FEBS Letters 580 (2006) 3943–3947

GRP78-binding protein regulates cAMP-induced glial fibrillary acidic protein expression in rat C6 glioblastoma cells

Kentaro Oh-hashi[a], Yoko Hirata[a], Hisashi Koga[b,c], Kazutoshi Kiuchi[a,*]

[a] Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Chiba Industry Advancement Center, 2-6 Nakase, Mihama-ku, Chiba 261-7126, Japan
[c] Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan

Received 28 April 2006; accepted 8 June 2006

Available online 21 June 2006

Edited by Lukas Huber

Abstract **We previously reported that a novel GRP78-binding protein (GBP) is predominantly expressed in rat brain and its expression declines through the aging process. To characterize its biological function, we established C6 glioblastoma cells that stably overexpressed GBP. Stable overexpression of GBP attenuated cAMP-induced expression of the glial fibrillary acidic protein (GFAP) gene, which was accompanied by a decrease in cAMP-induced signal transducer and activators of transcription 3 (STAT3) phosphorylation. Other distinct cAMP-induced events, including a transient reduction in extracellular signal-regulated protein kinase phosphorylation and a slowdown in cell proliferation, were hardly affected by GBP overexpression. Most importantly, treatment with siRNA against endogenous GBP markedly downregulated GBP expression in C6 glioblastoma cells, and dramatically augmented cAMP-induced *GFAP* mRNA expression in parallel with hyper-phosphorylation of STAT3. These results suggest a novel function of GBP in regulating *GFAP* gene expression via STAT3 phosphorylation.**

Keywords: cAMP; Glial fibrillary acidic protein; Glioblastoma; GRP78-binding protein; Signal transducer and activators of transcription 3

1. Introduction

Previously, we identified a novel 78 kDa glucose-regulated protein (GRP78)-binding protein (GBP) gene encoding 1021 amino acids, which was predominantly expressed in rat brain among various other tissues using PCR-selected cDNA subtraction [1]. *GBP* mRNA is already expressed in the E12 rat brain, and gradually increases in expression level to reach a peak during postnatal 0–2 weeks and decreases with age. GBP-overexpression in Neuro2a cells slightly, but significantly, suppressed serum starvation-induced cell death. GBP transiently expressed in COS7 cells is predominantly located in the ER among other intracellular organelles. However, the biological function of GBP cannot be deduced by amino acid sequence analysis since it contains no known domains.

The various kinds of cells in the central nervous system including neuronal and glial lineages are considered to arise from common precursor cells, which possess the potential to differentiate into multiple types of cells [2,3]. Until now, various mediators present in the environment of the developing brain have been reported to determine the fate of the multi-potential precursor cells [4–6]. During development of brain, astrocyte differentiation occurs primarily at postnatal stages. Extracellular mediators such as bone morphogenetic protein-2, ciliary neurotrophic factor, pituitary adenylate-cyclase activating protein, and the interleukin-6 (IL-6) family of cytokines are reported to promote astrocyte differentiation [4,7–11]. The IL-6 family of cytokines transduces its signals to activate a downstream transcription factor, signal transducer and activators of transcription 3 (STAT3) [12]. During differentiation of astrocytes from neuronal progenitor cells, activated STAT3 recognizes the specific sequence on the glial fibrillary acidic protein (GFAP, an astrocyte marker) promoter and activates its transcription [8,9,11,13,14].

The rat C6 glioblastoma cell line is well characterized as a model of astrocyte differentiation after treatment with intracellular cAMP elevating stimuli [14–17]. Recently, Takanaga et al. reported that cAMP-induced GFAP expression in C6 glioblastoma cells is mediated by a STAT3-dependent pathway [14]. Considering that the expression of *GBP* mRNA in rat brain reaches a peak postnatally, we speculated that GBP might be involved in glial differentiation and influence the gene expression of *GFAP* during this period. In this report, we find a novel function of GBP in regulating GFAP expression in C6 glioblastoma cells.

