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Expression of human β-defensin 1 is regulated *via* c-Myc and the biological clock

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ABSTRACT

Human β-defensin 1 (hBD-1) is an important antibacterial polypeptide whose expression is not induced by infection or inflammation. Our objective was to study the regulation of hBD-1 expression. Recently, we found that albumin up-regulated hBD-1 as well as c-Myc expression, suggesting that c-Myc may regulate hBD-1 expression *via* a non-inflammatory pathway. Direct evidence for the involvement of c-Myc was achieved by the inhibition of hBD-1 expression in the presence of a specific c-Myc inhibitor. Since both c-Myc and CLOCK:BMAL1 heterodimer, the complex of the core clock mechanism, bind to E-box (5′-CACGTG-3′) and E-box-like sequences to activate transcription, we studied whether hBD-1 expression was also regulated by the biological clock. Synchronization of HCT-116 cells by dexamethasone showed oscillation of hBD-1 and c-myc mRNA indicating that both are clock-controlled output genes. Using transfections and luciferase reporter assays in human embryonic kidney (HEK-293) cells, we found that hBD-1 promoter was induced by CLOCK:BMAL1 co-expression. hBD-1 promoter truncation and mutagenesis analyses revealed that the distal E-box-like binding sequence was the target of both CLOCK:BMAL1 and c-Myc for hBD-1 expression. This activation was abolished when CRY1 was co-expressed in these cells. Thus, hBD-1 expression is mediated by c-Myc and the CLOCK:BMAL1 heterodimer, whereas CRY1 expression represses this complex. These changes in hBD-1 levels lead to its circadian oscillation.

1. Introduction

Innate and adaptive immunity are two essential elements of host defense. One mechanism of the innate immunity is the secretion of broad-spectrum antimicrobial substances, such as cathelicidins and small cationic polypeptides named defensins (Ganz, 2003; Froy, 2005). The two main defensin subfamilies, α- and β-defensins, differ in length and pairing of the six cysteines (Selsted and Ouellette, 2005). α- and β-defensins are salt-sensitive and the direct antimicrobial effect occurs in vacuoles of phagocytes and on mucosal epithelia, where there is low ionic strength (Goldman et al., 1997; Yang et al., 2002).

Human β-defensins include six members (hBD-1 to hBD-6) that are expressed in a wide variety of tissues (Lehrer and Ganz, 2002; Ganz, 2003). hBD-1, which was originally isolated from the plasma of patients with end-stage renal disease (Bensch et al., 1995), is the most important antimicrobial peptide in human epithelia against infection, as its expression is constitutive in most tissues (Lehrer and Ganz, 2002; Ganz, 2003). Human colon epithelial cell lines, such as HT-29 or Caco-2, constitutively express hBD-1 and its expression is not up-regulated after stimulation with IL-1α, TNF-α, IFN-γ, LPS, *Salmonella dublin*, or *Escherichia coli*. Similar levels of hBD-1 mRNA were also found in intestinal biopsies taken from healthy and inflamed colonic mucosa of Crohn's disease and ulcerative colitis patients (O'Neil et al., 1999; Wehkamp et al., 2002; Lehrer and Ganz, 2002; Froy, 2005). Although in most cases hBD-1 expression is constitutive, there are some instances in which hBD-1 expression has been shown to be up-regulated, e.g., in monocytes exposed to LPS, or IFN-γ; in uterine epithelial cells stimulated with Toll-like receptor 3 agonists [poly(I:C) or double-stranded RNA], in pulmonary gland epithelial cells exposed to bacilli Calmette–Guerin cell wall components, and in human colon adenocarcinoma (HCT-116) cells exposed to bovine serum albumin (BSA) (Duits et al., 2002; Fang et al., 2003; Zhu et al., 2003; Schaefer et al., 2005; Froy, 2005; Sherman et al., 2006).

Circadian rhythms in mammals are regulated by the master clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Reppert and Weaver, 2002). A critical feature of circadian timing is the ability of the clockwork to be reset by light (Lucas et al., 2001). Environmental light, perceived by the retina, entrains

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the SCN clock to the 24-h day. Synchronization among SCN neurons leads to coordinated circadian outputs from the nuclei, ultimately regulating rhythms in physiology and behavior (Liu et al., 1997; Herzog et al., 1998). Other peripheral clocks have been found in other tissues, such as the liver, digestive system, etc. (Lee et al., 2001; Froy and Chapnik, 2007). A number of genes constitute the biological clock. *Clock*, the first clock gene identified in mammals (Vitaterna et al., 1994), encodes the transcription factor CLOCK that dimerizes with BMAL1 to activate transcription by binding to E-box (5′-CACGTG-3′) and E-box-like enhancer sequences. Thus, CLOCK and BMAL1 constitute the positive limb of the clock (Reppert and Weaver, 2002). *Per1* and *Per2* together with *Cry1* and *Cry2* are induced by the heterodimer CLOCK:BMAL1, but once the proteins are produced they inhibit transcription and serve as the negative feedback loop of the clock (Reppert and Weaver, 2002; Kume et al., 1999).

Previously, we have shown that BSA up-regulated hBD-1 expression in HCT-116 cells. Analysis of the 5 kbp hBD-1 promoter region revealed several putative binding sites for c-Myc (Sherman et al., 2006), a transcription factor that plays a key role in cell proliferation and apoptosis (Evan and Vousden, 2001). BSA-treated HCT-116 cells exhibited also higher levels of c-Myc indicating that BSA may induce hBD-1 transcription and secretion *via* a non-inflammatory pathway involving c-Myc (Sherman et al., 2006). As mammalian components of innate immunity are regulated by the circadian clock (Froy et al., 2005; Froy and Chapnik, 2007) and c-Myc binding site is the E-box sequence that is needed for CLOCK:BMAL1 binding, we examined the role of the circadian clock and c-Myc in regulating hBD-1 expression. Herein we show that both the biological clock and c-Myc dictate hBD-1 expression from an E-box-like sequence on hBD-1 promoter.

