Molecular and Cellular Endocrinology 314 (2010) 118–127

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Identification and characterization of the hypoxia-responsive element in human stanniocalcin-1 gene

A.Y.S. Law, L.Y. Ching, K.P. Lai, Chris K.C. Wong*

Department of Biology, Hong Kong Baptist University, Kowloon Tong, Hong Kong, Hong Kong

ARTICLE INFO

Article history:
Received 23 April 2009
Received in revised form 9 July 2009
Accepted 10 July 2009

Keywords:
FIH
p300
siRNA
Promoter

ABSTRACT

In this study, we aimed to identify the hypoxia-inducible factor-1 (HIF-1) binding motif in human STC1 gene promoter and to characterize the associated gene transactivation mechanism. Using normoxic human nasopharyngeal cancer cells (CNE2), we manipulated the stability of HIF-1α protein by over-expressing HIF-1α or the silencing of prolyl hydroxylase-2 (PHD2), to illustrate HIF-1 activation of STC1 promoter-driven luciferase activity. Subsequently luciferase activities of the deletion and mutated STC1 promoter constructs were investigated in HIF-1 overexpressed cells. The data revealed the presence of an authentic HRE motif in STC1 gene. This result was further supported by the chromatin immunoprecipitation (ChIP) assay. Using a similar experimental treatment, however, had no significant effect on the expression level of STC1 mRNA and protein. Moreover the activation of STC1 expression can be restored by the silencing of "factor inhibiting HIF-1" (FIH-1) in either HIF-1 overexpressed or PHD2 silenced cells. The data implied that the HIF-1-mediated STC1 gene expression required the recruitment of p300. This presumption was confirmed by the use of p300 inhibitor, chetomin and HIF-1α/p300 re-ChIP assay. Collectively our data provide the first evidence to show that STC1 is a FIH-inhibited gene with a functional HRE motif located at the upstream region between −2322/−2335. The data support the need for further investigation to reveal if STC1 can be used as a novel tumor marker for HIF-1 induction and for the monitoring of anti-angiogenic therapy.

1. Introduction

Stanniocalcin-1 (STC1) is a hypocalcemic glycoprotein hormone originally discovered in teleost fishes (Gerritsen and Wagner, 2005). The hormone is known to act in an endocrine fashion for the regulation of plasma Ca^{2+} homeostasis. The mammalian form of STC1 has been identified in the last decade and the hormone may have evolved to act as an autocrine/paracrine factor (De Niu et al., 2000; Ishibashi and Imai, 2002). The expression of the mammalian STC1 is found to be regulated in numerous developmental and pathophysiological processes, including pregnancy, lactation, organogenesis, and cerebral ischemia (Chang et al., 1995, 2003; Deol et al., 2000; Ishibashi and Imai, 2002; Olsen et al., 1996; Stasko and Wagner, 2001a,b; Stasko et al., 2001; Varghese et al., 1998, 2002; Wagner and DiMattia, 2006; Zhang et al., 1998, 2000). Recently there is growing evidence to suggest that the mammalian STC1 is involved in carcinogenesis (Chang et al., 2003). Considerable numbers of studies have demonstrated that hypoxia and/or VEGF can activate STC1 gene expression in several cancerous tissues and cell-line models (Fujiwara et al., 2000; Ismail et al., 2000; Liang and Richardson, 2003; Okabe et al., 2001; Watanabe et al., 2002; Welcsh et al., 2002; Yeung et al., 2005) and angiogenic endothelial cells (Bell et al., 2001; Gerritsen et al., 2002; Kahn et al., 2000; Liu et al., 2003; Wary et al., 2003). The use of STC1 expression as a prognostic marker for human breast, hepatocellular colorectal cancers and melanoma has also been suggested (Findeisen et al., 2008; Fujiwara et al., 2000; McCudden et al., 2004; Paulitschke et al., 2009; Wascher et al., 2003).

