Cellular Signalling 44 (2018) 158–170

Contents lists available at ScienceDirect

Cellular Signalling

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

HBP1 phosphorylation by AKT regulates its transcriptional activity and glioblastoma cell proliferation

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ARTICLE INFO

Keywords:
HBP1
AKT
Phosphorylation
Glioblastoma multiforme
Proliferation
Transcription

ABSTRACT

The HMG-box protein 1 (HBP1) is a transcriptional regulator and a potential tumor suppressor that controls cell proliferation, differentiation and oncogene-mediated senescence. In a previous study, we showed that AKT activation through the PI3K/AKT/FOXO pathway represses HBP1 expression at the transcriptional level in human fibroblasts as well as in cancer cell lines. In the present study, we investigated whether AKT could also regulate HBP1 directly. First, AKT1 phosphorylated recombinant human HBP1 *in vitro* on three conserved sites, Ser380, Thr484 and Ser509. In living cells, we confirmed the phosphorylation of HBP1 on residues 380 and 509 using phospho-specific antibodies. HBP1 phosphorylation was induced by growth factors, such as EGF or IGF-1, which activated AKT. Conversely, it was blocked by treatment of cells with an AKT inhibitor (MK-2206) or by AKT knockdown. Next, we observed that HBP1 transcriptional activity was strongly modified by mutating its phosphorylation sites. The regulation of target genes such as DNMT1, P47phox, $p16^{INK4A}$ and cyclin D1 was also affected. HBP1 had previously been shown to limit glioma cell growth. Accordingly, HBP1 silencing by small-hairpin RNA increased human glioblastoma cell proliferation. Conversely, HBP1 overexpression decreased cell growth and foci formation. This effect was amplified by mutations that prevented phosphorylation by AKT, and blunted by mutations that mimicked phosphorylation. In conclusion, our results suggest that HBP1 phosphorylation by AKT blocks its functions as transcriptional regulator and tumor suppressor.

1. Introduction

The HMG-box protein 1 (HBP1) is a ubiquitous transcriptional regulator that belongs to the high mobility group (HMG) family of DNA-binding proteins [1]. HBP1 represses the transcription of target genes, such as cyclin D1 (*CCND1*), the NADPH oxidase subunit P47phox (*NCF1*), DNA-methyltransferase 1 (*DNMT1*) and N-Myc (*MYCN*) by binding to promoter cis-regulatory elements [2–5]. HBP1 also decreases gene expression indirectly by inhibiting transcription factors, such as c-MYC and TCF4 [4,6,7]. Most HBP1 target genes are related to the cell cycle, which is potently inhibited by HBP1 activation, as demonstrated in numerous cell lines [3,8–10]. In mice, HBP1 deficiency shortens the neuronal cell cycle during cortical development [11]. HBP1 is also required for cell cycle arrest upon Ras-induced premature senescence [12]. In this process, HBP1 up-regulates $p16^{INK4A}$ (*CDKN2A*) expression, suggesting that it can also act as a transcriptional activator [13].

In agreement with its role as a cell cycle inhibitor, HBP1 was suggested to act as a tumor suppressor. The *HBP1* gene maps to chromosome 7q31, a region that is frequently deleted in myeloid cancers and contains several tumor suppressor candidates [14,15]. Alterations of the *HBP1* gene were suggested in invasive breast cancers, but this has not been confirmed yet in large scale breast cancer sequencing studies [16]. HBP1 expression is also decreased in a subset of breast cancers and several other tumor types, compared to matched normal tissues. In glioma, for instance, HBP1 is down-regulated by two micro-RNAs, miR-96 and miR-155, resulting in increased cell proliferation [17,18]. We showed that HBP1 transcription is also inhibited by growth factor signaling *via* the PI3K-AKT oncogenic pathway in multiple cell types [8].

