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Ganglioside GT1b in Rat Brain Binds to p58, a Brain-Specific Sodium-Dependent Inorganic Phosphate Cotransporter: Expression Cloning with a Specific Monoclonal Antibody to Ganglioside GT1b-Binding Protein

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To evidence the notion that gangliosides involve neuronal cell interactions in the brain, we surveyed the presence of ganglioside-binding proteins in membrane lysates of adult rat cerebellum. Three proteins (p58, p90, and p160) were identified as GT1b-binding proteins by incubation of the blot of the membrane lysate with GT1b micelles. We generated a monoclonal antibody (mAb) specific to the polypeptide portion of the GT1b-binding proteins (YAK-2). The YAK-2 mAb specifically reacted with all three proteins on blots of proteins pretreated under nonreducing conditions for SDS-PAGE, but reacted mainly with p58 under reducing conditions, showing that p90 and p160 are oligomeric forms of p58. The binding activity of the YAK-2 mAb was completely inhibited by the presence of GT1b micelles, indicating the specificity of YAK-2 mAb for p58 and its oligomers. Immunohistochemical investigations revealed that both p58 and GT1b colocalize within the granular layer of adult rat cerebellum. Expression cloning of p58 cDNA was performed using YAK-2 mAb, and five putative clones were obtained. Among them, the nucleotide sequence of one cDNA completely matched that of rat brain-specific sodium-dependent inorganic phosphate cotransporter (rBNPI), a 61 kDa membrane protein. COS7 cells were transfected with a Flag-chimeric construct containing the rBNPI/p58 cDNA, and the membrane lysate was subjected to immunoprecipitation with anti-Flag antibody. One protein (64 kDa) was detected only with YAK-2 mAb, and the membrane lysate specifically bound to GT1b micelles. Taking together, we propose that rBNPI/p58 functions as a GT1b-binding protein in neuronal cells.

Key words: ganglioside, GT1b, monoclonal antibody, rat brain, rBNPI.

Construction of the neuronal network is widely accepted as one of prerequisites for the exertion of normal brain functions. Construction occurs in the embryonic brain during morphogenetic processes in a spatiotemporally regulated manner, and comprises many biological events including neuronal cell division, migration, neurite extension, fasciculation, target recognition, synaptogenesis, and myelination. Most events are reported to be regulated by neuronal cell adhesion molecules on the cell surface (*1–5*). The adhesion molecules participating in neuro-neuronal and neuro-glial cell interactions can be classified as follows: *N*-cadherin, a member of the cadherin superfamily, that is specifically located at interneuronal synapses, and which not only functions as an adhesion molecule of the synaptic junction, but also modifies synaptic plasticity (*6–10*); NCAM, a member of the immunoglobulin superfamily (*11*), is located on neurons and concerned with neuronal migration and fasciculation (*12*); and myelin associated glycoprotein (MAG) (*13*), which belongs to the sialoadhesin superfamily and is involved in myelination (*14, 15*). Recent molecular biological approaches have provided some insight into the molecular structures and mechanisms involved in establishing functional cell junctions by means of neural cell adhesion molecules, and prompted us to speculate on the presence of other cell recognition systems during neuronal cell adhesion, considering the complexity and dynamic nature of brain morphogenesis.

Gangliosides, sialic acid–containing glycosphingolipids, are plasma membrane constituents that are widely distributed among vertebrates, and especially abundant in verte-

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Abbreviations: CBB, Coomassie Brilliant Blue; DIG, digoxigenin; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MAG, myelin associated glycoprotein; PBS, phosphate-buffered saline; rBNPI, rat brain-specific sodium-dependent inorganic phosphate cotransporter. Gangliosides are named according to the nomenclature of Svennerholm (*51*). Otherwise, the nomenclature used follows the IUPAC-IUB recommendation (*52*).

brate brain (*16*). They are thought to be involved in a variety of biological functions including cell–cell interactions, cell differentiation, and cell growth (*17*). Carbohydrate antigens also play functional roles in cell–cell interactions as ligands of cell adhesion molecules with lectin recognition domains, such as selectin (*18–22*) and the sialoadhesin super family (*23*). Recently, in brain, MAG was demonstrated to bind to gangliosides having 2,3-linked sialic acid on a Gal(β1-3)GalNAc core structure such as GD1a, GT1b, and GQ1b (*24*). Among these glycoconjugates, we used immunohistochemical techniques to determine that gangliosides are restricted to neuronal cells and layers in the rat central nervous system. Especially, b-series gangliosides such as GD1b, GT1b, and GQ1b are exclusively located on synapse-related regions in adult rat brain (*25, 26*), and their expression during the development of rat cerebellum converges into the synapse-related regions (*27*), suggesting that gangliosides are involved in some stage-specific biological functions in cerebellum morphogenesis. The results raise the possibility that these gangliosides play some essential roles as ligands for neuronal cell interactions.

