TFII-I knockdown alone (Figure 2C). Knockdown of TFII-I decreased promoter activity in HEK-293 cells grown in FBS or SFM, (Figure 2E). Combined TFII-I knockdown with CN/NFAT inhibition by FK506 was resulted in a significantly greater decrease only in HEK cells growing in FBS (Figure 2F).

NFAT and TFII-I mediated upregulation of endogenous calbindin-D28k

MIN6 cells grown in 10%FBS or SFM for up to 48 hrs showed a significant increase in calbindin-D28k expression -fold in SFM compared to FBS (Figure 3A). The mRNA level in SFM was significantly reduced by FK506 (Figure 3A). Serum deprivation also increased calbindin-D28k protein levels (Figure 3B).

Using CHIP assay in MIN6 cells, we found that both -NFATc2 (Figure 4A) and anti-NFATc1 (Figure 4B) bind to the distal (-3036/-2826) and the proximal promoter (-36/+139) especially when in SFM treated cells. ChIP assay, using a TFII-I antibody, showed constitutive recruitment of TFII-I in the calbindin-D28k proximal promoter region (-36/+139) in all treatments (Figure 4C) indicating its importance for basal transcription of the gene. However, TFII-I and NFATc did synergize on the calbindin promoter activity.

Discussion

In the present study, we have shown that the calbindin-D28k promoter is activated by serum deprivation or charcoal stripping of serum, conditions associated with inhibition of growth

factor signaling and induction of apoptosis [8]. In beta cell lines endogenous expression of calbindin-D28k protein and mRNA were increased in response to serum deprivation. In the upstream and downstream regions of the transcription start site, we identified regulatory regions that are critical to calbindin-D28k promoter activity. TFII-I was critical to the promoter activity under the basal conditions (10%FBS) and serum restricted conditions. Ectopic overexpression of NFAT increased promoter activity while CN/NFAT pharmacological inhibition decreased calbindin expression and promoter activity. NFAT recruitment to the calbindin-D28k promoter regions increased when the cells were grown in serum free medium.

Calcium is an important regulator of apoptotic signaling [26]. Previous studies have shown the cytoprotective effect of calbindin in preventing apoptosis induced by TNF- α treatment [2] through inhibition of caspase 3 activity. Compared with wild type mice, calbindin-D28k KO embryo showed a significant increase in number of TUNEL-positive cells with upregulation of pro-apoptotic proteins and ER stress marker Grp78/Bip. Our results provide a transcriptional mechanism whereby calbindin-D28k may be regulated during cellular stress.

The molecular cloning and characterization of the proximal promoter of mouse calbindin-D28k gene has allowed the identification of transcriptional regulatory regions [25,28,29,30]. Most studies focused on regulation by steroid hormones or calcium. Little is known about the effect of serum deprivation on transcriptional regulation of calbindin-D28k expression. Serum deprivation has been used as an *in vitro* model of ischemia and growth factor deprivation inducing intracellular reactive oxygen species [31]. In our study there was an inverse dose dependent relationship between FBS concentration and promoter activation. Other investigators have shown that serum deprivation causes alteration of intracellular calcium concentration by release of calcium ions from endoplasmic reticulum and activation of apoptotic factors such caspases in hippocampal neuroblast and fibroblastic cells which express calbindin-D28k [32,33]. In this context the calcium buffering and anti-apoptotic effects of calbindin-D28k may be cytoprotective. NFAT is a key transcriptional factor involved in intracellular calcium signaling pathways. High calcium concentration in MC4, preosteoblast cells, increased the expression of NFATc1 and NFATc3 [15]. Calbindin-D28k has not been suggested as an NFAT target gene. In our study co-transfection of the D28k promoter NFATc1 and NFATc2 resulted in significantly higher promoter activity alone. Treatment with the NFAT inhibitor FK506 blunted the promoter activity. ChIP analysis showed strong recruitment of NFATc1 and NFATc2 to the +1/+139 and -140/+139 regions, recruitment to the latter was greater. In endogenously expressing cells, calbindin-D28k expression increased in SFM and was inhibited by FK506. Stronger interaction of NFAT with DNA was observed when cells grown under SFM condition. Our results are consistent with a model in which serum deprivation causes increased intracellular calcium concentration with subsequent NFAT activation by calcineurin and transcriptional activation of the calbindin-D28k gene.