Abbreviations: DNMT1, DNA-methyltransferase 1; FOXO, Forkhead box O transcription factors; GST, glutathione *S*-transferase; HBP1, HMG-box protein 1; HEK, human embryonic kidney; HMG, high mobility group; HPLC, high performance liquid chromatography; KD, kinase dead; MS, mass spectrometry; MTT, methyl thiazolyl tetrazolium; PBS, phosphate-buffered saline; PFK2, phosphofructosekinase-2; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKB, protein kinase B; PRAS40, proline-rich AKT substrate of 40 kDa; RB, retinoblastoma protein; rps6, ribosomal protein S6; TCF4, transcription factor 4; UPLC, ultra-performance liquid chromatography

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https://doi.org/10.1016/j.cellsig.2018.01.014
Received 10 October 2017; Received in revised form 22 December 2017; Accepted 10 January 2018

This effect depends on the inactivation of FOXO transcription factors by AKT phosphorylation, which prevents *HBP1* promoter activation [8,19].

Interestingly, AKT was also suggested to phosphorylate HBP1, in a study by Cantley and colleagues, who identified a number of potential AKT substrates using a library screening [20]. In the present study, we investigated the phosphorylation of HBP1 by AKT. We found that AKT phosphorylated HBP1 directly, which reduced its transcriptional activity and promoted glioblastoma cell proliferation and transformation. Our data provide a new mechanism by which AKT regulates HBP1 and the cell cycle.

2. Materials and methods

2.1. Cell culture

The human embryonic kidney (HEK) 293T cells (obtained from ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Basel, Switzerland) with 10% fetal bovine serum (FBS). The glioblastoma U87 and A172 cells, a kind gift from Pr. B. Van den Eynde (Ludwig Cancer research, Brussels, BE), were cultured in Iscove-modified Dulbecco's medium (IMDM, Gibco, Life technologies, Grand Island, NY, USA) supplemented with 10% FBS.

2.2. Reagents and materials

MK-2206 was purchased from Selleckchem (Houston, TX, USA) and [γ-^{32}P] ATP was from Perkin Elmer (Waltham, MA, USA). Anti-RxRxxp (S/T) (reference #9611), anti-RRxp(S/T) (#9624), anti-total AKT (#9272), anti-pSer473 AKT (#9271), anti-total ribosomal protein S6 (#2317) and anti-pSer240/Ser244 rpS6 (#5364) antibodies were purchased from Cell signaling Technology (Danvers, MA, USA). Anti-pThr246 PRAS40 (#07-888) and anti-total PRAS40 (#05-988) were from Millipore (Billerica, MA, USA). Anti-HBP1 antibodies (A-5, sc-376831 and 11746-1-AP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Proteintech (Rosemont, IL, USA) respectively. Anti-β-actin (A-5441) was from Sigma (Saint-Louis, MO, USA). SignalSilence Akt siRNA I was purchased from Cell signaling Technology (#6211) and a non-targeting siRNA (#4390846) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant active ΔPH AKT1 S473D protein was from MRC PPU Reagents and Services (University of Dundee, Scotland, UK).

The cDNA of human HBP1 (ENST00000222574.8, NP_036389) was cloned in pEF-MYC-CYTO vector (Invitrogen, Life technologies) between the *Nco*I and *Xho*I restriction sites and in pBabe-puro between the *Bam*HI and *Sal*I restriction sites. HBP1 fragments (residues 1 to 200, named ΔCT and residues 201 to 514, named ΔNT) were generated by PCR amplification and introduced by restriction (*Nco*I, *Xho*I) in the pEF-MYC-CYTO vector. The DNMT1 promoter (−222 to +37 pb) and the P47phox promoter (−1472 to −1126 pb) were amplified by PCR using human genomic DNA (from K562 cells) as a template and subcloned into pGL3-Basic luciferase vector using *Xho*I and *Hin*dIII restriction sites. pCMV5-HA-AKT1-CA (T308D, S473D and G478S) and pCMV5-GST-AKT1-KD (K179A,T308A and S473A) vectors were a kind gift from Pr. D. Alessi (University of Dundee, UK). pCMV-HA-hRB was purchased from Addgene (plasmid #58905) [21]. Two shRNAs constructs for HBP1 (shHBP1-2, TRCN0000229982, Sigma) (shHBP1-74, TRCN0000015274, Thermo Fisher Scientific) in pLKO.1 lentiviral vector were used on the basis of their previously described efficiency [8]. A negative shRNA control (scramble) was obtained from Addgene (plasmid #1864) [22]. All the constructs were verified by sequencing.

