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

Kentaro Oh-hashi[1], Yoko Hirata[1], Hisashi Koga[2, 3] and Kazutoshi Kiuchi[1]*

[1] Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan; [2] Chiba Industry Advancement Center, 2-6 Nakase, Mihama-ku, Chiba 261-7126, Japan, [3] Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan

*Corresponding author: Kazutoshi Kiuchi, Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan. Tel; +81-58-293-2651; Fax; +81-58-230-1893; E-mail: kiuchi@biomol.gifu-u.ac.jp

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.

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 STAT3 phosphorylation. Other distinct cAMP-induced events, including a transient reduction in Erk phosphorylation and a slowdown in cell proliferation, were hardly affected by GBP overexpression. Most importantly, treatment with siRNA against endogenous GBP markedly down-regulated 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; GFAP; glioblastoma; GRP78-binding protein (GBP); STAT3

1. Introduction

Previously, we identified a novel 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, 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 IBMX were purchased from Sigma (St. Louis, MO, USA). Antibodies against STAT3, 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).

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 aa – 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 μl 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, D_0) was defined as 100%, respectively.

2. 6. Reverse transcription-polymerase chain reaction (RT-PCR)

To estimate the mRNA level of each gene by 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$^-$ reverse transcriptase (RT) (Invitrogen) as previously described (18). Specific DNAs were mixed and amplified with the PCR reaction 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'; *G3PDH* 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 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 nonspecific 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

phosphorylation levels of Erk in both of the cell lines were reduced within 0.5 h and partially recovered 24 h after forskolin/IBMX treatment. The phosphorylation pattern of Erk up to 24 h was similar between the two cell lines.

3.3. Suppression of intrinsic GBP augments cAMP-induced GFAP mRNA expression

Finally, we examined whether the suppression of intrinsic GBP enhances *GFAP* gene induction in C6 glioblastoma cells. The amount of endogenous *GBP* in C6 glioblastoma cells transfected with siRNA against rat GBP was less than half of that of control cells transfected with nonspecific siRNA. These phenomena occurred independently of forskolin/IBMX stimulation (Fig. 4A). On the contrary, GBP-specific siRNA transfection facilitated *GFAP* gene expression and STAT3 phosphorylation only when receiving the cAMP-elevating stimuli (Fig. 4B). These results imply that GBP negatively regulates cAMP-induced *GFAP* transcription through the STAT3 signaling pathway in C6 glioblastoma cells.

4. Discussion

GBP is predicted to have two transmembrane regions, a proline- and a glutamic acid-rich region, but its sequence offered few clues to its possible functions. Consistent with our previous results, we estimated the expression level of *GBP* mRNA in primary cultured neurons, astrocytes and microglial cells prepared from rat embryonic cerebral cortex by RT-PCR. Among the three types of primary cultured cells, the highest expression of *GBP* mRNA was observed in primary cultured neurons whereas lower amounts of *GBP* mRNA were detected in the other two cell types (data not shown). Considering that *GBP* mRNA is detected in E12 brain and reaches a peak around postnatal 0 – 2 weeks, GBP may take part in the development and maintenance of various types of cells in the brain.

In this study, we demonstrated that GBP plays a crucial role in GFAP expression in C6 glioblastoma cells and negatively regulates *GFAP* transcription only when the cAMP concentration in the cells is elevated. Stable expression of the mouse GBP homologue, KIAA1583, in C6 glioblastoma cells also reduced cAMP-induced *GFAP* mRNA expression (data not shown). On the contrary, the suppression of intrinsic GBP was found to augment the expression of cAMP-induced *GFAP* mRNA. In regard to this augmentation, the IL-6 family of cytokines is reported to enhance GFAP transcription

through the STAT3 binding site on the GFAP promoter (13, 14). STAT3 is a common downstream transcription factor of the IL-6 family of cytokines, and participates in various glial functions including differentiation, inflammation, and proliferation (8, 9, 11, 20). Takanaga *et al*. reported that cAMP-induced GFAP expression in C6 glioblastoma cells is mediated by autocrine IL-6 (14) signaling. In our present study, the remarkable increased expression level of *GFAP* mRNA resulting from siRNA-induced down-regulation of GBP was accompanied by hyper-phosphorylation of STAT3. On the other hand, the cAMP-induced STAT3 phosphorylation was diminished in C6 glioblastoma cells by overexpressing GBP. These results suggest that GBP might act upstream of STAT3 pathway, however, the precise mechanisms remain to be determined.