In the present study, based on the binding of ganglioside micelles to blots of rat cerebellum membrane lysates, we found the presence of a GT1b-binding protein, p58, and two of its putative oligomeric forms. By expression cloning using a monoclonal antibody specific to p58, the GT1b-binding protein was identified to be a rat brain-specific sodium-dependent inorganic phosphate cotransporter (rBNPI), which has already been cloned by Ni *et al.* (*28*).

EXPERIMENTAL PROCEDURES

Animals—Female Wistar rats were purchased from Japan Clea (Higashimurayama). Female BALB/c mice, 6 to 7-wk-old, were obtained from Shizuoka Laboratory Animal Center (Hamamatsu).

Cells and Cell Culture—Mouse myeloma cells (PAI) were cultured in RPMI 1640 supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 μM 2-ME, 1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. COS7 cells were cultured in DMEM containing 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS.

Gangliosides and Anti-Ganglioside Monoclonal Antibodies (mAbs)—Bovine GM1, GD1a, GD1b, GT1b, and GQ1b were purchased from Sigma (St. Louis, MO). Ganglioside-specific mAbs used were GMB16 (IgM), GMR17 (IgM), GGR12 (IgG2b), GMR5 (IgM), and GMR13 (IgM) specific for GM1, GD1a, GD1b, GT1b, and GQ1b, respectively (*29, 30*).

Preparation of Membrane Fractions and Lysates—Adult rat cerebellum was homogenized in 10 volumes of 50 mM Tris-HCl (pH 7.4) containing 1mM phenylmethylsulfonyl fluoride, 1mM EDTA, 10 μg/ml aprotinin, and 1mM iodoacetoamide. The homogenates were centrifuged at 12,000 ×*g* for 20 min at 4°C, and the precipitates were resuspended in homogenizing buffer and centrifuged again under the same conditions. The resulting precipitates were used as cerebellum cell membrane lysate. For the preparation of membranes from cultured cells, cells were homogenated in 10 volumes of homogenization buffer supplemented with 0.15 M NaCl, and centrifuged at 600 ×*g* for 10 min at 4°C. The supernatants were further centrifuged at 12,000 ×*g* for 20 min at 4°C, and the precipitates were used as the cell membrane lysate from cultured cells.

Membrane lysates were prepared by lysing the membranes with 0.15 M NaCl, 50 mM Tris-HCl (pH 7.4) containing 1% NP-40 on ice for 20 min. Protein concentration was estimated with bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL).

Generation of mAb—Female BALB/c mice (6–7-wk-old) were immunized three times in the footpad at 5 to 7 d intervals with 50 μl immunogen/footpad prepared by mixing the same volume of cerebellum cell membrane lysate (100 μg protein/ml) and a complete adjuvant Titer Max (CytRx, Norcross, GA), followed by a booster injection without adjuvant. Three days after the final booster immunization, poplitial lymph node cells were fused with PAI myeloma cells using polyethylene glycol 4000 (Merck, Darmstadt, Germany) as described previously (*31*). Hybridoma cells were screened as described below. The mAb isotypes were determined with a mouse mAb isotyping kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Western Blotting—The cerebellum cell membrane lysate (20 μg protein/lane) processed under nonreducing conditions without boiling was separated by SDS-PAGE (*32*) in a 10% polyacrylamide gel and electroblotted onto PVDF membranes (Immobilon; Nippon Millipore, Tokyo) according to Towbin *et al.* (*33*). The blotted membrane blocked with 1% skim milk in PBS for 1 h was incubated with hybridoma culture supernatant for 1 h, and then with peroxidase-conjugated goat anti–mouse Ig (Amersham Pharmacia Biotech; 2,000-fold diluted) for 1 h. Bands were detected by a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech) according to the manufacturer's protocol.

For sodium metaperiodate oxidation of membrane constituents, the blots of the cerebellum cell membrane lysates were treated with 25 mM $NaIO_4$ in PBS for 30 min at room temperature in the dark, and probed with hybridoma culture supernatant as described above.