Currently STC1 receptor has not yet been cloned, however, using both electron microscopy and receptor binding studies, McCudden et al. (2002) revealed that mitochondrion is the cellular target of the hormone. Since STC1 is identified as one of the target genes in hypoxia and was demonstrated to be a stimulator of mitochondrial respiration, these observations have prompted a question regarding the possible role of STC1 to HIF-1-mediated Warburg effect in solid tumors (Chang et al., 2003; Maxwell et al., 2001). Using murine model, a possible biological function of STC1 was reported (Westberg et al., 2007a,b). In their studies, STC1 expression was able to provoke tolerance development in mouse brains and hearts against ischemia. The data provide an insight into the possible role of STC1 in cell survival at hypoxic conditions. Recently Block et al. (2008) demonstrated that STC-1 reduced the number of apoptotic lung cancer epithelial cells, following hypoxia. In concurring with their findings, we have reported that STC1 expression can be reg-

* Corresponding author. Tel.: +852 3411 7053; fax: +852 3411 5995.
E-mail address: ckcwong@hkbu.edu.hk (C.K.C. Wong).

0303-7207/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mce.2009.07.007

ulated by p53 and NFκB in apoptotic nasopharyngeal and colon cancer cells (Lai et al., 2007; Law et al., 2008b). In considering the important roles of the transcriptional factors (i.e. p53 and NFκB) and mitochondria in tumor hypoxia (Gogvadze et al., 2008; Royds et al., 1998; Hammond and Giaccia, 2006), the induction of STC1 in hypoxic tumor microenvironments has suggested its possible role in tumor progression.

With the benefit of hindsight, we are interested in the understanding of the transcriptional regulation of STC1 in hypoxic cancer cells. By using small interference RNA approach, we previously demonstrated the involvement of HIF-1 in the regulation of STC1 expression, using nasopharyngeal cancer cell-lines (Yeung et al., 2005). In another previous report by Manalo et al. (2005), it was shown that the overexpression of HIF-1α upregulated STC1 expression in arterial endothelial cells. However, these data have inferred but did not prove the direct transactivation role of HIF-1α in the regulation of STC1 expression. Additionally no information of HIF-1 binding motif on STC1 gene promoter region has been reported. In the present study, we aimed to test whether HIF-1 is involved in the transactivation of human STC1 gene promoter. With the use of a luciferase reporter system and promoter mutagenesis, we demonstrated that the upstream region between −2322/−2335, contains an authentic HIF-1 binding motif that mediates increased STC1 promoter activity. ChIP assays were conducted to illustrate the binding of HIF-1α to STC1 gene promoter. In addition our data demonstrated that p300 was required for a productive interaction with HIF-1 and the upregulation of STC1 mRNA and protein expression.

2. Materials and methods

2.1. Effects of HIF-1α overexpression to STC1 gene promoter-driven luciferase activity and STC1 expression in normoxic CNE2 cells

The human nasopharyngeal carcinoma cell-line, CNE-2 were grown in RPMI 1640 supplemented with 10% FBS (HyClone®, Perbio) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) (Invitrogen). The day before transfection, CNE2 cells were plated into 6-well tissue culture dishes at a density reaching 70–80% confluence by the time of transfection. Transfection was performed using LipofectAMINE™ 2000 reagent (Invitrogen). For STC1 promoter luciferase assay, the pGL3-basic vector (Promega) or the pGL3-basic vector containing ∼3 kb of human STC1 promoter was cotransfected with the wild-type form of HIF1α (pCMV-HIF-1α, OriGene) and an internal control, pRL-SV40 plasmid (Promega). For the study of STC1 deletion constructs, the cells were cotransfected with pCMV-HIF-1α and pGL3-promoter vector (Promega) or pGL3-promoter vector containing 1.8–2.7, 1.8–2.3 or 2.3–2.7 kb fragment of human STC1 promoter (−2724/−1821, −2325/−1821, −2724/−2305). Six hours after transfection, the medium was replaced by a complete medium and the cells were then incubated overnight in 5% CO_2/95% air at 37 C. The cells were then lysed in a passive lysis buffer (Promega) for Western blotting and luciferase assay. Firefly and renilla luciferase activities were sequentially measured in a same sample using the Dual-Luciferase reporter assay system (Promega) and an Infinite™ F200 luminometer (TECAN).

