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Research Article

Adaptation of HepG2 cells to a steady-state reduction in the content of protein phosphatase 6 (PP6) catalytic subunit

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ABSTRACT

Protein phosphatase 6 (PP6) is a ubiquitous Ser/Thr phosphatase involved in an array of cellular processes. To assess the potential of PP6 as a therapeutic target in liver disorders, we attenuated expression of the PP6 catalytic subunit in HepG2 cells using lentiviral-transduced shRNA. Two PP6 knock-down (PP6KD) cell lines (90% reduction of PP6-C protein content) were studied in depth. Both proliferated at a rate similar to control cells. However, flow cytometry indicated G2/M cell cycle arrest that was accounted for by a shift of the cells from a diploid to tetraploid state. PP6KD cells did not show an increase in apoptosis, nor did they exhibit reduced viability in the presence of bleomycin or taxol. Gene expression analysis by microarray showed attenuated anti-inflammatory signaling. Genes associated with DNA replication were downregulated. Mass spectrometry-based phosphoproteomic analysis yielded 80 phosphopeptides representing 56 proteins that were significantly affected by a stable reduction in PP6-C. Proteins involved in DNA replication, DNA damage repair and pre-mRNA splicing were overrepresented among these. PP6KD cells showed intact mTOR signaling. Our studies demonstrated involvement of PP6 in a diverse set of biological pathways and an adaptive response that may limit the effectiveness of targeting PP6 in liver disorders.

Introduction

The reversible phosphorylation of cellular proteins is controlled by the activities of both protein kinases and protein phosphatases. The protein serine/threonine phosphatases are subdivided into four broad classes: type1 (PP1), type 2A (PP2A), type 2B (PP2B) and type 2C (PP2C). All four classes play an important role in the regulation of a broad range of cellular proteins that include metabolic enzymes, ion channels, hormone receptors, cytostructural proteins and components of kinase cascades [1].

Protein phosphatase 6 (PP6) is classified as a PP2A-type phosphatase based on its sequence homology to PP2A and its

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sensitivity to protein phosphatase inhibitors such as okadaic acid, microcystin-LR, and calyculin A [2]. The PP6 holoenzyme is proposed to be a heterotrimer consisting of a catalytic subunit (PP6-C), a SAPS (Sit4 Associated Protein Subunit; SAPS1, SAPS2, or SAPS3), and an ankyrin repeat domain containing subunit (ANKRD28, ANKRD44, or ANKRD52) [3]. The SAPS have been shown to bind directly to PP6-C [4]. ANKRD28, the only ANKRD subunit to be studied to any degree, has been shown to bind to all of the SAPS [3]. In general, phosphatase regulatory subunits control enzyme activity, confer substrate specificity, and target the enzyme to specific cellular locations. While this is presumed to be the case for PP6, the precise role of its regulatory subunits is yet to be fully characterized.

PP6-C is the human homologue of the yeast phosphatase, Sit4, with which it shares 61% amino acid identity [4]. Studies in *Saccharomyces cerevisiae* have implicated Sit4 and its regulatory subunits SAP155, SAP185, SAP190, and SAP4 in G1 to S progression [5]. PP6 has been shown to function similarly in human cancer cells [6,7]. Other studies in yeast have shown that PP6 contributes to the response to mitochondrial DNA damage [8]. In addition, PP6 in yeast plays a role in signaling by the target of rapamycin (TOR), a key nutrient-sensing kinase [9]. Activation of TOR is associated with the inhibition of Sit4 by its association with regulatory subunits, including TAP42, the mammalian homologue of which is α4, and the SAP proteins [10]. This may account for a mechanism by which TOR can enhance protein phosphorylation through inhibition of a phosphatase. PP6 has been shown to functionally substitute for Sit4 mutations in *S. cerevisiae* and the Sit4 homolog ppe1 in fission yeast [11]. Deletion of the SAP or Sit4 genes in *S. cerevisiae* results in increased sensitivity to rapamycin and defects in the expression of certain TOR-regulated genes [10]. It has further been reported that human SAPS, when expressed in *S. cerevisiae*, are able to physically interact with Sit4, and that the expression of either SAPS2 or SAPS3, but not SAPS1, can rescue the growth defect and rapamycin sensitivity of yeast cells lacking all four yeast SAPs. This effect was dependent on the presence of Sit4 [12].