Recently, it was reported that apoptosis signal-regulated kinase 1 represses GFAP promoter activity through a p38 MAPK-dependent and STAT3-independent pathway in adult hippocampus-derived progenitor cells (21). However, a p38 MAPK inhibitor did not restore the lower expression level of cAMP-induced *GFAP* mRNA in GBP-overexpressing cells in our preliminary experiments, which is consistent with the previous report that the p38 MAPK inhibitor had no effect on cAMP-induced GFAP promoter activity in C6 glioblastoma cells (14). In addition, phosphatidylinositol

3-kinase (PI3K) is reported to be required for cAMP-induced *GFAP* expression in C6 glioblastoma cells (22), whereas protein kinase B (PKB), a downstream target of PI3K, is reported to negatively regulate this expression in the same cells (23). Thus, the precise mechanisms by which PI3K and/or PKB regulate GFAP expression are still unclear. Further characterization of GBP may uncover a complex link between GBP, PI3K-PKB and STAT3 in the regulation of GFAP expression in C6 glioblastoma cells.

In conclusion, we have demonstrated a novel function of GBP in regulating cAMP-induced *GFAP* transcription through the STAT3 signaling pathway in C6 glioblastoma cells. We believe that further studies to understand how GBP regulates STAT3 phosphorylation may reveal novel functional domains of GBP. STAT3 is reported to participate not only in the expression of various genes, such as inflammatory cytokines and adhesion molecules, but also in apoptosis and differentiation of neurons and their progenitor cells (20, 24 – 26). Therefore, further characterization of GBP might provide new insights into its crucial roles in the developing brain and neurodegenerative diseases.

Acknowledgements

This research was supported in part by the Sasagawa Scientific Research Grant from The

Japan Science Society and grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] Oh-hashi, K., Naruse. Y., Amaya. F., Shimosato, G., and Tanaka, M. (2003) Cloning and characterization of a novel GRP78-binding protein in the rat brain. J. Biol. Chem. 278, 10531-10537.

[2] McKay R. (1997) Stem cells in the central nervous system. Science 276, 66-71.

[3] Anderson D. J. (2001) Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron 30, 19-35.

[4] Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D. (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. 10, 3129-3140.

[5] Edlund ,T., and Jessell, T.M. (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell 96, 211-224.

[6] Nakashima, K., and Taga, T. (2002) Mechanisms underlying cytokine-mediated cell-fate regulation in the nervous system. Mol. Neurobiol. 25, 233-244.

[7] Gross, R.E., Mehler, M.F., Mabie, P.C., Zang, Z., Santschi, L., and Kessler, J.A. (1996) Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron 17, 595-606.

[8] Bonni, A., Sun, Y., Nadal-Vicens, M., Bhatt, A., Frank, D.A., Rozovsky, I., Stahl, N., Yancopoulos, G.D., and Greenberg, M.E. (1997) Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 278,

477-483.

[9] Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., and Taga, T. (1999) Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science 284, 479-482.

[10] Vallejo, I., and Vallejo, M. (2002) Pituitary adenylate cyclase-activating polypeptide induces astrocyte differentiation of precursor cells from developing cerebral cortex. Mol. Cell Neurosci. 21, 671-683.

[11] Song, M.R., and Ghosh, A. (2004) FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat. Neurosci. 7, 229-235.

