Molecular cloning of a novel GRP78 binding protein

Cloning and characterization of a novel GRP78 binding protein in the rat brain

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The abbreviations used are: BODIPY, dipyromethene difluoride; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GRP78, 78kDa glucose regulated protein; GST, glutathione S-transferase; Hsc73, heat shock cognate protein 70; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends.
Summary

The full-length cDNA clone of a novel GRP78 binding protein, GBP, was isolated from rat brain using PCR-selected cDNA subtraction. GBP was predominantly expressed in neuronal cells among various brain tissues. GBP mRNA was already detected in the E12 brain, and then gradually increased to reach a peak within P0 – 2 weeks after birth. GBP expression in the brain decreased age-dependently to approximately 30% of the postnatal level at 12 months. GBP encoded 1021 amino acids and was predicted to have two transmembrane regions and glutamic acid- and proline-rich regions. Since the sequence of GBP offered few clues to the possible function, we performed a GST-tagged GBP pull down assay in PC12 lysates and identified GRP78, one of the heat shock proteins, as a counterpart. Observation of COS7 cells expressing GFP- or myc-tagged GBP showed that GBP was localized in the ER-Golgi domain where BOJIPY-labeled Brefeldin A accumulated. To investigate a biological role for GBP, we established Neuro2a cells stably expressing myc-tagged GBP. Overexpression of GBP did not affect cell growth or morphological features but attenuated the time-dependent decrease in cell viability caused by serum deprivation compared with control cells. After 48 h of serum starvation, Neuro2a cells overexpressing GBP were resistant to the cell death induced by serum
withdrawal. These results suggest that GBP would have a relevant functional role in embryonic
and postnatal development of the brain.

Introduction

The central nervous system is established through various developmental steps, which
include determination of cell fate to the neuronal lineage, proliferation and differentiation of
precursor cells, migration to defined regions, and formation of neuronal interactions. Most
neurons derived at the embryonic stage construct a number of synapses during the early
postnatal development of the brain, which accompanies programmed cell death of excessively
produced neurons in order to form a proper neuronal network (1-3). A number of molecules
such as humoral factors (4, 5), receptors (6, 7), adhesion molecules (8, 9) and transcriptional
factors (10, 11) have been reported to participate in brain development at various stages. The
molecular mechanisms for the formation of structural and functional nervous systems are
complex and our knowledge is limited. Therefore, we consider that many as yet unidentified
genes would contribute to the regulation of brain development at the embryonic and postnatal stages.

In the present study, we describe the isolation and characterization of a novel gene enriched in the embryonic and postnatal stages of rat brain development. Our cloned gene encoded an ER-Golgi localized protein which was associated with GRP78, one of the heat shock proteins. We suggest that our cloned GRP78 binding protein, GBP, plays a role in the functional development of neurons.

Experimental procedures

_Molecular cloning of rat GBP_ – The expressions of mRNAs in the hypothalamus, including the suprachiasmatic nucleus, of postnatal rats (2 weeks after birth) and adult rats (10 weeks) were compared by PCR-selected cDNA subtraction according to the manufacturer’s instructions (CLONTECH) (12).

Briefly, poly A+ RNA (0.5 µg) was extracted from the brains at each age, converted to double stranded cDNA (ds cDNA) by incubation with reverse transcriptase, 5’-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3’ (10 µM), and 5’-
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AAGCAGTGGTAACAACGCAGAGTACN\textsubscript{3}N-3’ (10 μM). The ds cDNA was digested to blunt-ended fragments by treatment with Rsa I, and only ds cDNA from 2 week-rats was ligated with oligonucleotides: 5’-

CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCAGGT-3’ (adapter1) or 5’-

CTAATACGACTCACTATAGGGCACTGGTGCGGCCCGAGGT-3’ (adapter2R). For the 1st hybridization, each adapter-ligated cDNA was respectively hybridized with an excessive amount of cDNA from adult rats. Following the 2nd hybridization by combination with each 1st hybridized solution, 2 week-specific cDNAs were amplified by PCR using the 1st primer: 5’-

CTAATACGACTCACTATAGGC-3’, 2nd primer1: 5’-TCGAGCGCCGCCCGGAGGT-3’ and 2nd primer2: 5’-AGCGTGGTGGCAGCCGAGGT-3’. The secondary PCR products were cloned using the T/A cloning system and sequenced on both strands using an ABI PRISM dye terminator cycle sequencing kit (PerkinElmer) with T7 and SP6 primers. To obtain the full-length cDNA of GBP, we produced 5’-RACE and 3’-RACE PCR products from rat forebrain RNA and sequenced them as described above.

