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Regulation of expression of 1,25D_3-MARRS/ERp57/PDIA3 in rat IEC-6 cells by TGFβ and 1,25$(OH)_2D_3$

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ABSTRACT

We examined the transcriptional regulation of expression of the redox-sensitive Membrane-Associated-Rapid Response, Steroid-binding (1,25D_3-MARRS) protein specific for 1,25$(OH)_2D_3$ in a rat small intestinal cell line, IEC-6, that demonstrates rapid responses to 1,25$(OH)_2D_3$. 1,25D_3-MARRS binds and is activated by 1,25$(OH)_2D_3$, but is not itself up-regulated by treatment with 1,25$(OH)_2D_3$, nor is there a Vitamin D response element (VDRE) in its proximal promoter. We previously reported that transforming growth factor β (TGFβ) increased steady state levels of 1,25D_3-MARRS transcript and protein approximately two-fold [Rohe B, Safford SE, Nemere I, Farach-Carson, MC. Identification and characterization of 1,25D_3-membrane-associated rapid response, steroid (1,25D_3-MARRS)-binding protein in rat IEC-6 cells. Steroids 2005;70:458–63]. To determine if this up-regulation could be attributed to the function of a highly conserved consensus smad 3 binding element present in the proximal promoter of the 1,25D_3-MARRS gene, we created a promoter-reporter [SEAP] construct that was responsive to TGFβ (200 pM). Deletion or mutation of the smad 3 element greatly reduced the response of the 1,25D_3-MARRS promoter to TGFβ. Subsequent studies found that the smad 3 response element is bound by a protein found in the IEC-6 nuclear extract, most likely smad 3. Interestingly, although 1,25$(OH)_2D_3$ alone did not increase expression of the 1,25D_3-MARRS promoter-reporter, co-treatment of transfected IEC-6 cells with 1,25$(OH)_2D_3$ and TGFβ shifted the dose–response curve to a lower effective concentration (100 pM peptide). We conclude that TGFβ is a transcriptional regulator of 1,25D_3-MARRS expression via a functional smad 3 element and that cross-talk with non-classical 1,25$(OH)_2D_3$-stimulated pathways occurs. The findings have broad implications for redox-sensitive signaling phenomena including those that regulate phosphate transport in the intestine.

1. Introduction

The recently identified 1,25D_3-MARRS (*m*embrane-*a*ssociated-*r*apid *r*esponse, *s*teroid-*b*inding) protein, identical to ERp57/PDIA3, is a key regulator of 1,25$(OH)_2D_3$-stimulated intestinal phosphate absorption in chickens [1]. The protein contains two thioredoxin domains that are characteristic of members of the redox-sensitive family of proteins that include protein disulfide isomerase (PDI) and ERp72 [2]. We recently reported [3] that oxidation of 1,25D_3-MARRS with H_2O_2 gradually (10–20 min) reduced 1,25$(OH)_2D_3$ binding to 1,25D_3-MARRS protein. Consistent with this, feeding animals

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with diets enriched in the antioxidants Vitamins C and E doubled net phosphate absorption *in vivo* as compared to chicks fed a control diet [3]. Finally, antioxidant diets increased binding of [^{3}H]1,25(OH)$_2$D$_3$ to 1,25D$_3$-MARRS, leading us to suggest that 1,25D$_3$-stimulated phosphate transport in the intestine is sensitive to redox-sensitive transcription- and membrane-initiated signaling pathways.

Very little is known of the mechanisms that regulate expression of the 1,25D$_3$-MARRS receptor itself, although its levels are associated with the amount of phosphate that can be transported in response to 1,25(OH)$_2$D$_3$ [1,4]. To this end, we analyzed the immediate proximal promoter [within 1000 bp] of the transcription start site of the 1,25D$_3$-MARRS receptor gene which lies at chromosome 3q35 in the rat. Examination of this region revealed a smad 3 consensus element [GTCTGGCCC] but no consensus VDRE. Consistent with this, we found that treatment of IEC-6 cells withTGFβ1, but not 1,25(OH)$_2$D$_3$, doubled steady-state transcript and protein levels of the 1,25D$_3$-MARRS receptor [5] Stimulation was dose-dependent with a maximum increase occurring at 200 pM TGFβ1.

Rapid actions of 1,25(OH)$_2$D$_3$ have been shown to alter gene transcription via mechanisms independent of a VDRE, and may involve activation of Ca^{2+} signaling [6], a phenomenon recently named "excitation–transcription coupling" [7]. In these cases, the seco-steroid hormone also alters membrane ion permeability, characteristically shifting the threshold of activation for voltage sensitive ion channels toward the resting membrane potential, a so called "left shift" [8,9]. Such action is postulated to cooperatively sensitize peptide hormone receptors to their ligands, typically reducing the concentration of peptide hormone needed for cell activation. This type of mechanism defines a form of receptor "cross-talk" that creates a functional synergy between steroid and peptide hormones [10].