Evidence for analogous involvement of PP6 in TOR signaling in mammalian cells has been lacking. However, Wengrod et al. [13] recently showed that PP6 is required for the pharmacological induction of autophagy by pharmacologic inhibition of mTORC1, an action that also required GCN2 (general control nonrepressed 2) and eukaryotic initiation factor 2α (eIF2α). As noted above, PP6 has been implicated in the regulation of cell growth and proliferation, which may reflect its involvement in signaling by the mammalian homolog of the Target of Rapamycin, mTOR. As reviewed by Douglas et al. [14], PP6 has an established role in the cell response to DNA damage [15]. This aspect of PP6 function likely reflects its ability to modulate signaling by DNA-dependent protein kinase [16] as well as its interactions with Aurora A kinase [17,18]. More recent studies suggest a broader role for PP6 in endoplasmic reticulum-to-Golgi trafficking [19], pre-mRNA splicing [20], formation of adherens junctions through interaction with E-cadherin [21], control of apoptosis in immune cells [22], response to mitochondrial DNA damage [8], and modulation of signaling through the Hippo pathway [23]. With regard to its role in liver cells, PP6 has been identified as a tumor suppressor based on its ability to induce G1/S cell cycle arrest in two human hepatocellular carcinoma (HCC) cell lines [7]. Additional data on a hepatic role for PP6 are lacking.

In recent years, while protein kinases have been widely targeted in the development of drug therapies for a broad spectrum of disease entities, the therapeutic targeting of protein phosphatases has only begun to emerge [24]. Given the diversity of functions served by PP6, the present studies were undertaken to explore the role of PP6 in determination of cell phenotype, regulation of gene expression, and involvement in signal transduction pathways in liver cells. Our goal was to explore the potential for PP6 to serve as a therapeutic target in liver disorders. We chose to study HepG2 cells in which PP6-C expression was stably attenuated in an effort to mimic the effects of tonic administration of a specific phosphatase inhibitor.

Material and methods

Cell culture

HepG2 cells were obtained from the American Type Culture Collection. Low passage cells were maintained in minimal essential medium with Earle's salts, L-glutamine and non-essential amino acids (Invitrogen, Carlsbad, CA) supplemented with 1 mM sodium pyruvate, 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin.

To generate cells lines in which PP6-C was reduced in expression (PP6-C knock-down; PP6KD), HepG2 cells were seeded into 96-well plates and transduced with lentiviral particles (multiplicity of infection of 5) corresponding to different short hairpin RNA (shRNA) constructs targeted to the PP6-C gene (Mission shRNA; Sigma-Aldrich, St. Louis, MO). Procedures followed the manufacturer's protocol. Cell lines were selected in 2 μg/ml puromycin and surviving colonies of cells were expanded as mass cultures in puromycin. Five individual shRNA lentiviral particle constructs were tested. The Mission lentiviral designations were as follows: Cell line 16.5, TRCN0000279890; 17.5, TRCN0000279949; 18.5 TRCN0000297274; 19.5, TRCN0000379835; 20.5, TRCN0000379918). Except where noted, all studies were carried out on cell lines 18.5 and 19.5. Mission TRC2 non-target shRNA control transduction particles were used for control transduction.

Western immunoblotting of phosphatase subunits and other signaling proteins

Cell lines were plated at 1×10^6 cells per 10 cm tissue culture plate and allowed to proliferate for 48 h. Samples were prepared using 0.5 ml per plate of a lysis buffer containing 10 mM Tris-base, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 1% Triton X-100, freshly added protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 34.4 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, and the phosphatase inhibitor microcystin-LR (500 nM). Samples were allowed to incubate on ice for 30 min followed by centrifugation at 16,000*g* for 10 min at 4 °C. Protein concentration of the lysates was measured using the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL).

Western immunoblotting, image acquisition and quantification of results were carried out as described previously [25]. Primary antibodies were obtained from the following sources: PP6-C, Millipore, Billerica, MA; SAPS1, 4E-BP1 and p-PKCα$^{(Ser657)}$, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; SAPS2 and SAPS3, Bethyl