2.3. Site-directed mutagenesis

All the mutants were generated by using the QuickChange™ XL-II kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. The different mutations were verified by DNA sequencing. Mutagenic primers were synthesized by Eurogentec (Ougrée, Belgium) and are listed in Suppl. Table 1.

2.4. Cell transfection, cell lysis, immunoprecipitation and immunoblotting

HEK 293T cells were transfected with the calcium phosphate method as follows. The day before transfection, cells were seeded in 10 cm diameter dishes. Plasmid DNA (10 μg) was diluted in 404 μl of water and was mixed with 360 μl of BBS buffer (50 mM *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethane-sulfonic acid pH 7, 280 mM NaCl, 1.5 mM Na_2HPO_4) and 36 μl $CaCl_2$ 2.5 M (final volume of 800 μl). Plasmid DNA (10 μg) and AKT siRNA (200 pmol) were transfected using TurboFect™ transfection reagent from Thermo Fisher Scientific. The solution was incubated for 20 min at room temperature. Precipitates were added to cell medium. Four hours after transfection, cells were washed once with PBS and incubated in medium with 10% FBS or serum-starved during 24 h, as described [23]. Next, the medium was removed and the cells were washed in cold PBS before lysis in buffer (25 mM Tris/HCl pH 7.4, 150 mM NaCl, 6 mM EDTA, 10% glycerol and 1% Triton X-100) containing protease inhibitors (1 mM Pefabloc and 1 μg/ml aprotinin). The cells were incubated on ice for 20 min. Extracts were cleared by centrifugation (10,000 × *g* × 10 min at 4 °C) and protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). In immunoprecipitation experiments, 1 mg of lysate proteins was incubated overnight at 4 °C with 1 μg of the anti-total HBP1 (N-terminal HBP1, #sc-376831, unless otherwise stated). In co-immunoprecipitation experiments, cells were lysed with RIPA buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate and 1% NP-40). The lysate was precleared with protein A/G beads (Thermo Fisher Scientific) and incubated overnight at 4 °C with 1 μg the anti-total HBP1 antibody (N-terminal HBP1, #sc-376831, unless otherwise stated) or anti-total AKT. For Immunoprecipitations and co-immunoprecipitations, the antibody complexes were collected by adding protein A/G beads for 90 min at 4 °C, washed extensively, and then analyzed by western blotting. Protein extracts were loaded on 10% polyacrylamide gels. Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes, which were then blocked in 5% of fat-free milk powder. The membranes were incubated overnight at 4 °C with the indicated primary antibodies, and then washed extensively before and after incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Anti-RxRxxp(S/T), anti-RRxp(S/T), anti-pSer473 AKT, anti-total AKT, anti-total HBP1 (11746-1-AP, Proteintech), anti-pSer240/Ser244 rpS6, anti-total rpS6, anti-pThr246 PRAS40 and anti-total PRAS40 antibodies were used at dilution of 1:1000. Anti-total HBP1 (A-5, Santa Cruz) and anti-β-actin were used at dilutions of 1:500 and 1:5000 respectively. Immunodetection was performed using chemiluminescence (Western blot Luminol Reagent, Santa Cruz).

2.5. Expression and purification of human recombinant HBP1

The cDNA sequence of human HBP1 was subcloned into the pGEX-6P-1 expression vector using the *Bam*HI and *Xho*I restriction sites. This vector was used to overexpress glutathione *S*-transferase (GST) fusion protein in BL21 *E. coli* strain. A starter culture (2 l) was grown at 37 °C until the OD_{600} reached ~0.6. The expression of GST-tagged HBP1 was then induced by addition of 1 mM isopropylthiogalactoside overnight at 18 °C. Bacteria were pelleted by centrifugation (5000 × *g* for 10 min at 4 °C), resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% 2-mercaptoethanol, 0.01% Brij 35, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine chloride, 1 μg/ml leupeptin and 1 μg/ml aprotinin), and homogenized using a French press. The bacteria lysate was then centrifuged (17,000 × *g* for 20 min at 4 °C) and passed through a 45 μm filter (Millex-HA, Merck-Millipore) to load a cleared lysate onto a glutathione (GSH)-Sepharose column