2. Materials and methods

2.1. Cell culture and treatments

Human colon carcinoma cells (HCT-116) and human kidney 293 cells (HEK-293) were maintained at 37 °C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Sigma, Israel) and 10% heat-inactivated fetal bovine serum (Biological Industries, Israel). HCT-116 cells were grown to 80% confluence and the medium was then replaced by DMEM as control or DMEM supplemented with fatty acid-free Fraction V BSA (Sigma, Israel) in the absence or presence of 64 μM of the c-Myc inhibitor (*Z*,*E*)-5-(4-ethylbenzylidine)-2-thioxothiazolidin-4-one for 6 h (Calbiochem, Germany). After 6 h, media were collected and cell total RNA was extracted. For circadian rhythms measurements, HCT-116 cells were grown to 80% confluence and synchronized by addition of 40 μM dexamethasone. After 2 h, the medium was changed to DMEM and 10% heat-inactivated fetal bovine serum. Cells were harvested every 4 h and total RNA was extracted.

2.2. RNA extraction and quantitative real-time polymerase chain reaction

For hBD-1, *hPer-1*, and c-myc gene expression analyses, total RNA was extracted from HCT-116 cells using TRI Reagent (Sigma, Israel). Total RNA was DNase I-treated using RQ1 DNase (Promega, USA) for 2 h at 37 °C, as was previously described (Froy et al., 2006). 2 μg of DNase I-treated RNA were reverse-transcribed using MMuLV reverse transcriptase (Promega, USA) and random hexamers. 1/20 of the reaction was then subjected to quantitative real-time PCR using the Sybr Green Master kit (Applied Biosystems, USA) and the ABI Prism 7300 Sequence Detection System. Primers for hBD-1, *hPer-1*, and c-myc were tested alongside the normalizing gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

2.3. Western blot assays

After stimulation of HCT-116 with BSA for 6 h, supernatants were removed and cells were harvested, centrifuged at 12,000 rpm for 3 min, and washed three times in phosphate-buffered saline (PBS). Cells were lysed in lysis buffer [20 mM Tris–HCl pH 7.8, 100 mM NaCl, 5% glycerol, and protease inhibitors (Sigma, Israel)]. Protein concentration of extracts was estimated using the Bradford reagent (Bio-Rad, USA) with BSA as standard. Equal amounts of protein were loaded onto a 10% acrylamide:bisacrylamide gel and blotted onto a Hybond-C nitrocellulose filter (Amersham Biosciences, UK). The nitrocellulose blot was then processed as was described (Sherman et al., 2006) using rabbit anti-human c-Myc (Cell Signaling, USA) 1:1000 in blocking solution or mouse anti-human actin (MP Biomedicals, France) 1:5000 in blocking solution. Staining was then visualized using the Renaissance-enhanced chemiluminescence (ECL) kit and ECL Hyperfilm (Amersham Biosciences, UK). Densitometry was performed using an imaging densitometer to quantify the intensity of bands.

2.4. Promoter isolation, truncation, and mutagenesis

For genomic DNA isolation, HCT-116 cells were lysed in proteinase K buffer [10 mM Tris–HCl pH 8, 100 mM NaCl, 50 mM EDTA, 0.5% SDS and 250 μg/ml proteinase K], and incubated at 50 °C for 5 h. The mixture was centrifuged at 14,000 rpm for 5 min at 4 °C. Equal volume of isopropanol was added to the supernatants and the mixture was centrifuged at 14,000 rpm for 15 min at room temperature. The supernatant was discarded and the precipitate was washed with 75% ethanol and resuspended in water. Template genomic DNA was reacted by PCR with three groups of oligonucleotide primers: (1) primers designed according to the 1117 bp full promoter of the hBD-1 (GenBank accession no. NT_023736.16) containing three E-box-like sequences (hBD-1 F 5′-gcggtaccctggatctctctctgtctttctctg-3′, hBD-1-R 5′-gcaagcttagaggcttccagaggctggagcgtc-3′). (2) Primers designed to delete the distal (149 bp downstream of the 5′-end of the 1117 bp template) E-box-like sequence, thus generating a short promoter region of 367 bp (hBD-1-Δdis) (hBD-1-Δdis-F 5′-gcggtaccaccagcttagagccgagcggcccc-3', hBD-1-Δdis-R 5′-gcaagcttagaggcttccagaggctggagcgtc-3′). (3) A primer designed to mutate the distal E-box-like sequence 5′-CATGTG-3′ to 5′-CCTGCG-3′ (hBD-1-mut-R 5′-gcagccgcagggcatgagac-3′). For the mutagenesis, we performed PCR using Taq Mix Purple (Lamda Biotech, USA), as has recently been described (Chapnik et al., 2008), generating hBD-1-mut.

2.5. Transfections and luciferase reporter assay

For luciferase reporter assays, gel-purified promoter sequences were ligated into the Kpn I/Hind III sites of the pGL3-Basic vector (Promega, USA) upstream of the luciferase gene. HEK-293 cells were transfected using jetPEI reagent (Polyplus Transfection, USA) according to the manufacturer's instructions. 48 h after transfection, cells were washed with PBS and harvested in Reporter Lysis Buffer (Promega, USA). Luciferase activities were assayed with the Dual-Luciferase Reporter Assay System (Promega, USA) using a Tristar LB-941 (Berthold Technologies, Germany) luminometer. β-Galactosidase was co-transfected and used as a normalizer. The amount of DNA per well was adjusted with the pcDNA3.1 empty vector.