2. Materials and methods

2.1. Materials

Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, MO, USA). Antibodies against STAT3, extracellular signal-regulated protein kinase (Erk) and their phosphorylated forms were purchased from Cell Signaling (Beverly, MA, USA). Anti-Myc monoclonal antibody (9E10) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

*Corresponding author. Fax: +81 58 230 1893.
E-mail address: kiuchi@biomol.gifu-u.ac.jp (K. Kiuchi).

Abbreviations: Erk, extracellular signal-regulated protein kinase; GBP, GRP78-binding protein; GFAP, glial fibrillary acidic protein; GRP78, 78 kDa glucose-regulated protein; IBMX, 3-isobutyl-1-methylxanthine; STAT3, signal transducer and activators of transcription 3

0014-5793/$32.00
doi:10.1016/j.febslet.2006.06.028

2.2. Preparation of anti-GBP polyclonal antibody

The rabbit polyclonal anti-GBP antibody was prepared against a 137 amino acid synthetic peptide corresponding to the C-terminal amino acid residues of the mouse GBP homologue (KIAA1583, 882–1018 aa).

2.3. Cell culture and generation of cells stably expressing Myc-tagged GBP

Rat C6 glioblastoma cells were maintained in F-10 Nutrient Mixture (HAM) medium (Invitrogen) containing 7% fetal calf serum and 3% horse serum. For generation of stable GBP overexpressing and control cell lines, Myc-tagged GBP or empty vector as a control was transfected into C6 glioblastoma cells by Lipofectamine-Plus reagent according to the manufacturer's instructions. The transfected cells were selected and maintained in the presence of G418.

2.4. Morphological study

C6 glioblastoma cells culture were fixed with 4% paraformaldehyde for 5 min at room temperature and treated with PBS containing 0.2% TritonX-100. After washing with PBS, cells were stained with rhodamine-phalloidin (Molecular probes) to detect F-actin and were observed with a fluorescence microscope (Olympus).

2.5. Cell proliferation assay

Cell proliferation was quantified by the WST-1 assay. In brief, C6 glioblastoma cells in 96-well plates were treated with 10 μM forskolin, 10 μM forskolin/100 μM IBMX (F/I) or vehicle for 24 h. Ten microliters of WST-1 solution was added per well at the beginning of the last hour. The difference in absorbance at 450 and 620 nm was measured as an indicator of cell proliferation. Each value of absorbance in control and GBP-overexpressing cells with no incubation (at 0 h, D0) was defined as 100%, respectively.

2.6. Reverse transcription-polymerase chain reaction

To estimate the mRNA level of each gene by reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from cells lysed with Trizol (Invitrogen), and converted to cDNA by reverse transcription using random ninemers to prime superscript III RNase$^-$ RT (Invitrogen) as previously described [18]. Specific DNAs were mixed and amplified with the PCR mixture (EX Taq PCR kit, Takara). The RT-PCR primers used in this study were as follows: *GBP* sense primer, 5′-GGCTCCCAGTGCTTCAGAGA-3′; *GBP* antisense primer, 5′-GATCCTCTCCATGTAGTTCCGAA-3′; *GRP78* sense, 5′-ACCAATGACCAAAACCGCCT-3′; *GRP78* antisense, 5′-GAGTTTGCTGATAATTGGCTGAAC-3′; *GFAP* sense, 5′-CCAAGATGAAACCAACCT-3′; *GFAP* antisense, 5′-CGCTGTGAGGTCTGGCTT-3′; *G3PDH* sense, 5′-TCCACCACCCTGTTGCTGTA-3′; *3PDH* antisense, 5′-ACCACAGTCCATGCCATCAC-3′; β-*Actin* sense, 5′-TGTATGCCTCTGGTCGTACC-3′; β-*Actin* antisense, 5′-CAACGTCACACTTCATGATGG-3′. The typical reaction conditions were 0.5 min at 96 °C, 0.5 min at 60 °C, and 0.5 min at 72 °C. The results represent 19–34 cycles of amplification, after which cDNAs were separated by electrophoresis on 2.0% agarose gels and visualized using ethidium bromide. Experiments were repeated and reproducibility was confirmed.

