Biochemical Pharmacology 93 (2015) 110–124

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Oncogenic c-Myc and prothymosin-alpha protect hepatocellular carcinoma cells against sorafenib-induced apoptosis

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ARTICLE INFO

Article history:
Received 7 September 2014
Accepted 24 October 2014
Available online 3 November 2014

Keywords:
Apoptosis
Hepatocellular carcinoma
c-Myc
Prothymosin
Sorafenib.

ABSTRACT

Prothymosin alpha (PTMA) is overexpressed in various human tumors, including hepatocellular carcinoma (HCC). The significance of PTMA overexpression and its underlying mechanism remain unclear. We show here that silencing PTMA sensitizes HCC cells to the kinase inhibitor sorafenib. In contrast, ectopic expression of PTMA induces cell resistance to the drug. While inhibitors targeting JNK, ERK or PI3K reduce PTMA expression, only ERK activation is suppressed by sorafenib. In addition, inhibition of ERK produces a dramatic decrease in both endogenous PTMA level and promoter activation. Ectopic expression of active MKK1/2 considerably induces PTMA expression. We also identify a sorafenib-responsive segment lying 1000–1500-bp upstream of the PTMA transcription start site and observe that it is controlled by c-Myc and ERK. Mutation in the PTMA promoter at the predicted c-Myc binding site and silencing of c-Myc both abrogate sorafenib's effect on PTMA transcription. We also find that silencing PTMA potentiates Bax translocation to mitochondria in response to sorafenib and this is associated with increased cytochrome c release from mitochondria and enhanced caspase-9 activation. These results indicate that PTMA is positively regulated by the oncoprotein c-Myc and protects HCC cells against sorafenib-induced cell death, thus identifying PTMA as a new target for chemotherapy against HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a highly prevalent, treatment-resistant malignancy with a multifaceted molecular pathogenesis. HCC is the third highest cause of cancer-related deaths worldwide [1]. In Taiwan, HCC is one of the most frequent and devastating malignancies, representing 83% of all cancer cases. HCC patients often respond poorly to current drug therapies. Early diagnosis is difficult, leading to poor prognosis and high mortality rates.

Sorafenib was initially identified as a potent inhibitor of Raf1 kinase *in vitro* [2,3] and was later described as a multi-kinase inhibitor targeting the RAF/MEK/ERK pathway as well as vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR)-β, KIT, FLT-3, RET and Wnt/β-catenin [4–6]. Cellular signaling of the Raf-1 and vascular endothelial growth factor (VEGF) pathways has been implicated in the molecular pathogenesis of HCC [7–10], thereby providing a rationale for using sorafenib in clinical settings. However, it remains unclear whether sorafenib inhibits kinase-regulated genes in HCC cells.

Alterations in several important cellular signaling pathways are associated with hepatocarcinogenesis. Genes upregulated by these signaling pathways are potential candidates to prevent the anti-apoptotic effects and chemoresistance observed during HCC therapy. We previously demonstrated that HURP (hepatoma upregulated protein), a protein overexpressed in human hepatocellular carcinoma [11], represents an important target of sorafenib in HCC cells cultured *in vitro* [12].

Abbreviations: ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; ERK, extracellular regulated protein kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; HURP, hepatoma upregulated protein; JNK, c-Jun N-terminal kinases; Luc, luciferase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PTMA, prothymosin alpha; PVDF, polyvinylidene fluoride; qRT-PCR, quantitative real-time reverse transcription-PCR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; VDAC, voltage-dependent anion channels.

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http://dx.doi.org/10.1016/j.bcp.2014.10.012
0006-2952/

Prothymosin alpha (PTMA) plays essential roles in cell proliferation [13,14], transcriptional regulation [15,16], chromatin remodeling [17,18], oxidative stress response [19] and apoptosis [20,21]. Furthermore, this protein is highly expressed in various tumor tissues, including breast cancer and hepatocarcinoma [22–28]. Accordingly, PTMA is used as a cancer prognostic marker [22,24,27,29–32].