*Cell lines, cell culture and transfection* – Neuro2a and COS7 cells were cultured in
DMEM supplemented with 10% fetal bovine serum (FBS), and PC12 cells were cultured in DMEM supplemented with 5% FBS and 5% calf serum. Transfection with each plasmid (2 - 6 µg) was performed using Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. Stable cell lines transfected with each plasmid were selected and maintained by G418 (CLONTECH) at concentrations of 0.5 - 2.0 mg/ml.

Northern blotting and RT-PCR analysis – Total RNA (5 – 10 µg) from various rat tissues was purified, resolved by agarose gel electrophoresis under denaturing conditions, and blotted onto nylon membranes. To investigate the expression of GBP mRNA in the various brain regions and in the whole brain at indicated ages, we purchased RNA-blotted membrane from Seegene Co. Ltd. An α-[32P]-radiolabeled rat GBP cDNA fragment containing nucleotides 2972-3248 was hybridized in the CLONTECH hybridization solution. After washing, the membranes were exposed to a Fuji imaging plate and analyzed with BAS2000 (Fuji Film, Japan). In order to control for the amount of RNA present, the membranes were stripped and re-hybridized with a G3PDH cDNA probe.

RT-PCR was also performed to estimate the mRNA levels of various tissues and cell
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lines as described previously (13). Briefly, 0.5 µg of total RNA was converted into cDNA by reverse transcriptase and specific DNAs were amplified by PCR using Taq polymerase. The PCR primers used in this study were as follows: GBP sense primer, 5’-

GGCTCCCAGTGCTTCA GAGA-3’; GBP antisense primer1, 5’-

TCCAGGGGCCTGGAATCCGG-3’; GBP antisense primer2, 5’-

GATCCTCTCATGTAGTTCCGAA-3’; GBP antisense primer3, 5’-

TGCAGTTCACACTACAGGC-3’; G3PDH sense primer, 5’-

TCCACCACCTGTGCTGTA-3’; G3PDH antisense primer, 5’-

ACCACAGTCCATGCCCATC-3’. The level of GBP mRNA in tissues and cell lines was detected using GBP sense primer and GBP antisense primer1 or 2, T7 primer and GBP antisense primer3 were used to detect the level of myc-tagged GBP mRNA in Neuro2a cells. The typical reaction conditions were 0.5 min at 95°C, 0.5 min at 60°C, and 1 min 72°C. The results shown represent 18-30 cycles of amplification. After amplification, the cDNAs were separated by electrophoresis in 2.0% agarose gels and visualized using ethidium bromide.

*In situ hybridization* – Antisense and sense RNA probes were prepared by in vitro
transcription of an RT-PCR amplified fragment of GBP cDNA (nucleotides 1122 to 3248) subcloned into pBSSK(+) and synthesized from these cDNA templates with T3 or T7 RNA polymerase and a digoxigenin (DIG) labeling mixture (Roche) (14). Postnatal (2w) and adult (10w) rat brains were perfused and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C and coronal sections (30 μm in thickness) were cut on a cryostat. The sections were treated with 0.1 mg/ml proteinase K (Sigma), 10 mM Tris-HCl buffer (pH=7.4) and 10 mM EDTA for 10 min at 37°C, 4% paraformaldehyde in 0.1 M PB for 5 min, and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in hybridization buffer containing a DIG-UTP-labeled GBP riboprobe for 12 h at 60°C. Following the hybridization, the sections were washed sequentially in 2×SSC containing 50% formamide, RNase solution and 0.4×SSC. Finally, they were processed for DIG-coloring steps using an anti-DIG antibody, 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as described previously (14).

Fluorescence microscopy– COS7 cells were seeded on glass coverslips and transfected with the indicated plasmid as described above. For the detection of cells expressing myc-tagged
GBP, cells were fixed with PBS containing 4% formaldehyde for 15 min. After washing, the cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min and incubated with an anti-myc antibody for 1 h. The cells were then incubated with an FITC-conjugated anti-mouse IgG as the secondary antibody. The ER and Golgi apparatus were visualized by staining with BODIPY-Brefeldin A for 30 min just before fixation with PBS containing 4% formaldehyde (16).