Ganglioside Micelle Binding Assay—The cerebellum cell membrane lysate was processed for electroblotting as described above. The blotted membrane blocked with blocking medium was incubated for 45 min at room temperature with 10^{-5} M ganglioside micelles, which were prepared by ultrasonication of dried gangliosides in PBS at 150 W for 45 s. The PVDF membrane was incubated first with the corresponding ganglioside-specific mAb for 1 h, then with peroxidase conjugated goat anti–mouse IgM or IgG $F(ab')_2$ (Pierce; 2,000-fold diluted), and subjected to ECL detection.

Inhibition of mAb Binding to Membrane Lysates by Ganglioside Micelles—Aliquots of rat cerebellum membrane lysate (25 μg/well) were added to 96-well microtiter plates and incubated overnight at 4°C. After washing with 1% BSA in PBS, 100 μl of a solution containing equal volumes of ganglioside micelles and mAb at various concentrations was placed in each well and the mixtures were incubated for 45 min. The plates were incubated with peroxidase-conjugated goat anti–mouse IgM $F(ab')_2$ (2,000-fold diluted), and color developed for peroxidase as described previously (*34*).

Immunohistochemical and Immunocytochemical Analyses—Frozen sections of adult rat cerebellum (7 μm thickness) were mounted on glass slides, dried at room tem-

perature for 4 h, and fixed in acetone for 5 min at 4°C. The sections were blocked with 1% BSA in PBS and incubated with GMR5 mAb for 1 h. After washing, the sections were incubated with FITC-conjugated goat anti–mouse IgM $F(ab')_2$ (CAPPEL, West Chester, PA; 100-fold diluted) for 45 min at room temperature. Then, the stained sections were incubated with YAK-2 mAb for 1 h, and then with rhodamine-conjugated anti–mouse IgM $F(ab')_2$ (TAGO, Buringame, CA; 100-fold diluted). The tissue sections were examined under a microscope (DMRE; Leica, Heidelberg, Germany) equipped with a confocal laser scanning system (TCS-SP; Leica).

The COS7 transfectants on Lab-Tek chamber slides (Nunc, Nupervill, IL) were treated with or without 4% paraformaldehyde in PBS for 5 min, and blocked with 5% BSA in PBS for 30 min. The samples were incubated with an anti-Flag mAb (anti-FLAG M2 mAb; Eastman Chemical, Rochester, NY) or with YAK-2 mAb for 1 h, and then with FITC-conjugated goat anti–mouse IgG or IgM $F(ab')_2$ (diluted at 100-fold) for 1 h. The stained cells were examined under a light microscope (Axiophot; Zeiss, Oberkochen, Jena, Germany) equipped with an epifluorescence apparatus.

Screening and PCR—A rat brain λgt11 cDNA library (Clontech, Palo Alto, CA) was used for cDNA screening. The cDNA library containing 50,000 recombinants was plated with Y1090 *E. coli* on 90 mm dishes. The plates were covered with Hybond-C membranes (Amersham Pharmacia Biotech) presoaked in 10 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The membranes were blocked with 1% skim milk in PBS for 1 h, incubated for 1 h with YAK-2 mAb, and then with peroxidase-conjugated goat anti–mouse IgM $F(ab')_2$ (Pierce) for 1 h. The detection was by ECL as described above. Five putative clones were identified from 5×10^5 plaques. The phage DNAs were isolated and digested with *Eco*RI to check the insert size (about 1.1–1.8 kbp length).

Bacterially expressed proteins of the selected clones were expressed in Y1089 *E. coli* in NZCYM medium containing 50 μg ampicillin/ml. During the mid-log growth phase, the recombinant proteins were induced with 10 mM IPTG for 3 h. The recombinant proteins from each clone were subjected to SDS-PAGE and electroblotting to measure ganglioside micelle binding.

The cDNA clone fragments were subcloned into the *Eco*RI site of Bluescript II SK^- and sequenced by Sanger's dideoxy chain termination method using Thermo Sequenase fluorescent labeled primer cycle sequencing with 7-deaza-dGTP (Amersham Pharmacia Biotech).