[12] Heinrich, P.C., Behrmann, I., Muller-Newen, G., Schaper, F., and Graeve, L. (1998) Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem. J. 334, 297-314.

[13] Yanagisawa, M., Nakashima, K., Arakawa, H., Ikenaka, K., Yoshida, K., Kishimoto, T., Hisatsune, T., and Taga, T. (2000) Astrocyte differentiation of fetal neuroepithelial cells by interleukin-11 via activation of a common cytokine signal transducer, gp130, and a transcription factor, STAT3. J. Neurochem. 74, 1498-1504.

[14] Takanaga, H., Yoshitake, T., Hara, S., Yamasaki, C., and Kunimoto, M. (2004)

cAMP-induced astrocytic differentiation of C6 glioma cells is mediated by autocrine interleukin-6. J. Biol. Chem. 279, 15441-15447.

[15] Benda, P., Lightbody, J., Sato, G., Levine, L., and Sweet, W. (1968) Differentiated rat glial cell strain in tissue culture. Science 161, 370-371.

[16] Roymans, D., Vissenberg, K., De Jonghe, C., Grobben, B., Claes, P., Verbelen, J. P., Van Broeckhoven, C., and Slegers, H. (2000) Nucleoside diphosphate kinase beta (Nm23-R1/NDPKβ) is associated with intermediate filaments and becomes upregulated upon cAMP-induced differentiation of rat C6 glioblastoma. Exp. Cell Res. 261, 127-138.

[17] Wang, L., Liu, F., and Adamo, M.L. (2001) Cyclic AMP inhibits extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt pathways by inhibiting Rap1. J. Biol. Chem. 276, 37242-37249.

[18] Oh-hashi, K., Naruse, Y., and Tanaka, M. (2002) Intracellular calcium mobilization induces period genes via MAPK pathways in NIH3T3 cells. FEBS Lett. 516, 101-105.

[19] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of

microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

[20] Park, E.J., Ji, K.A., Jeon, S.B., Choi, W.H., Han, I.O., You, H.J., Kim, J.H., Jou I., and Joe, E.H. (2004) Rac1 contributes to maximal activation of STAT1 and STAT3 in IFN-γ-stimulated rat astrocytes. J. Immunol. 173, 5697-5703.

[21] Faigle, R., Brederlau, A., Elmi, M., Arvidsson, Y., Hamazaki, T.S., Uramoto, H., and Funa, K. (2004) ASK1 inhibits astroglial development via p38 mitogen-activated protein kinase and promotes neuronal differentiation in adult hippocampus-derived progenitor cells. Mol. Cell Biol. 24, 280-293.

[22] Roymans, D., Vissenberg, K., De Jonghe, C., Grobben, B., Claes, P., Verbelen, J. P., Van Broeckhoven, C., and Slegers, H. (2001) Phosphatidylinositol 3-kinase activity is required for the expression of glial fibrillary acidic protein upon cAMP-dependent induction of differentiation in rat C6 glioma. J. Neurochem. 76, 610-618.

[23] Van Kolen, K., and Slegers, H. (2004) P2Y12 receptor stimulation inhibits beta-adrenergic receptor-induced differentiation by reversing the cyclic

AMP-dependent inhibition of protein kinase B. J. Neurochem. 89, 442-453.

[24] Kim, S.Y., Park, H.J., Choi, J.S., Lee, J.E., Cha, J.H., Choi, Y.S., Cho, K.O., Chun, M.H., and Lee, M.Y. (2004) Ischemic preconditioning-induced expression of gp130 and STAT3 in astrocytes of the rat hippocampus. Brain Res. Mol. Brain Res. 129, 96-103.

[25] Yamashita, T., Sawamoto, K., Suzuki, S., Suzuki, N., Adachi, K., Kawase, T., Mihara, M., Ohsugi, Y., Abe, K., and Okano, H. (2005) Blockade of interleukin-6 signaling aggravates ischemic cerebral damage in mice: possible involvement of Stat3 activation in the protection of neurons. J. Neurochem. 94, 459-468.