For the determination of STC1 expression, pCMV-HIF-1α or pCMV-empty vector was transfected into the cells. The cells were then lysed in TRIZOL solution (Invitrogen) for RNA extraction or in a lysis buffer for Western blotting.

2.2. Site-directed mutagenesis

The HRE motif in the pGL3-STC1$_{(2.3–2.7\,kb)}$-SV40 vector and the pGL3-STC1$_{(3\,kb)}$ vector were mutated by using the QuickChange site-directed mutagenesis kit (Stratagene). Two distinct point mutations were generated. The motif of 5-ggacaTCGTGttct-3 at −2322/−2335 was replaced by 5-ggacaTAATGttct-3. The mutations were verified by sequencing.

2.3. Effects of hypoxia to the expression of STC1 and the binding of HIF-1α to STC1 gene promoter

CNE-2 were seeded at a density of 5×10^4 or 1×10^5 per well in 12-well or 6-well plates (Falcon). The cells were incubated in 5% CO_2 at 37 °C. After overnight incubation, the culture was maintained either in normoxic or hypoxic condition for 24 h as described previously (Yeung et al., 2005). To achieve condition of O_2 depletion, the culture was incubated in an air-tight modular chamber (Billups-Rothenberg Inc., Del Mar, CA) infused with a preanalyzed gas mixture (5% CO_2/95% N_2). The pO_2 was measured by a gas analyzer mounted with an O_2 sensor (Quest Technologies, Oconomowoc, WI). The O_2 content in the chamber was maintained in a range of 1–3%. In some experiment, the cells incubated in the hypoxic chamber were cotreated with an inhibitor of p300, chetomin (50 nM, Calbiochem) for 24 h. In removing the culture plates from the chamber, the cells were immediately lysed by TRIZOL solution (Invitrogen) or cell lysis buffer to prevent cell reoxygenation. Total RNA and cell lysates were collected for real-time PCR assay and Western blotting. For ChIP assay, the cells were fixed in 1% paraformaldehyde (Sigma). For re-ChIP assay, the cells were incubated in 2 mM of disuccinimidyl glutarate (Sigma) for 45 min before a postfixation with paraformaldehyde. In some experiment, NFκB silencing was conducted in hypoxic cells using human NFκB specific siRNA duplex (siRNA$_{NFκB\text{-}p65}$) or siCONTROL® Non-Targeting siRNA duplex (siRNA$_{CTRL}$) (Dharmacon) using siLectFect™ (BioRad).

2.4. Effects of PHD-2 silencing to HIF-1α protein stabilization and STC1 expression in normoxic CNE2 cells

For PHD-2 silencing, the cells were transfected with human PHD-2 specific siRNA duplex (siRNA$_{PHD\text{-}2}$) or siRNA$_{CTRL}$ (Dharmacon) using siLectFect™ (BioRad). At 24 h of post-transfection, total RNA and cell lysates were collected for real-time PCR assay and Western blotting, respectively, for the determination of the expression levels of STC1, HIF1α and PHD-2. Messenger RNA level of vascular epithelial growth factor (VEGF), the typical HIF-1 responsive gene, was measured.

Effects of HIF-1α overexpression and FIH-1 silencing to STC1 expression, promoter activity and p300 binding to STC1 gene in normoxic CNE2 cells. In this experiment, the cells were transfected with either siRNA$_{CTRL}$ or human FIH-1 specific siRNA duplex (siRNA$_{FIH\text{-}1}$) (Dharmacon), using siLectFect™ (BioRad). After overnight incubation, the cells were transfected with pCMV-HIF-1α, pCMV-empty vector and/or pGL3-STC1$_{(2.3–2.7\,kb)}$-SV40 plasmid, using LipofectAMINE™ 2000 reagent. At 24 h of post-transfection, total RNA or cell lysates were collected to determine the expression levels of STC1, HIF1α, FIH-1 or luciferase activity. Some of the cells were fixed for HIF-1α and p300 re-ChIP assays. In another experiment, double silencing using siRNA$_{FIH\text{-}1}$ and siRNA$_{PHD\text{-}2}$ were conducted in normoxic CNE2 cells.

