Cellular Signalling 26 (2014) 1725–1734

Contents lists available at ScienceDirect

Cellular Signalling

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

Reduced phosphorylation of Stat3 at Ser-727 mediated by casein kinase 2 — Protein phosphatase 2A enhances Stat3 Tyr-705 induced tumorigenic potential of glioma cells

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ARTICLE INFO

Article history:
Received 7 January 2014
Received in revised form 4 April 2014
Accepted 4 April 2014
Available online 12 April 2014

Keywords:
pStat3^{S727}
pStat3^{Y705}
CK2
PP2A
Glioma

ABSTRACT

Signal transducer and activator of transcription 3 (Stat3) is a transcription factor that is involved in cell survival and proliferation and has been found to be persistently activated in most human cancers mainly through its phosphorylation at Tyr-705. However, the role and regulation of Stat3 Ser-727 phosphorylation in cancer cells have not been clearly evaluated. In our findings, correlation studies on the expression of CK2 and Stat3 Ser-727 phosphorylation levels in human glioma patient samples as well as rat orthotopic tumor model show a degree of negative correlation. Moreover, brain tumor cell lines were treated with various pharmacological inhibitors to inactivate the CK2 pathway. Here, increased Stat3 Ser-727 phosphorylation upon CK2 inhibition was observed. Overexpression of CK2 (α, α′ or β subunits) by transient transfection resulted in decreased Stat3 Ser-727 phosphorylation. Stat3 Tyr-705 residue was conversely phosphorylated in similar situations. Interestingly, we found PP2A, a protein phosphatase, to be a mediator in the negative regulation of Stat3 Ser-727 phosphorylation by CK2. In vitro assays prove that Ser-727 phosphorylation of Stat3 affects the transcriptional activity of its downstream targets like SOCS3, bcl-xl and Cyclin D1. Stable cell lines constitutively expressing Stat3 S727A mutant showed increased survival, proliferation and invasion which are characteristics of a cancer cell. Rat tumor models generated with the Stat3 S727A mutant cell line formed more aggressive tumors when compared to the Stat3 WT expressing stable cell line. Thus, in glioma, reduced Stat3 Ser-727 phosphorylation enhances tumorigenicity which may be regulated in part by CK2–PP2A pathway.

1. Introduction

Glioblastoma multiforme (GBM) is the most aggressive and malignant form of brain tumor in human with very short patient survival [1]. Amongst the many causal reasons of GBM, alterations and perturbations in the molecular signaling pathways play a major role. Several signaling pathways have been implicated in the progression of GBM including phosphoinositide-3-kinase (PI3K), Ras-MAPK and tyrosine kinase signaling pathways viz. EGFR and VEGFR [2].

Apart from these conventional pathways, kinases like CK2 are now emerging as crucial molecules for GBM progression [3]. CK2 is a ubiquitous and highly conserved serine/threonine kinase with two alpha (catalytic) and two beta (regulatory) subunits [4]. This protein has been involved in the regulation of cellular growth and proliferation, transformation, apoptosis and senescence [5,6]. Increased expression and activity of CK2 in several cancers mark it as an important target for specific therapies [6]. Elevated levels of this protein have also been reported in human GBM tumor specimens where inhibition of CK2 could sensitize these cells to tumor necrosis factor-α induced apoptosis [7]. A CK2 inhibitor has been reported to induce antitumor activity in mouse xenograft model of human glioblastoma [8] while apigenin, another CK2 inhibitor, was found to modulate DNA damage response of GBM cells though it could not radiosensitize them [9]. CK2 can also induce glioma cell invasion by mediating α-catenin phosphorylation

Abbreviations: Stat3, signal transducer and activator of transcription 3; CK2, casein kinase 2; PP2A, protein phosphatase 2A; pStat3^{S727}, phosphoStat3 Ser-727; pStat3^{Y705}, phosphoStat3 Tyr-705; GBM, glioblastoma multiforme; EV, empty vector; qRT-PCR, quantitative real time polymerase chain reaction; WCL, whole cell lysate; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; IP, immunoprecipitation; FACS, fluorescence activated cell sorting; RT, room temperature; PBS, Phosphate Buffered Saline; PI, propidium iodide; CPCSEA, committee for the purpose of control and supervision on experiments on animals.

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http://dx.doi.org/10.1016/j.cellsig.2014.04.003

and translocation of β-catenin into the nucleus thereby increasing its transcriptional activity [10].

In addition to several substrates already existing for CK2, novel interacting partners are now emerging. It has been recently reported that CK2 activates the Jak–Stat signaling pathway by associating with Jak1 and Jak2 but not Stat3 in myelo-proliferative disorders [11]. Stats are latent transcription factors that mediate cytokine- and growth factor-directed transcription. Stat3 is persistently activated in many human cancers, including glioma, leukemia, myelomas, breast, prostate, pancreatic, lung and ovarian cancers. In tumors, active Stat3 is required for either transformation, enhancing proliferation or blocking apoptosis [12]. The role of Stat3 in GBM pathogenesis is well documented [13–15]. Though Stat3 transcriptional activity is reflected by phosphorylation at both its tyrosine and serine residues, $pStat3^{Tyr705}$ has been implicated in most cases of disease progression [16,17]. Elevated levels of serine phosphorylated Stat3 in GBM have been reported in a single study [18] but the mechanism of activation is poorly understood.

In this study, we focus on the regulation of Stat3 Ser-727 phosphorylation by CK2 in glioma. This regulation was found to be negative and led to increased Stat3 transcriptional activity. Here, PP2A was found to maintain the decreased phosphorylation levels of Stat3 Ser-727 which is controlled by CK2. Reduced Ser-727 phosphorylation of Stat3 with concomitant increase in Tyr-705 phosphorylation leads to enhanced rate of proliferation and invasive property of glioma cells thereby establishing Ser-727 residue of Stat3 to be antagonistic to tumor formation. CK2, thus, crosstalks with the Stat3 signaling pathway through PP2A leading to the progression of more aggressive forms of glioma.