[26] Yadav, A., Kalita, A., Dhillon, S., and Banerjee, K. (2005) JAK/STAT3 pathway is involved in survival of neurons in response to insulin-like growth factor and negatively regulated by suppressor of cytokine signaling-3. J. Biol. Chem. 280, 31830-31840.

Legend for Figures

Fig. 1 Establishment of C6 glioblastoma cells stably overexpressing GBP. (A) C6 glioblastoma cells stably expressing Myc-tagged GBP (Myc-GBP) or cells stably transfected with the empty vector (mock) as a control were established by selection with G418 and the expression of Myc-tagged GBP in the cells was detected by immunoprecipitation of the Myc-epitope and western blotting as described in materials and methods. (B) Control (mock; a, b, c, d) and GBP-overexpressing cells (GBP; e, f, g, h) were treated with 10 μM forskolin/100 μM IBMX (F/I) (c, d, g, h), or vehicle (-) (a, b, e, f) for 24 h and their morphological changes were observed as described in materials and methods. Phase contrast (a, c, e, g) and F-actin staining (b, d, f, h). (C) Control (mock) and GBP-overexpressing cells (GBP) were treated with 10 μM forskolin (F), 10 μM forskolin/100 μM IBMX (F/I), or vehicle (-) for 24 h, and the reduction in levels of WST-1 was determined as described in materials and methods. Values represent means ± SEM of triplicate cultures and are expressed as the percentage of the cells before incubation (0 h, D_0).

Fig. 2 GBP-overexpression attenuated cAMP-induced *GFAP* mRNA expression in C6 glioblastoma cells. (A) Control and GBP-overexpressing cells were treated with 10 μM forskolin to detect expression levels of *GFAP*, *GBP* and *GRP78* genes at the indicated time. (B) Control and GBP-overexpressing cells were treated with 10 μM forskolin (F), 10 μM forskolin/100 μM IBMX (F/I), or vehicle (-) for 24 h to detect expression levels of *GFAP* and *GBP* genes. Total RNA was isolated from the cells and their expression levels in each clone were analyzed by RT-PCR as described in materials and methods.

Fig. 3 GBP-overexpression suppressed cAMP-induced STAT3 phosphorylation in C6 glioblastoma cells. Control (mock) and GBP-overexpressing cells (GBP) were treated with 10 μM forskolin/100 μM IBMX (F/I) for the indicated time. Cell lysates of each clone were analyzed by western blotting using antibodies against phospho-Erk1/2, Erk1/2, phospho-STAT3, STAT3 and Myc-epitope as described in materials and methods.

Fig. 4 Down-regulation of GBP by siRNA accelerated cAMP-induced *GFAP* mRNA expression in C6 glioblastoma cells. (A) Thirty six hours after transfection of siRNA against GBP or control siRNA, cells were treated with 10 μM forskolin/100 μM IBMX (F/I) or vehicle (-) for 20 h to detect expression levels of *GFAP*, *GBP* and *β-Actin* mRNAs in cells treated with each siRNA. Total RNA was isolated from the cells and their expression levels in each case were analyzed by RT-PCR as described in materials and methods. (B) Phospho-STAT3, STAT3 and intrinsic GBP in each cell lysates were analyzed by western blotting as described in materials and methods.