We showed that TF-II is important for the basal transcription of the calbindin gene. TFII-I, was initially identified as initiator of transcription in many genes, however later studies have reported that phosphorylation of TFII-I by mitogenic stimuli such as serum, EGF or PDGF,

induced multifunctional transcription factors that up-regulate transcription of a diverse range of genes [21]. Other studies have also reported that the endoplasmic reticulum stress caused by calcium depletion leads to enhanced TFII-I tyrosine phosphorylation and increased cell survival [20]. Although in the present study we did not monitor the TFII-I expression, we showed by ChIP assay that MIN6 cells grown under serum deprivation stress conditions had a higher magnitude of TFII-I interaction with the proximal promoter. Our results also showed D28k promoter activity was strongly decreased by siRNA knockdown of TFII-I. Interestingly, our results indicate both TFII-I and NFAT seem to be important for full promoter function. Perhaps most importantly the TFII-I recognition site CTCTCA, present at +115/+120, is within +1/+139 shown to be necessary for promoter activation and within a 23bp region shown to be very important for maximal up-regulation during serum deprivation.

In conclusion, this is the first description of NFAT and TF-II in the transcriptional regulation of calbindin-D28k expression. It is also the first description of the critical role of sequences downstream from the start site, including a 23nt sequence at the proximal end of the calbindin-D28k promoter. Combined with other reports in the literature, our results indicate the expression of calbindin-D28k is physiologically inducible under extracellular stress conditions that may cause the release or changes in intracellular calcium level. Although, there are several TFII-I and NFATs binding sites present in both distal and proximal promoter we found these two transcription factors are more active in binding to the proximal promoter under our experimental conditions. Because deletion mutants lacking portions of the distal end of the promoter were less active in serum deprivation stress, we also hypothesize that the interaction of both distal and proximal promoter elements with downstream region is required to maintain the full promoter function.

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Highlights

- Calbindin-D28k promoter activity in kidney and pancreatic beta cells increases response to serum withdrawal.
- Cis-elements downstream from the start site in (+2/139) are necessary for promoter activity.
- A 23nt from +117/+139 is critical for basal transcription.
- Transcriptional control of calbindin-D28k expression is regulated in part by NFAT and TFII-I recruited to specific regions of the proximal murine calbindin-D28k promoter.
- Discovery of a novel regulatory mechanism of calbindin-D28k expression by CN/NFAT may provide new methods to modulate cell survival in response to cytotoxic stress.

Figure 1.

The 3.2kb calbindin-D28k promoter activity is regulated by serum deprivation. (A) Schematic of evolutionarily conserved putative cis elements in the 3.2kb calbindin D28k promoter and truncated promoters. (B) Calbindin D28k promoter activity in MDCK cells after treatment with various serum concentrations for 16 hours relative to control 10%FBS treatment. (C) Promoter activity in HeLa cells transfected with various deletion constructs and grown in SFM for 24 hrs. (D) HEK-293 cells grown in 96-well plates in phenol-free DMEM supplemented with 5% FBS and transfected with various deletion constructs and grown in 5% csFBS phenol free DMEM. Fold change is relative to control empty basic vector. * $p < 0.05$, ** $p < 0.001$ relative to full length 3.2 Kbp promoter.

Figure 2.

NFATs and TFII-I activate the calbindin-D28k promoter (A) Hela cells transfected with the 3.2kbp calbindin-D28k promoter with the NFATc1 and NFATc2 expression vector and treated with vehicle or FK506 for 16 hours. (B) Representative immunoblot of TFII-I knock-down by siRNA in Hela cells followed by FK506 treatment. (C) Transient transfection of Hela cells with TFII-I siRNA for 72hrs and the 3.2kbp promoter transfection with and without the CN/NFAT inhibitor FK506 the last 24hrs. Fold activation relative to pGL3 basic vector for each culture condition. (D) HEK-293 cells co-transfected with the 3.2kbp calbindin-D28k promoter and an NFATc2 expression vector then treated with vehicle or FK506 for 16 hours. (E) ChIP assay using NFATc2 in HEK-293 cells cultured in SFM media. (F) HEK cells grown in FBS or SFM transfected with the 3.2kbp calbindin-D28k promoter and TFII-I siRNA treated with vehicle or FK506 for 16 hours. * $p < 0.05$, and ** $p < 0.05$ relative to si control (D28k 3.2Kbp promoter (Pr) siCtrl), NS: not statically significance. + $p < 0.05$ versus FK506 or TFII-I siRNA alone.

Figure 3. SFM induces endogenous calbindin-D28k in β -like cells. (A) Calbindin D28k gene expression relative to cyclophilin B in MIN6 cells under 10% FBS or SFM medium without and with 1 μ M FK506 after serum withdrawal. * $p < 0.05$ relative to FBS control. (B) Calbindin D28k protein expression assessed by immunoblotting from MIN6 cells lysate after SFM.

Figure 4.

Cooperative regulation of D28k promoter by NFAT and TFII-. (A) ChIP assay using NFATc2 and NFATc1 (B) in MIN6 cells cultured 10% serum, csFBS or SFM media by qPCR analysis using primers binding to mouse calbindin-D28K promoter. (C) ChIP assay of TFII-I to the calbindin-D28k proximal promoter in MIN6 cells in 10% serum or SFM medium.