Northern Blot Analysis—cDNA subcloned into Bluescript II SK^- was linearized with *Kpn*I and used as a template for the synthesis of a DIG-labeled riboprobe. A DIG-labeled antisense riboprobe (877 base) was synthesized with T3 RNA polymerase using a DIG RNA labeling kit (Roche Diagnostics; Mannheim, Germany) according to the manufacturer's protocol. Hybridization was carried out on a Multiple Tissue Northern Blot membrane (Clontech) according to Iwatsuki *et al.* (*35*). In brief, the membrane was hybridized with the DIG-labeled antisense riboprobe in 5×SSC containing 50% formamide, 1.5% SDS, and 1% blocking reagent (Roche Diagnostics), washed twice with 2×SSC–0.1% SDS at room temperature for 5 min, and then washed twice with 0.1×SSC–0.1% SDS at 68°C for 1 h. The membrane was blocked with blocking buffer at room temperature for 1 h, after which anti-DIG antibody was added (Roche Diagnostics). The membrane was subjected to chemiluminescent detection with CDP-star (TROPIX, Bedford, MA).

Transient Expression—A pFlag-CMV5a mammalian expression vector (Eastman Kodak, New Haven, CT), which has a downstream Flag amino acid sequence, was used. A fragment including the open reading frame of rBNPI (designated #2-1*; 1,717 bp) was prepared by PCR with KOD polymerase (Toyobo, Tokyo) and sense primer (GGAATTCGGCACAGCCACAGCCACCATGGAGTT) and antisense primer (ATAGTTTAGCGGCCGCACGTGGTGAGTAGTCCCGGACA). C-Flag-CMV5a-2-1* was generated by ligation of the PCR product into the *Eco*RI (5′ site) and *Not*I (3′ site) sites of the pFlag-CMV5a vector.

COS7 cells were plated on a 90 mm plastic dish (Becton Dickinson, Franklin Lakes, NJ). After incubation overnight, the cells were transfected with 5 μg of C-Flag-CMV5a-2-1* or pFlag-CMV5a (mock) using Lipofectamine (Gibco BRL, Rockville, MD). After transfection, the cells were cultured in DMEM for 2 d, and processed for immunoprecipitation and immunocytochemistry.

Immunoprecipitation—One hundred microliters of rabbit anti-FLAG antibody (ZYMET, San Francisco, CA; 10 μg/ml) and 20 μl of Protein G-Sepharose (Amersham Pharmacia Biotech) were incubated for 4 h at 4°C. The preparation was washed with PBS, 250 μl of membrane lysate from the transfected cells was added, and the mixture was incubated at 4°C overnight with gentle mixing. The samples were washed with lysis buffer, and then 30 μl Laemmli sample buffer (without 2-ME) was added to elute the bound antigen. The eluates were subjected to Western blotting with mAbs against anti-Flag and YAK-2 as described above.

RESULTS

Presence of GT1b-Binding Proteins in Adult Rat Brain—In order to survey the presence of ganglioside-binding proteins in rat cerebellum, we examined ganglioside micelle binding to blots of the membrane lysate. When the blots were probed with GT1b micelles, the micelles bound strongly to three molecules, p58, p90, and p160 (Fig. 1). The micelle binding was not affected by the presence or absence of 25 mM EDTA (not shown). Although the GT1b micelles apparently bound additional molecules (44 and 75 kDa proteins), the appearance of the bands was not reproducible in repeated experiments. A 54 kDa protein also showed nonspecific binding to GT1b micelles, as evidenced by the band detectable without GT1b micelles (Fig. 1). When GD1a and GD1b micelles were used, the labeled proteins were detected as a smear at extremely high molecular masses (Fig. 1). In the case of incubation with GM1 and GQ1b micelles, no proteins with binding affinities to the micelles were found (Fig. 1). These results indicate that rat cerebellum expresses some proteins that bind to GT1b as analyzed by the ganglioside micelle binding assay; we focused on the GT1b-binding proteins p58, p90, and p160 for further analysis.