In all cases, the cells were mounted in Vectashield (Vector) and observed by fluorescence microscopy (OLYMPUS, Japan).

**GST pull-down assay** – PC12 cells in 10 cm dishes were pre-incubated with methionine-free medium for 60 min, and then 25 μCi of [35S]-methionine was added. After 6 h incubation, the cells were lysed with lysis buffer (Tris-HCl (pH=8.0), 150mM NaCl, 1mM EDTA, 1% Nonidet P40, 1mM PMSF and protease inhibitors) and centrifuged at 15000 rpm for 10min. The collected supernatants were rotated with the C-terminal region of GBP-fused GST- or GST-immobilized beads overnight at 4°C (15). After extensive washing with lysis buffer, the proteins bound to each resin were eluted with 50mM Tris-HCl (pH=8.0) containing 10mM GSH
and separated by 8% SDS-PAGE. After drying, the gels were exposed to a Fuji imaging plate and analyzed with BAS2000. To extract and analyze the proteins bound to GST-fused GBP, SDS-PAGE gels were stained with Sypro Ruby (Molecular Probes) or Coomassie Brilliant Blue (CBB) and the stained regions were digested in trypsin solution at 35°C for 20h for analysis by LC/MS/MS (Aproscience, Japan).

*Western blotting and co-immunoprecipitation* – COS7 cells in 10 cm dishes were transfected with pcDNA3.1/GBP-myc.his (Invitrogen) and resuspended in lysis buffer (20mM Tris-HCl (pH=8.0), 150mM NaCl, 1mM EDTA, 1mM PMSF and protease inhibitors) containing 1% Triton-X100 or Nonidet P40 for 20 min on ice. After centrifugation at 15000 rpm, the detergent-soluble fraction was collected. The detergent-resistant fraction was resuspended and sonicated in an equal amount of lysis buffer. An aliquot of each sample was mixed with an equal amount of SDS sample buffer and boiled for 3 min. Western blotting was performed as described previously using the anti-myc monoclonal antibody 9E10 (1:500) (Santa Cruz).

For co-immunoprecipitation, COS7 cells in 10 cm dishes were transfected with
pcDNA3.1/myc. his. or pcDNA3.1/GBP-myc.his. together with pflag-GRP78. At 24h after the transfection, the cells were dissolved in lysis buffer and debris was discarded after centrifugation. Anti-myc or anti-flag M2 monoclonal antibodies (Sigma) were added to the whole cell lysates and rotated overnight at 4°C. Then 20 μl of protein G-sepharose beads were added and rotated for a further 1 h. After washing the beads four times with 20mM Tris-HCl (pH=8.0) containing 150mM NaCl, 1mM EDTA, 1mM PMSF, 0.2% Nonidet P40 and protease inhibitors, the beads were resuspended in SDS sample buffer, boiled and analyzed by western blotting. Each protein was respectively detected with the anti-myc antibody or anti-flag antibody.

**Measurement of cell viability** - Cell viability was determined by the MTT assay as described previously (17). Briefly, each Neuro2a cell line stably transfected with myc-tagged GBP or empty vector (3×10^4 cells/ well) was cultured in the presence or absence of 10% FBS for the indicated time, and then MTT solution was added to each well and incubated for 4h. The cells were lysed with 0.04M HCl in isopropanol and the difference in absorbance between 595 and 655 nm was measured as an indicator of cell viability.
Results

Molecular cloning of GBP - To identify novel genes in the hypothalamus during postnatal brain development, the mRNA expressions in the forebrains of postnatal day (P) 14 and adult rats at 10 weeks were compared by PCR-selected differential screening since retinal axons grow into the hypothalamus (retinohypothalamic tract) during the postnatal stage, and this reaches a peak at around 2 weeks (18, 19). We screened the partial sequence of a novel gene, which was expressed at a higher level in postnatal brain compared with adult brain. To obtain the full-length cDNA of this gene, we produced 5'-RACE and 3'-RACE PCR products from the rat forebrain RNA, and fused them together. The nucleotides and deduced amino acid sequence of this rat cDNA are shown in Fig. 1. According to a search with BLAST, this sequence was almost 90% identical to a mouse one (the accession number; BC006896). A related sequence in human (the accession number; AK023577) was partial and about 80% identical to rat GBP (nucleotides 1077 to 3066). The cDNA encoded 1021 amino acids with a predicted molecular mass of 110 kDa. A motif search of this protein with PROSITE predicted two transmembrane regions (amino acid residues (aa) 14-40 and 852-868), a proline-rich region (aa 739-780) and a
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glutamic acid-rich region (aa 809-831). In addition, several N-glycosylation, N-myristoylation and phosphorylation sites were deduced (data not shown).