2.5. Chromatin immunoprecipitation (ChIP) and re-ChIP assays

ChIP assay was conducted using the ChIP assay kit according to the manufacturer's instruction (upstate) and rabbit antibody against human HIF-1α protein (Abcam®) as described in our previous studies (Law et al., 2008a,b). The re-ChIP experiment was performed with modifications of the procedure described by Metivier et al. (2003). Briefly HIF-1 ChIP complexes were eluted by incubation in 25 μl of 10 mM dithiothreitol (Calbiochem) for 30 min at 37 C. After centrifugation, the supernatant was diluted with a re-ChIP buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA and 1% Triton X-100, pH 8.0). The diluted complexes were then subjected to immunoprecipitation by mouse antibody against human p300 (BD Pharmingen). The immunoprecipitated chromatin was analyzed in triplicate by PCR using the primers (GATAGTAAGTGAGCAAAATAAGAA-forward and CTCCGTTGCTCT-CACCAG AA-reverse), located at the upstream region, between bases −2413/−2305, of human STC1 gene promoter.

2.6. RNA extraction, PCR product verification and real-time PCR

Cells were dissolved in TRIZOL Reagent (GIBCO/BRL). Total RNA was extracted according to the manufacturer's instructions. The RNA A_{260}/A_{280} ratios were between 1.6 and 1.8. Complementary DNA (cDNA) was synthesized from 1 μg of total cellular RNA using the iScript™ cDNA synthesis kit (Bio-Rad). Real-time PCR was conducted with the condition of 95 °C for 3 min and 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min. The primers were designed on the basis of the published sequence of human STC1 [CACACCCACGAGCTGACTTC-forward and TCTCCCTGGTTATGCACTCTCA-reverse], PHD-2 [GGGAAGATGGAGAAC-CTGC TG-forward and CACACGAGCTTGTGCTTCTTC-reverse], FIH-1 [GCAGTTATAGC TTCCCGACTAGG-forward and [ATTTCAGGGCAGGATACACAAGAT-reverse], VEGF [CGAAACCATGAACTTTCTGC-forward and CCTCAGTGGGCACACACTCC-reverse], and actin [GACTACCTCATGAAGATCCTCACC-forward and TCTCCTTAATGT CACGCACGATT-reverse]. Quantitated standards (10^4 to 10^8) and sample cDNAs were analyzed with the iCycler iQ real-time PCR detection system using iQ™ SYBR® Green Supermix (Bio-Rad). The copy number for each sample was calculated and all the data were normalized to actin. The occurrence of primer-dimers and secondary products were inspected using melting curve analysis. Control amplifications were done either without RT or without RNA. Following PCR amplification, the reaction products were resolved at 100 V on a 1% agarose gel with 0.5 μg/ml ethidium bromide. All glass- and plastic-ware were treated with diethyl pyrocarbonate and autoclaved.

2.7. Western blot analysis

The treated cells were washed with 2–3 changes of cold PBS. Adherent cells were scraped from the plastic surface and transferred to a microcentrifuge tube. The cells were pelleted and resuspended in a cold lysis buffer containing 250 mM Tris/HCl, pH 8.0, 1% NP-40 and 150 mM NaCl. After 10 min incubation on ice, the lysed cells were pelleted and the supernatants were assayed for protein concentration (DC Protein Assay Kit II, Bio-Rad Pacific Ltd.). Samples were subjected to electrophoresis in NuPage 4–12% Bis-Tris gradient gels (Invitrogen). Gels were blotted onto PVDF mem-