2. Materials and methods

2.1. Cell culture, transfection and drug treatments

The human cell lines HEK293 (embryonic kidney), U87MG, DBTRG-05MG (glioma) and rat cell line C6 (glioma) used in the study were obtained from ATCC (Manassas, VA, USA) and NCCS (Pune, India) respectively. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2000 units/l penicillin and 2 mg/l streptomycin (Invitrogen, Life Technologies, Grand Island, NY, USA). All cells were maintained at 37 °C in a humid incubator with 5% CO_2. Transfections of different DNA constructs were performed using either Attractene (Qiagen, Gaithersburg, MD, USA) according to manufacturer's instructions or calcium phosphate protocol as described previously [19]. The following drugs/inhibitors, dissolved in DMSO, were used in this study: TBCA (10 μM), LY294002 (20 μM), U0126 (15 μM), and calyculin A (100 nM) (Calbiochem, Darmstadt, Germany), D-sorbitol (0.5 M) and okadaic acid (1 μM) (Sigma Aldrich, Seelze, Germany). Incubation times are indicated in the figure legends. Stable C6 cells were selected using either neomycin (2 mg/ml) or hygromycin (500 μg/ml) depending on the plasmid used.

2.2. Expression plasmids

pGZ21dx-GFP-CK2α, pCDNA3.1 H(+)-Stat3 WT and pCDNA3.1-Stat3-(S727A) were previously described [19,20]. pGZ21dx-GFP-PP2A-Cα plasmid was cloned from HEK293 cells using the primers PP2Acα-F: 5′-AATGAATTCATGGACGAGAAGGTGTTC-3′ and PP2Acα-R: 5′-AATG TCGACCAGGAAGTAGTCTGGGGT-3′. pRS2-(CK2alpha′,CK2beta) and pRS3-(CK2alpha, CK2beta) constructs were obtained from Addgene (Cambridge, MA, USA). All constructs were verified by sequencing.

2.3. Cell lysis, immunoprecipitation and immunoblot analysis

Whole-cell lysates (WCLs) were prepared by lysing the cells in ice-cold buffer as described earlier [21]. SDS-PAGE and Western blot analyses were performed using standard procedures. For coimmunoprecipitation experiments, 1 mg of total protein from lysates was subjected to immunoprecipitation as described previously [22]. Antibodies for $pStat3^{Y705}$, $pStat3^{S727}$, Stat3, CK2α, SOCS3 and Bcl-xl were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies for MMP9, MMP2, VEGF, Actin and Lamin B were from Santa Cruz Biotechnology (Dallas, Texas, USA). Blots were evaluated by using Enhanced Chemi-luminescence (ECL) reagent according to the manufacturer's protocol (GE Healthcare, Pittsburgh, USA).

2.4. Quantitative real time PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, NY, USA) according to the manufacturer's instructions. For each sample, 2 μg of RNA was converted to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Glen Burnie, Maryland, USA) and was subsequently used for qRT-PCR analysis using Power SYBR Green Master Mix on 7500 Fast real time PCR system (Applied Biosystems, Foster City, CA, USA). 18S rRNA (for human glioma cells) and RPL19 (for C6 glioma cells) served as internal controls (normalization) and calibrator controls were chosen appropriately.

Listed below are the primers (human and rat) for the qRT-PCR analysis:

bcl-xl: F-5′-ACCCCAGGGACAGCATATCA-3′,
R-5′-TGCGATCCGACTCACCAATA-3′

SOCS3: F-5′-GGCCACTCTTCAGCATCTC-3′,
R-5′-ATCGTACTGGTCCAGGAACTC-3′

Cyclin D1: F-5′-CCGTCCATGCGGAAGATC-3′,
R-5′-GAAGACCTCCTCCTCGCACT-3′

MCL1: F-5′-AAAGAGGCTGGGATGGGTTT-3′,
R-5′-CAAAAGCCAGCAGCACATTC-3′

18S: F-5′-GCTTAATTTGACTCAACACGG-3′,
R-5′-AGCTATCAATCTGTCAATCCTGTC-3′

BCL2: F-5′-CCGGGAGAACAGGGTATGATAA-3′,
R-5′-CCCACTCGTAGCCCCTCTG-3′

Myc: F-5′-TGTATGTGGAACGGCTTCTC-3′,
R-5′-CCTGGTAGGGGTCCAGCTTC-3′

RPL19: F-5′-ATCGCCAATGCCAACTCT-3′,
R-5′-GAGAATCCGCTTGTTTTTGAA-3′.

2.5. Luciferase reporter assay

c-jun promoter region (−1780 to +731) containing Stat3 binding site (GAS sequence − TTCCCGGAA) was amplified from genomic DNA using sense oligonucleotide: 5′-GAGAATTCCAAGTTCAGAAGCAG-3′ and antisense oligonucleotide: 5′-GAGCTACCCGGCTTTGAAAAGT-3′ containing XhoI and HindIII restriction sites respectively. The resulting fragment was inserted into pGL3-basic plasmid to generate pGL3–c-jun construct. HEK293 or C6 cells were transiently transfected with the pGL3–c-jun construct along with *Renilla* luciferase vector (pRL-TK) and respective gene constructs as per experimental interest. 36 h post-transfection, cells were treated with respective inhibitors or activators for the time periods indicated in the figure legend. Cells were harvested using Dual Luciferase Reporter Assay System (Promega, Madison, USA) following the manufacturer's protocol and measured in VICTOR X Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). Quantification was based on three independent experiments.