Enrichment of GBP mRNA in the rat brain - Northern blotting and RT-PCR analysis were performed to examine the distribution of GBP mRNA in tissues. As shown in Fig. 2 A, enriched expression of GBP mRNA was observed in the brain compared with other tissues. The expression in the lung was moderate and weak signals were detected in the heart, liver, kidney, spleen, testis and muscle. Within the brain, northern blotting of total RNA isolated from different brain regions showed that GBP was expressed at higher levels in the cerebral cortex, thalamus and cerebellum, and at lower levels in the pons, medulla oblongata and spinal cord (Fig. 2 B).

The temporal changes in the expression of GBP mRNA in the whole brain from the embryonic stage to the adult were examined (Fig. 3). The expression of GBP was faintly
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detected at E12 and gradually increased until P0. After birth, the GBP level was almost
maintained from E20.5 to 2 weeks (W2), and then continuously declined until 12 months (M12).
Densitometric analysis of northern blotting normalized by G3PDH mRNA intensity revealed
that the expression of GBP in the 12 month-old rat brain had decreased by about 70% compared
with that in postnatal brain.

[insert Fig. 3 near here]

To elucidate the expression pattern of GBP mRNA in embryonic, postnatal and adult
rat brain brains, we performed in situ hybridization histochemistry. The expression of GBP
mRNA was observed predominantly in the gray matter and the appearance and distribution of
the GBP-expressing cells suggested that GBP mRNA was expressed in neurons but not in glial
cells. GBP-expressing cells were detected in various regions of the rat forebrain, including the
hypothalamus, and cells strongly expressing GBP were observed in the thalamus, cerebral
cortex, amygdala and cerebellum (Fig. 4). In agreement with the results of the Northern blotting,
the expression of GBP mRNA in aged rat brain was ubiquitously decreased in comparison with
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that in postnatal developing brain.

[insert Fig. 4 near here]

*Immunostaining of COS7 cells transfected with GBP* – Next, we prepared myc- and GFP-tagged GBP constructs, and investigated the expression and intracellular localization in intact cells by immunoblotting and fluorescence microscopy. As shown in Fig. 5, myc-tagged GBP in COS7 cells was almost solubilized by 1% Triton X-100 or Nonidet P40, and bands corresponding to a 170kDa protein were detected by immunoblotting. COS7 cells transfected with myc-tagged GBP or GFP-tagged GBP showed similar localization of GBP (Fig. 5A and C). Furthermore, we confirmed that GFP-tagged GBP in COS7 cells almost overlapped with the fluorescent probe (BODIPY)-labeled Brefeldin A which specifically stains the ER and Golgi apparatus (Fig. 5C). To further characterize the localization of GBP, we constructed three deletion mutants, consisting of the N-terminal region (aa 1-411), the middle region (aa 375-817), and the C-terminal region (aa 666-1021), and established stable cell lines expressing full-length GBP or a mutant GBP. As shown in Fig. 6, only the C-terminal region of GBP among the three
mutants had a unique cellular localization and its pattern was similar to that of full-length GBP.

Association analysis between GBP and GRP78 – To investigate the roles of our cloned gene, we used affinity methods to identify its binding proteins. An affinity matrix was prepared by immobilization of a GST-GBP (aa 606-913) fusion protein on glutathione-sepharose beads, since the roles of this region were somewhat deduced by the observation of the COS7 cells transfected with the GFP-tagged GBP (aa 666-1021) plasmid as described above. A band corresponding to an approximately 45kDa protein was mainly detected by CBB staining although this cDNA encoded 248 amino acids with a predicted molecular mass of about 54 kDa (Fig. 7A). We considered that the GST-fused C-terminal region of GBP in E. coli would be digested during the extraction and immobilization to the GSH-sepharose beads. Incubation of [35-S]methionine-labeled PC12 cell lysates with the immobilized GST-GBP (606-1022)-fusion protein resulted in the binding of several proteins (Fig. 7B). Among these proteins, we identified two proteins with molecular masses of about 70 kDa from non-labeled PC12 cell
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lysates (see Experimental Procedures). LC/MS/MS analysis of the fragments of the lower and upper bands and comparison with the sequences in the GenBank Data Bank revealed almost complete identity to rat Hsc73 and GRP78, respectively.