2.7. Transfection of GBP siRNA

Stealth siRNA against rat GBP (sense, 5′-CCACCCUUCUGCUUCGACACAAUUU-3′, and antisense, 5′-AAAUUGUGUCGAAGCAGAAGGGUGG-3′) and a non-specific negative control siRNA were obtained from Invitrogen. In brief, C6 glioblastoma cells (2×10^5 cells per well in 6-well plates) were transfected with either control or stealth siRNA against rat GBP (100 nM) using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. Thirty-six hours after transfection, cells were treated with forskolin/IBMX or vehicle for 20 h.

2.8. Immunoprecipitation and Western blotting analysis

Phosphorylated and non-phosphorylated Erk, STAT3 and Myc-tagged GBP were analyzed by Western blotting. In brief, cells were lysed with 20 mM Tris buffer, pH 8.0, containing 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate and 1% (v/v) Triton X-100. The protein concentration was determined by the Bradford method [19]. Equal amounts of cell lysates were separated on 8.0% SDS–polyacrylamide electrophoresis gels, immunoblotted onto polyvinylidene difluoride membrane (Amersham) and identified by enhanced chemiluminescence using antibodies against GBP, Erk, STAT3 and Myc-epitope. For immunoprecipitation, cells were lysed with 20 mM Tris buffer, pH 8.0, containing 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin. After centrifugation, the lysates were immunoprecipitated with anti-Myc antibody and protein G sepharose FF (Amersham), and analyzed by Western blotting as described above.

3. Results

3.1. Stable overexpression of GBP attenuates cAMP-induced GFAP mRNA expression in C6 glioblastoma cells

To characterize biological functions of GBP, we first established C6 glioblastoma cells stably overexpressing GBP. As shown in Fig. 1A, the approximately 160 kDa protein band corresponding to Myc-tagged GBP in the lysates was detected by immunoprecipitation and Western blotting with anti-Myc antibody. As shown in Fig. 1B, morphological properties of GBP-overexpressing C6 glioblastoma cells were compared with those transfected with the empty vector (mock) as a control. GBP-overexpressing C6 glioblastoma cells had slightly enlarged cell bodies under resting conditions. Cyclic AMP-stimulus caused morphological changes such as process extension and spindle-shapes in control cells (mock), but these changes were rarely detected in GBP-overexpressing C6 glioblastoma cells. On the other hand, the cAMP-stimulus provoked a growth arrest in both of the cell types within 8 h according to the WST-1 assay (data not shown). The proliferation rate of GBP-overexpressing cells was slightly higher than that of control cells at 24 h after stimulation (Fig. 1C). In order to investigate whether the overexpressed GBP influences the expression levels of *GFAP* and *GRP78* genes, GBP overexpressing or control cells were treated with a cAMP-elevating reagent, forskolin and/or IBMX, and their expression levels were estimated using RT-PCR analyses. As shown in Fig. 2, *GFAP* mRNA was significantly induced 24 h after treatment with forskolin and/or IBMX in the control cells, whereas the GBP-overexpressed cells failed to express the *GFAP* gene. The expression levels of cell cycle and growth arrest-related genes such as *p21*, *p27*, *GADD45* and *GADD153* were also analyzed; however, they were negligibly affected by GBP-overexpression in C6 glioblastoma cells (data not shown).

3.2. Stable overexpression of GBP attenuates cAMP-induced STAT3 phosphorylation in C6 glioblastoma cells

As STAT3 activation is reported to play a crucial role in cAMP-induced GFAP expression in C6 glioblastoma cells [14], we investigated whether GBP-overexpression affects the cAMP-induced phosphorylation of STAT3 together with Erk phosphorylation. STAT3 phosphorylation in control cells was detected 24 h after forskolin/IBMX treatment, whereas in GBP-overexpressing cells this phosphorylation was markedly lower (Fig. 3). These results corresponded well with the faint induction of *GFAP* mRNA by forskolin/IBMX treatment in the GBP-overexpressing cells. On the other hand, the phos-