A cell-death-regulatory pathway consisting of PTMA and the tumor suppressor PHAP (putative HLA-DR-associated protein), with each protein playing a distinctive role in regulating apoptosome formation and activity, has been identified previously [20]. Inhibition screening studies performed using small molecules revealed that PTMA-mediated inhibition of apoptosome formation occurs through blockage of its interaction with Apaf-1 [33].

In a previous report, PTMA expression and localization were shown to vary during rat hepatocyte proliferation and apoptosis [34]. In addition, PTMA was found to be highly expressed in human HCC [23]. However, the mechanism underlying regulation of PTMA expression and the possibility that it might produce anti-apoptotic effects in HCC cells exposed to sorafenib have not been studied. High level of PTMA and c-Myc co-expression was reported in various human tumors, including HCC [35–37]. c-Myc was initially found to upregulate PTMA transcription [38], and c-Myc-binding sites were found in the proximal promoter and intron 1 of the PTMA gene [39–41]. However, regulation of PTMA by c-Myc and the relevance of this process in human HCC remain to be examined. In the present study, we show that PTMA is upregulated in HCC cells in a manner inversely dependent of sorafenib sensitivity. Silencing of PTMA enhances sorafenib-induced caspase activation, Bax translocation to the mitochondria and cytochrome c release into the cytosol. Notably, we identify a sorafenib-responsive element in the PTMA promoter and demonstrate that sorafenib inhibits PTMA expression at the transcriptional level through inactivation of p-ERK and c-Myc.

2. Materials and methods

2.1. Cell cultures and reagents

Primary liver Chang liver cells and hepatocellular carcinoma cells (Huh7, J7, SK-Hep1, and Mahlavu) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 U/ml; Gibco), and streptomycin (100 mg/ml; Gibco). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 (v/v) in air. The reagents used included antibodies against caspase-3, caspase-9, Akt, p-Akt, p-JNK, p-P38 (Cell Signaling, Danvers, MA, USA), PTMA, JNK, ERK, p-ERK, P38, IκB-α, P65, Elk-1, survivin, Mcl-1, Bcl-X_L, Bcl-2, Bax, Bad, cytochrome c, GAPDH, α-tubulin, lamin A/C, VDAC, HA, c-Myc, Max (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Flag (Sigma-Aldrich, St. Louis, MO, USA). Kinase inhibitors against NF-κB (Bay11-7082), JNK (SP600125), P38 (SB203580) (Santa Cruz Biotechnology), MEK/ERK (U0126) (Calbiochem, CA, USA), and PI3K (Wortmannin; Cell Signaling) were also used. Sorafenib (Bayer HealthCare AG, Berlin, Germany) was kindly provided by Dr. T.-C. Chang (Chang Gung Memorial Hospital, Taoyuan, Taiwan). The other chemicals were purchased from Sigma-Aldrich.