To investigate the association with Hsc73 or GRP78 in intact cells, COS7 cells were co-transfected with myc-tagged GBP and flag-tagged Hsc73 or GRP78. After 24 h of transfection, the cells were lysed and anti-myc antibody immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membrane, and immunoblotted with an anti-flag antibody. Under these conditions, flag-tagged GRP78 was identified in the anti-myc antibody immunoprecipitates (Fig. 7C). Therefore we named this novel gene, GRP78 binding protein (GBP). On the other hand, interaction with myc-tagged GBP and flag-tagged Hsc73 was very weak (data not shown). No signals were detected in immunoprecipitates without the anti-myc antibody or the lysates from empty vector-transfected cells.

[insert Fig. 7 near here]

*Overexpression of GBP attenuates serum starvation-induced cell death* – In order to
investigate a biological role for GBP, we established four Neuro2a cell lines stably expressing myc-tagged GBP or an empty vector. Two overexpressed cell lines markedly up-regulated the level of GBP mRNA, although Neuro2a cells did endogenously express some GBP (Fig. 8A). The band of myc-tagged GBP in Neuro2a cells corresponded to about 135kDa by immunoblotting, which was different from that in the COS7 cell lysates (Fig. 8B). Overexpression of GBP did not change the features or growth rate of Neuro2a cells. However, overexpression of GBP attenuated the time-dependent reduction in cell viability which was caused by serum deprivation (Fig. 8C). After 48 h of serum starvation, both Neuro2a cell lines overexpressing GBP were more resistant to the cell death induced by serum starvation (Fig. 8D).

[insert Fig. 8 near here]

**Discussion**

In the present study, we isolated and characterized a novel GRP78 binding protein, GBP, in the rat brain. GBP was predicted to have two transmembrane regions, a proline-rich
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region and a glutamic acid-rich region, but the sequence offered few clues to the possible function. In order to identify regions likely to be functionally important, we observed the intracellular localization of full length and deletion mutants of this gene in COS7 cells. We then found that GRP78, one of the heat shock proteins, was a GBP-binding protein by the GST pull down assay method using the identified region of GBP. Our results suggest that the C-terminal region of GBP would have some function in the localization to the ER-Golgi region and interaction with GRP78, but the precise functional domain in GBP remains to be determined. The bands of GBP corresponding to about 135 and 170kDa proteins in Neuro2a and COS7 lysates, respectively, were detected by immunoblotting although GBP encoded 1021 amino acids with a deduced molecular mass of 110 kDa. We consider that some sites in GBP would be individually modified by as yet unknown and different mechanisms in Neuro2a and COS7 cells since a motif search of GBP by PROSITE suggested that GBP contains several N-glycosylation, N-myristoylation and phosphorylation sites.

We demonstrated that GBP mRNA was predominantly expressed in the neurons, but not glial cells, of the rat brain, by northern blotting and in situ hybridization. GBP-expressing cells were widely detected in various regions of the rat brain such as the cortex, thalamus/
hypothalamus, amygdala and cerebellum. Interestingly, in the cerebellum, granule cells but not Purkinje cells expressed GBP mRNA (data not shown). GBP mRNA was already detected in the E12 brain, and it gradually increased to reach a peak within P0 – 2 weeks after birth. These observations indicate that GBP would play a role in the development of the embryonic and postnatal brain and the function of neuronal cells. Furthermore, the age-dependent decrease in GBP expression implies that GBP might affect neuronal susceptibility to various extra- and intra-cellular stresses to cause several neuronal disorders.