Generation and Characterization of mAb Specific for GT1b-Binding Proteins—To characterize GT1b-binding proteins, we generated mAbs specific for them by immunizing mice with the rat cerebellum membrane lysate, and

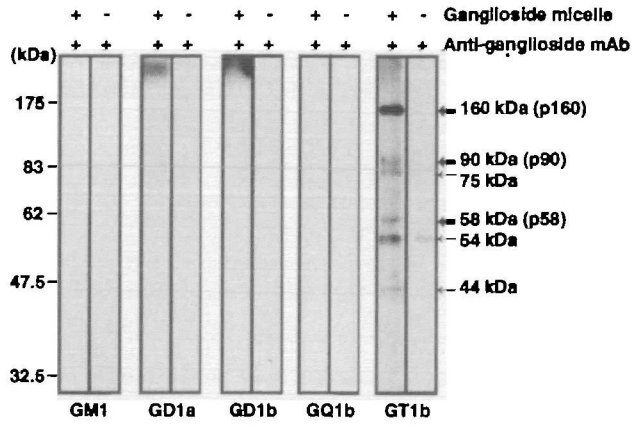

Fig. 1. **Presence of ganglioside-binding proteins in cerebellum cell membrane lysate as evidenced by the ganglioside micelle binding assay.** A cerebellum cell membrane lysate (20 μg/lane) pretreated under nonreducing conditions was separated by SDS-PAGE and transferred to PVDF membranes. The blots were incubated with (+) or without (–) the indicated ganglioside micelles and bands were detected with the corresponding anti-ganglioside mAbs. The thick and thin arrows indicate GT1b-binding proteins (p58, p90, and p160) and non-specific bands (44, 54, and 75 kDa), respectively.

obtained one hybridoma clone producing mAb, designated YAK-2 mAb (IgM). YAK-2 mAb reacted with three molecules, 58, 90, and 160 kDa, under nonreducing conditions (Fig. 2A). The recognition epitope of the YAK-2 mAb was confirmed to be within the protein portion of the molecules, because the reactivity of the YAK-2 mAb was not significantly affected by treatment of the blot with sodium metaperiodate (Fig. 2B). When the membrane lysate was heated in the presence of 2-ME prior to electrophoresis, p160 completely disappeared, and most of p90 also disappeared (Fig. 2C). Consistent with the disappearance and decrease in the molecules, the intensity of p58 was apparently strengthened by 2-ME treatment (Fig. 2C), suggesting that p90 and p160 are oligomeric forms of p58.

Next, in order to confirm that the molecules detected by the YAK-2 mAb are identical to the proteins that react with GT1b micelles, we assessed the inhibitory effect of ganglioside micelles on the binding of YAK-2 mAb to the membrane lysate. Among them, only GT1b micelles inhibited the binding of YAK-2 mAb to the membrane lysate (Fig. 3). These results clearly indicate that the YAK-2 mAb reacts specifically with the GT1b-binding protein p58, as well as with its putative oligomers.

Distribution of GT1b-Binding Protein—The distribution of the GT1b-binding protein was examined by Western blotting of membrane lysates from various organs with YAK-2 mAb. The protein was expressed strongly in cerebellum but weakly in cerebrum (Fig. 4A). The bands detected in heart and testis showed no specific binding to GT1b micelles (not shown), indicating that the GT1b-binding protein is expressed exclusively in brain.

The distribution of the GT1b-binding protein in the cerebellum was further examined by double-immunofluorescence staining with mAbs to YAK-2 and GMR5 (anti-GT1b mAb). YAK-2 mAb stained neuronal regions such as the molecular layer, Purkinje cell layer, and granular layer in adult rat cerebellum, but did not stain the white matter (Fig. 4B). GMR5 mAb stained all layers except the Purkinje cell layer (Fig. 4B; see also Ref. *26*). The results clearly indicate that the GT1b-binding protein and GT1b colocalize within the granular layer (Fig. 4B). The findings also indicate that the distribution of the GT1b-binding protein is neuronal cell-specific, as in the case of GT1b.

Molecular Cloning of a cDNA Encoding the GT1b-Binding Protein, p58, by YAK-2 mAb—To further characterize the neuronal cell–specific GT1b-binding protein, we performed expression cloning using YAK-2 mAb. Five putative clones (#1-4, #2-1, #3-3, #3-5, and #3-8) were obtained, of which the nucleotide sequence of clone #2-1, with a length