2.2. Plasmids and transfection

The pLKO-AS3w-puro expression vector was purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The pLKO-AS3w-eGFP-puro plasmid was used as a negative control. The DNA sequence containing PTMA's open-reading frame (ORF) was cloned from Mahlavu cells by using specific primers (forward primer with *Xho*I site, 5′-GCCTCGAGATGTCAGACG-CAGCCGTAGACACC-3′; reverse primer with *Xba*I site, 5′-TTTCTA-GACTAGTCATCCTCGTCGGTCTTCTG-3′), and ligated with the pGEM-T Easy vector (Promega, Madison, WI, USA) using the T4 DNA ligase. PTMA ORF was released from the pGEM-T Easy vector using the restriction enzymes *Xho*I and *Xba*I, and was inserted into the pCMV-Flag expression vector, resulting in pCMV-FlagPTMA. The FlagPTMA sequence was isolated from pCMV-FlagPTMA using specific primers (forward primer with *Nhe*I site, 5′-GCTAGCATAC-GACTCACTATAGGG-3′; reverse primer with *Pme*I site, 5′-GTTTAAACATTTAGGTGACACTATAG-3′), and ligated with the pGEM-T Easy vector (Promega). FlagPTMA sequence was released from the pGEM-T Easy vector using the restriction enzymes *Nhe*I and *Pme*I, and was inserted in the pLKO-AS3w-puro expression vector, resulting in pLKO-FlagPTMA. MKK1/2CA plasmids (constitutively active MKK1/2 vectors) were kindly provided by Dr. Yun-Wei Lin (National Chiayi University, Chiayi, Taiwan; originally obtained from Dr. Jia-Ling Yang, National Tsing Hua University, Taiwan). pcDNA3-Myc expression plasmid was offered by Professor K.-H. Lin, Chang Gung University. A 2500-bp functional promoter segment of the PTMA gene was isolated from Mahlavu genomic DNA by using PCR and a synthetic pair of oligonucleotides: forward primer with a 5′ *Kpn*I restriction enzyme site: 5′-GGGGTACCAGAACTGTCAAGTCTGGTTTTCA-3′; reverse primer with a 3′ *Bgl*II restriction enzyme site: 5′-CCAGATCTTAATATA-GATTAGTGGGCGGCG-3′. 5′-Truncated promoter segments of PTMA were constructed by PCR using the reverse primer described above but with different forward primers containing a 3′ *Kpn*I site: 2 K: 5′-GGGGTACCTAGTCCTCGGGAACAGCA-3′, 1.6 K: 5′-GGGGTACCAGACTGCGTGCTAAGCTC-3′, 1.5 K: 5′-GGGGTACC GTTTTCCCCATTCCCAGCT-3′, 1 K: 5′-GGGGTACCTGGCTCCAGA-GACGATGATT-3′, and 0.5 K: 5′-GGGGTACCAATCCTTGGTCCA AGGCG-3′. Mutant PTMA promoter was constructed by PCR-induced mutation using 500-bp segments (ranging from 1000- to 1500-bp upstream of the transcription start site) with synthetic oligonucleotides (forward primer: 5′-CCCACCTGAGCTAGATCG-3′ and reverse primer: 5′-CGATCTAGCTCAGGTGGG-3′) that target c-Myc binding sequence and with one nucleotide altered from G to C (underlined above). Promoter segments of PTMA were initially ligated with the pGEM-T Easy vector (Promega), followed by release from the pGEM-T Easy vector using the restriction enzymes *Kpn*I and *Sac*I, and finally inserted in the pGL3-basic reporter vector (Promega; kindly provided by our colleague Prof. K.-H. Lin at Chang Gung University), resulting in pGL3-PTMA (2.5 kb), pGL3-PTMA 2 K, pGL3-PTMA 1.6 K, pGL3-PTMA 1.5 K, pGL3-PTMA 1 K, pGL3-PTMA 0.5 K, and pGL3-PTMA-MycBS(mt). Plasmid construction and preparation was performed according to standard protocols [42]. Cells were transfected with plasmids using lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the instructions provided by the supplier. Transfected cells were incubated for 48 h for overexpression of the plasmids.

2.3. Quantitative real-time reverse transcription-PCR (qRT-PCR)

qRT-PCR, or in short qPCR, was performed on total RNA extracted with Trizol (Invitrogen) and 200 nM of primers as previously described [43]. The primers used were the following: PTMA, forward, 5′-CGAAATCACCACCAAGGACT-3′; reverse, 5′-GT CGGTCTTCTGCTTCTTGG-3′; and GAPDH, forward, 5′-TCCTGCAC-CACCAACTGCTT-3′; reverse, 5′-GAGGGGCCATCCACGTCTT-3′. All unknown samples and controls were done in triplicate. Relative quantification was calculated by the ΔΔCt method and normalized against GAPDH as described before [44]. Namely, the ΔCt for each candidate was calculated as ΔCt (candidate) = [Ct (candidate) − Ct (GAPDH)]. The relative abundance of the candidate gene X was shown as $2^{\Delta Ct(X) - \Delta Ct(GAPDH)}$.