GRP78 was originally identified because its expression is significantly increased in glucose-deprived cells. Similar to other heat shock proteins, such as Hsp40, Hsp70 and GRP98, GRP78 has been shown to be induced by a variety of stimuli, including an increase in intracellular calcium, oxidative stress and ER-stress (20-22). GRP78 has been reported to exist as an ER lumen protein, and to act as a molecular chaperon that regulates protein folding and translocation into the ER and protein secretion, and ER disruption occurred when mutated GRP78 was overexpressed (23-26). The localization of GBP in the ER-Golgi apparatus and its association with GRP78 in our experiments is consistent with the intracellular localization of GRP78. In the central nervous system, the levels of GRP78 are markedly induced in response
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to ischemia, axotomy and kainate-induced seizures (27, 28). Downregulation of the GRP78 level by treatment with GRP78 antisense caused sensitivity to a variety insults such as glutamate, Fe\^{2+}, and amyloid β-peptide (29). In particular, GRP78 has been suggested to suppress oxyradical accumulation and to stabilize mitochondrial functions in PC12 cells (29), but the precise mechanisms remain to be determined. Very recently, GRP78 has been reported to associate with ATF6 and caspase-7/12, respectively (22, 30) and suggested to regulate stress-related gene expression and apoptosis. Both ATF6 and caspases are anchored in the ER by binding to GRP78 under resting conditions but some ER-stresses cause cleavage of both proteins and translocation to the Golgi-nucleus and cytosol, respectively. In our preliminary experiments, the amount of flag-tagged GRP78 co-immunoprecipitated with myc-tagged GBP was hardly affected by treatment with thapsigargin, which causes ER-stress by inhibiting ER Ca\^{2+}-ATPase, suggesting that the association of GRP78 and GBP is constitutive. On the other hand, the expression of GBP mRNA in neuronal cell lines was hardly modulated by several stimuli which cause growth arrest and/or apoptosis (data not shown). The significance of GBP in vivo under pathological conditions remains to be determined although GBP might affect ER-Golgi-localized proteins, such as ATF-6 and caspase-7/12, by forming a complex with GRP78.
Furthermore, we found that stable overexpression of GBP in Neuro2a cells suppressed serum deprivation-induced cell death, which is consistent with the anti-apoptotic feature of GRP78. Serum deprivation would disturb a variety of cellular signaling pathways, such as MAP kinases and p53 cascades, to induce apoptosis. Since ATF-6 and caspase-12 are reported to be predominantly activated by ER-stress reagents, we consider that GBP would attenuate other serum deprivation-induced cascades among several death signaling pathways in Neuro2a cells.

In conclusion, we cloned a novel GRP78 binding protein (GBP), which is predominantly expressed in the rat brain, and possesses an anti-apoptotic property although it is not clear how overexpression of GBP in Neuro2a cells maintained cell viability after serum deprivation. Apoptotic cell death is accompanied by proliferation and differentiation of neurons and the formation of synapses at embryonic and postnatal stages. Neuronal dysfunction during aging and neuronal diseases is also associated with apoptotic cell death. Thus, further studies on the functions of GBP, including GRP78, would provide new insights to clarify not only the development and differentiation of neuronal cells at the embryonic and postnatal stages but also neurodegenerative disorders.
Acknowledgements- This study was supported by a grant from the Ministry of Education, Sports, and Culture in Japan.

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Legends for figures

Fig. 1. Nucleotides and predicted amino acid sequence of rat GBP. The nucleotides and predicted amino acid sequence of GBP cDNA are shown. Putative transmembrane (squares), proline-rich (underline) and glutamic acid-rich (double underline) regions are indicated.

Fig. 2. Tissue distribution of GBP mRNA by northern blotting. Total RNAs isolated from different rat tissues (A) (lane 1, whole brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, kidney; lane 6, spleen; lane 7, testis; lane 8, muscle), and different brain regions (B) (lane 1, olfactory; lane 2, cerebral cortex; lane 3, hippocampus; lane 4, thalamus; lane 5, hypothalamus; lane 6, midbrain; lane 7, cerebellum; lane 8, pons and medulla oblongata; lane 9, spinal cord) were hybridized with a radiolabeled 277bp fragment of GBP as described in the Experimental Procedures. The same membrane was rehybridized with a radiolabeled fragment of G3PDH.

Fig. 3. GBP mRNA levels during rat brain development. The expression levels of GBP mRNA in the rat brain at the indicated different embryonic (A) and postnatal (B) stages were
respectively analyzed by RT-PCR and Northern blotting as described in the Experimental Procedures. (C) The relative mRNA level of GBP in Fig. 3B was calculated by comparison of G3PDH-normalized values with the level of E20.5.