Figure(s)

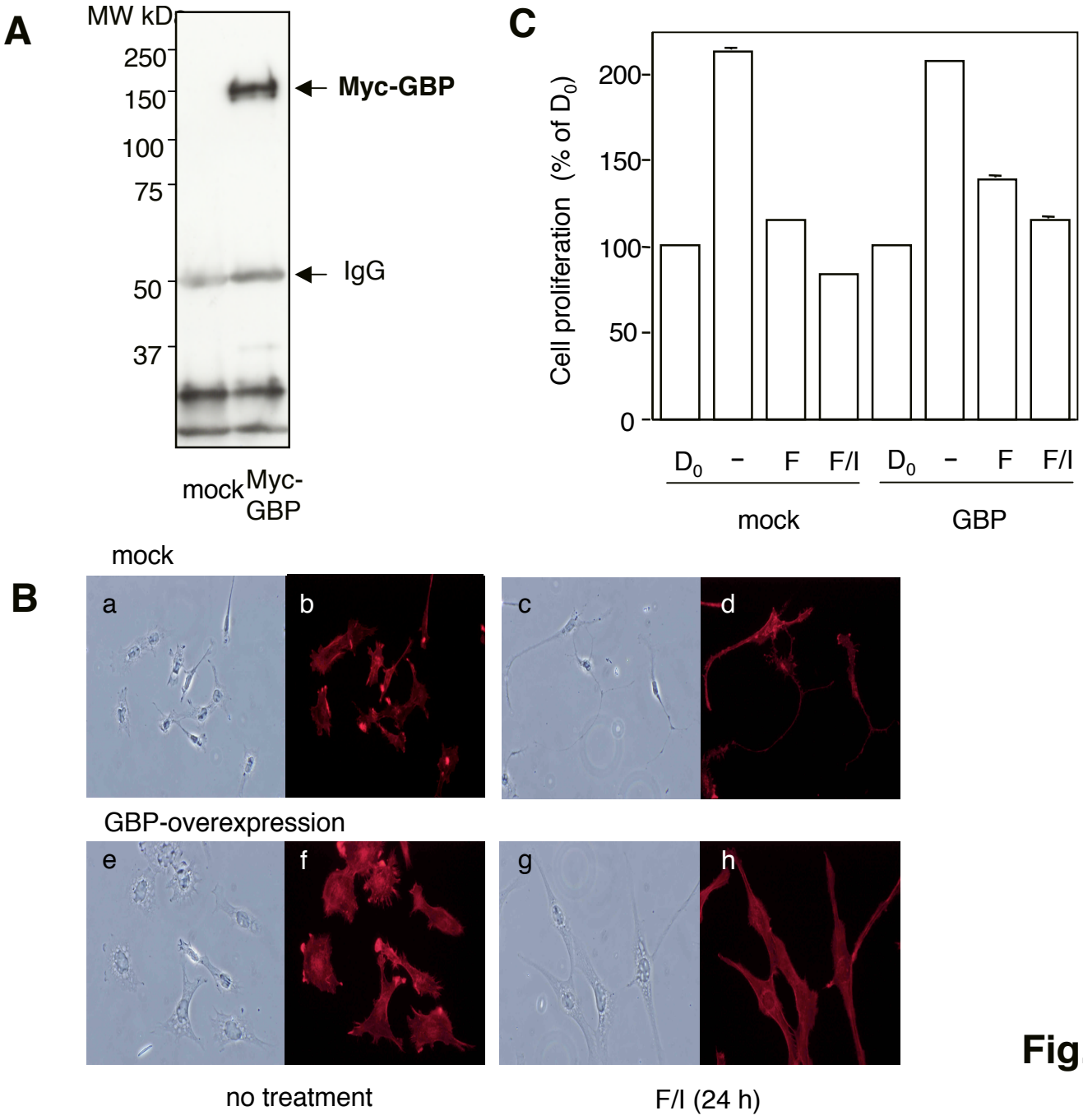

Fig. 1 Oh-hashi *et al.*