In this study, we used a promoter-reporter construct to analyze the response of the smad 3 element in the rat 1,25D$_3$-MARRS promoter to TGFβ1. We used a deletion/mutation approach to demonstrate the functionality of the proximal element, and surveyed cellular extracts for the presence of TGFβ1 pathway proteins and nuclear extracts for smad 3 binding proteins. Finally, we investigated the ability of 1,25(OH)$_2$D$_3$, when added with TGFβ1, to alter the dose response for increase of steady state levels of 1,25D$_3$-MARRS receptor expression.

2. Experimental

2.1. Cell culture

Rat IEC-6 cells were purchased from ATCC (Manassas, VA) and maintained in DMEM with 5% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco Invitrogen Corporation Carlsbad, CA) at 37 °C with 5% CO_2. Cells were grown to 80% confluency and passaged with trypsin/EDTA.

2.2. Hormone treatment

Cells were withdrawn from serum 24 h prior to treatment. Cells then were treated with TGFβ1 alone at the indicated concentrations or co-treated with TGFβ1 and 1 nm 1,25(OH)$_2$D$_3$ for 18 h.

2.3. ELISA

IEC-6 cells were withdrawn from serum in the medium for 48 h. After serum withdrawal, media was collected and analyzed by ELISA for TGFβ1 levels. All treatments were performed in serum free DMEM at the indicated concentrations.

2.4. Transfection

IEC-6 cells were allowed to attach and proliferate in six well culture plates for 24 h in media containing 5% (v/v) FBS, then withdrawn from serum for 24 h. One half hour prior to transfection, the cells were placed in Opti-MEM media (Gibco Invitrogen Corporation) and incubated under normal incubation conditions. Cells then were transfected using 4 μL of Lipofectamine 2000 kit and protocol (Gibco Invitrogen), and a total amount of 3 μg of DNA (1.5 μg of pEGFP-N3 for transfection efficiency detection and 1.5 μg of empty, control or promoter/reporter construct plasmid) per well.

2.5. RT-PCR

Cells were harvested at 80% confluency and total RNA was isolated by use of RNAeasy Mini-prep® kits and a protocol supplied by the manufacturer (Qiagen Valencia, CA). Five hundred ng of RNA were used in conjunction with Omniscript™ kit and a protocol provided by the manufacturer (Qiagen). Polymerase chain reaction (PCR) was performed with the cDNA template using Hot Star Taq™ polymerase kit and protocol (Qiagen). Samples then were identified by electrophoresis on 1.2% (w/v) agarose (Gibco Invitrogen) in TAE at 90 V for 45–60 min. Quantitative (Q, or real time)-PCR was performed as above using the Quantitect™ SYBR® Green kit and protocol (Qiagen). Primers used in this study include rat smad 2: 5′-TCTAACAGAACTGCCGCCT-3′, 5′-GGAAGGTCTCTCCAACCCTC-3′; rat smad 3: 5′-GCAGTGGCAATCACAGAGAA-3′, 5′-AACAGCCTGGGAGAGACTCA-3′; rat smad 4: 5′-GTTGCAGATAGCTTCAGGGC-3′, 5′-GGATCCACGTATCCATCCAC-3′; rat smad 7: 5′-GCACAAAGTGTTCCCTGGTT-3′, 5′-ACAACAAAACCGACCGAAAG-3′; rat SARA: 5′-CACGGGTGTGAAAGGAGATT-3′, 5′-AGTGCATCCCGGTAAAGTTG-3′; and Q-PCR primers, 1,25D$_3$-MARRS-RT: 5′-AAACTCAACTTTGCTGTAGCTAGCC-3′, 5′-TGCATGACAAACTTCTCTCCTTTAGC-3′.

2.6. Promoter/reporter constructs

Genomic DNA from IEC-6 cells grown to 80% confluence was extracted using the Genomic Tip® 20/G kit and protocol (Qiagen) for cultured cells using proteinase K at a 20 μg/μL final concentration as per kit instructions. Two primer sets were produced to generate a PCR product of 943 bp upstream of the 1,25D$_3$-MARRS transcriptional start site and a 3′ truncated PCR product of 867 bp removing the smad 3 response element. These products then were cloned individually into the pSEAP2Basic vector from BDBiosciences Clontech (San Jose, CA). The smad 3 mutant construct was produced by use of the Quikchange II XL