Fig. 4. Localization of GBP mRNA in the rat brain by in situ hybridization. GBP mRNA localization was detected by in situ hybridization with a DIG-labeled antisense RNA probe derived from the coding region of GBP. At 2 weeks, a number of GBP mRNA-expressing cells were widely distributed in the rat hypothalamus (a), thalamus (c) and cerebral cortex (e), and their expressions were stronger than those in adulthood (hypothalamus (b), thalamus (d) and cerebral cortex (f)). Fig. 4-g shows a higher magnification of the rectangle in (e). Control hybridization experiments were performed using a sense RNA probe (h). The scale bars represent 200 µm. OC, optic chiasm; 3V, third ventricle; LV, lateral ventricle.

Fig. 5. Cellular localization of GBP in COS7 cells. (A) COS7 cells were transiently transfected with myc-tagged GBP. 24 h later, the cells were fixed and incubated with an anti-myc monoclonal antibody. The cells were then incubated with an FITC-conjugated anti-mouse IgG
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and analyzed by fluorescence microscopy. (B) 24 h after transfection, COS7 cells were lysed with 1% Triton X-100 or Nonidet P40 (NP-40). After centrifugation, the supernatant (sup), pellet (p) and total homogenates (H) were immunoblotted as described in the Experimental Procedures. (C) 24 h after transfection with GFP-tagged GBP or GFP vectors, the cells were incubated with BODIPY-Brefeldin A for 30 min. The cells were fixed and analyzed by fluorescence microscopy.

Fig. 6. COS7 cells stably expressing GFP-tagged full-length, deletion mutants and empty vectors were analyzed by fluorescence microscopy. Each GFP-GBP fusion protein corresponding to the indicated residues (A) was stably expressed in COS7 cells and observed by fluorescence microscopy (B).

Fig. 7. Analysis of the association between GBP and GRP78. (A) The GST-fused C-terminal region of GBP expressed in E. coli was extracted by GSH-conjugated sepharose beads, resolved by SDS-PAGE and stained with CBB. (B) The GST-fused C-terminal region of GBP or GST immobilized beads was incubated with [35-S]methionine-labeled PC12 lysates as described in
the Experimental Procedures. After washing and elution, the proteins bound to each resin were resolved by SDS-PAGE and visualized with BAS2000. (C) COS7 cells were co-transfected with different combinations of expression constructs as indicated. Co-immunoprecipitation was performed using the anti-myc monoclonal antibody as described in the Experimental Procedures. Immunoprecipitates were analyzed using the anti-myc or anti-flag antibodies, respectively.

Fig. 8. Overexpression of GBP attenuates serum starvation-induced cell death in Neuro2a cells.

(A) Neuro2a cells stably expressing myc-tagged GBP (GBP-1 and –2) or empty vector (Con-1 and –2) were established by selection with G418 and the level of myc-tagged GBP (myc-GBP) and total GBP (GBP) mRNA in each clone was analyzed by RT-PCR. (B) The expression of myc-tagged GBP in Neuro2a cells was detected by immunoblotting. (C) Neuro2a cells stably expressing myc-tagged GBP (triangles) or empty vector (circles) were cultured with (filled symbols) or without 10% serum (open symbols) for the indicated times, and then the cell viability was measured using the MTT assay as described in the Experimental Procedures. Each value represents the percentage of the control value at day 0 (D0). (D) 2 days after serum starvation, the cell viability of each clone was measured by the MTT assay. Each value
represents the mean ± SD of four independent cultures, and is expressed as the percentage of
the cell viability of each clone cultured in the presence of serum for 2 days. *, The difference
from cells expressing the empty vectors (Con-1 and –2) was statistically significant by ANOVA
(p<0.01, Fisher PLSD).
**A**

GBP

G3PDH

**B**

GBP

G3PDH
A

myc-GBP

B

kDa
250
160
105
50

H sup p H sup p
(1% TritonX-100) (1% NP-40)

C

GFP-GBP  BODIPY-Bref erdin A  merged
A

Site residues

a  -
b  1 - 1018     GFP
c  1 - 411     GFP
d  375 - 817     GFP
e  666 - 1021     GFP

B

a)     b)

c)     d)     e)
A  

B  

C  

IP: anti-myc Ab  
IB: anti-myc Ab

myc-GBP  
-  +  +

anti-myc Ab  
-  +  +

IP: anti-flag Ab  
IB: anti-flag Ab

myc-GBP  
-  +
Cloning and characterization of a novel GRP78 binding protein in the rat brain
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J. Biol. Chem. published online January 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212083200

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