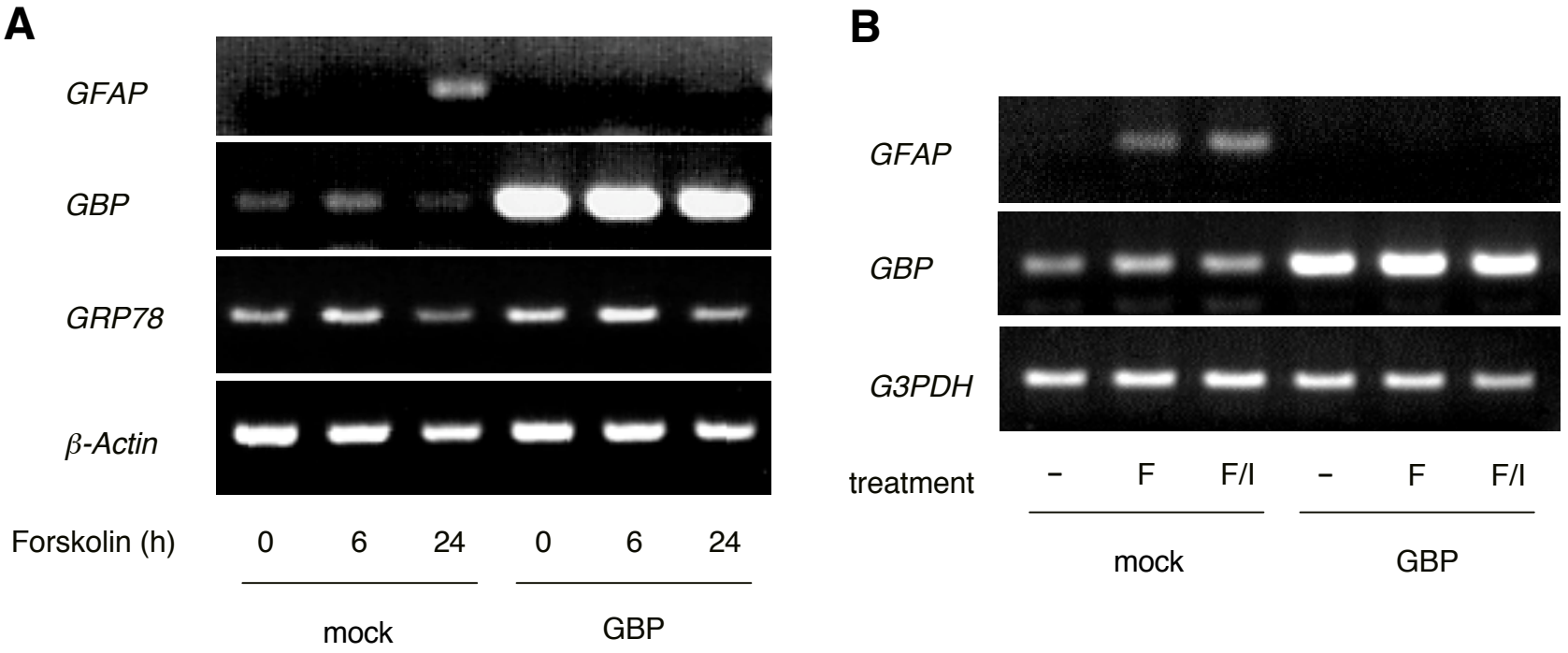

Fig. 2 Oh-hashi *et al.*

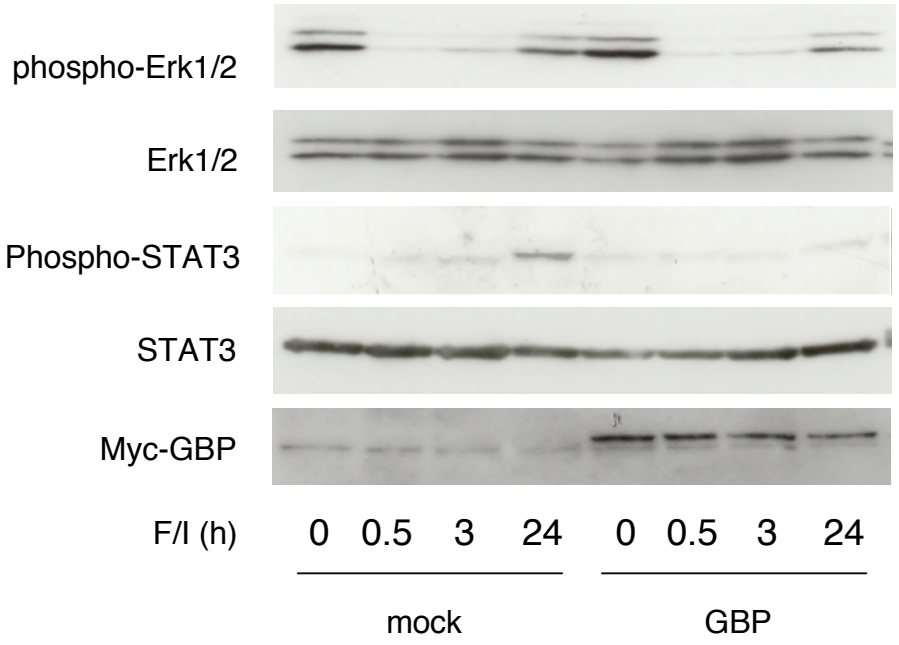

Fig. 3 Oh-hashi *et al.*

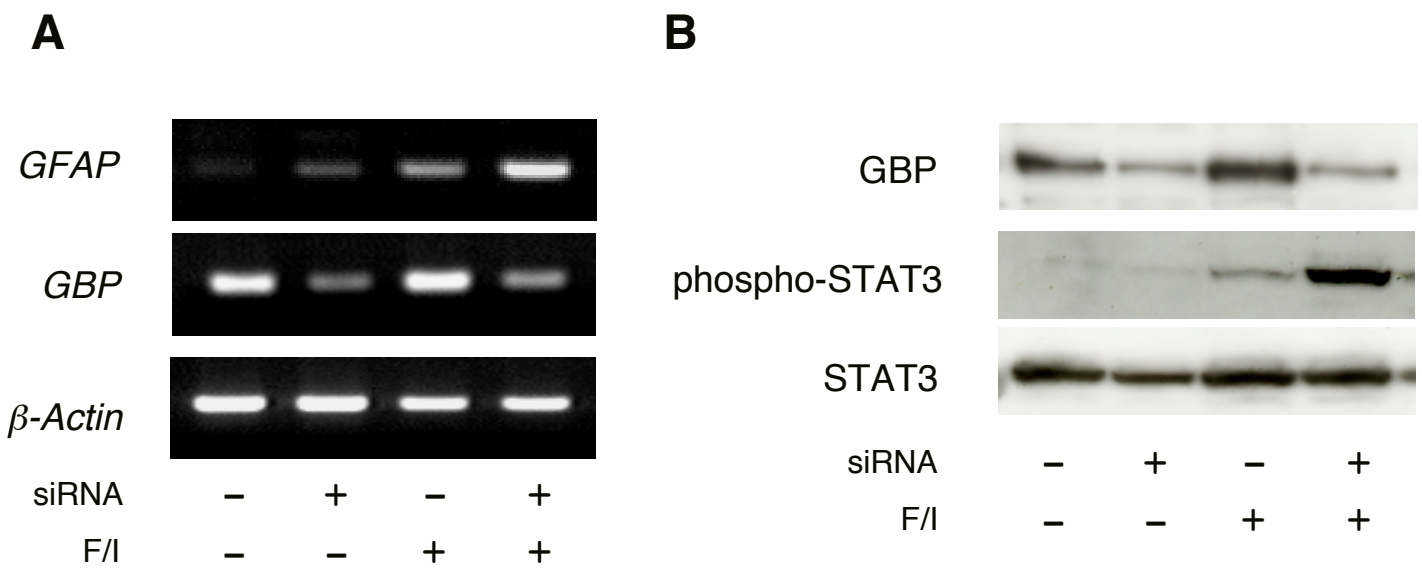

Fig. 4 Oh-hashi *et al.*