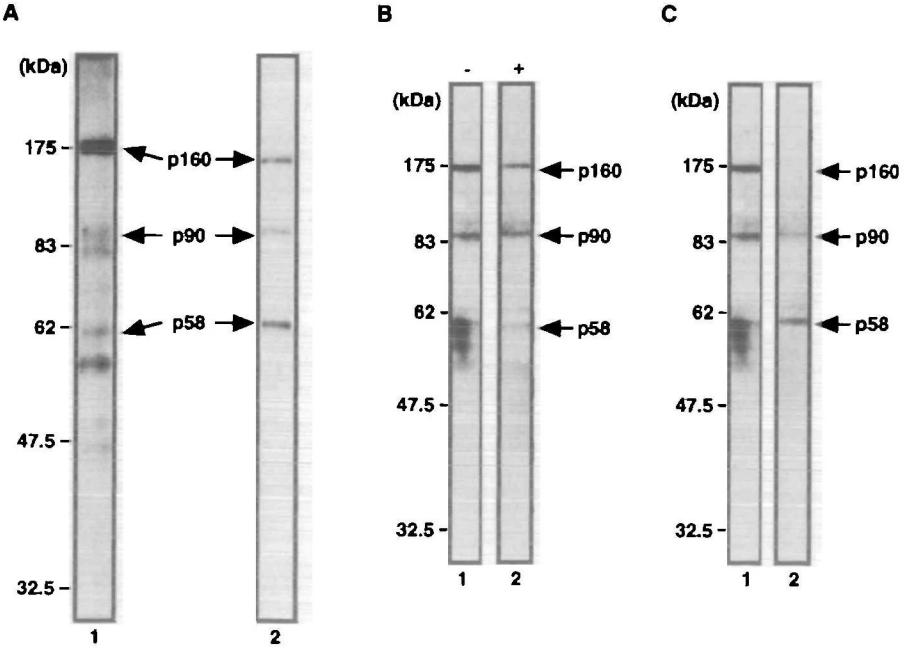

Fig. 2. **Western blot analysis of the molecules identified by the YAK-2 mAb.** (A) The cerebellum cell membrane lysate blot was subjected to binding assay with GT1b micelles (lane 1) or stained with YAK-2 mAb (lane 2). The mAb reacted specifically with three molecules (p58, p90, and p160). Arrows indicate the common bands (p58, p90, and p160) detected by both probes. (B) The cerebellum cell membrane lysate blot was subjected to SDS-PAGE following pretreatment of proteins with (+, lane 2) or without (–, lane 1) $NaIO_4$. The staining was not altered by $NaIO_4$ oxidation. (C) The cerebellum cell membrane lysate was subjected to SDS-PAGE under nonreducing (lane 1) or reducing (lane 2) conditions, and then stained with YAK-2 mAb. When the membrane lysate was treated under reducing conditions, two upper bands (p90 and p160) shifted to the lower position, p58.

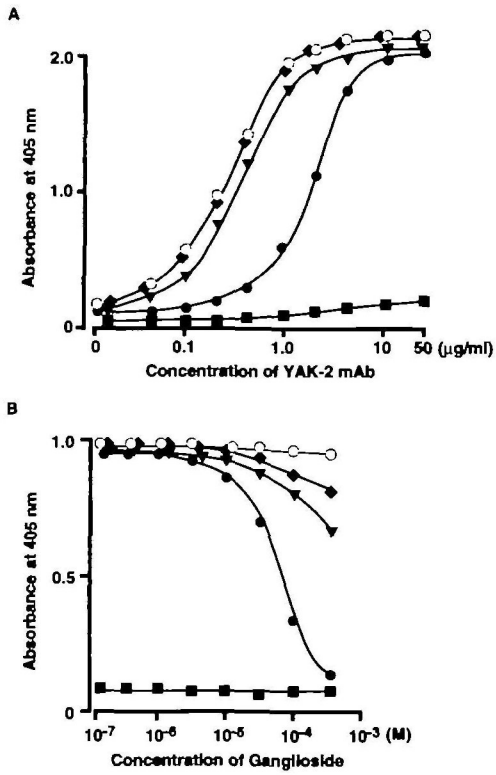

Fig. 3. **Inhibition of YAK-2 mAb binding to membrane lysate by ganglioside micelles.** *aliquots of cerebellum cell membrane* lysate (25 μg protein/well) on a microtiter plate were incubated with mixtures containing the same volume of ganglioside micelles and various concentrations of YAK-2 mAb (A), or YAK-2 mAb (5 μg/ml) and various concentrations of ganglioside micelles (B). The bound YAK-2 mAb was quantified by ELISA. YAK-2 mAb reacted with the membrane lysate in a dose-dependent manner (open circles). Only GT1b micelles (closed circles) inhibited binding to the membrane lysate in a dose-dependent manner. Other gangliosides, including GM1 and GD1b (closed diamonds) or GD1a and GQ1b (closed triangles), showed no inhibitory effect on binding. No binding activity was observed when mouse IgM was used instead of YAK-2 mAb (closed squares).

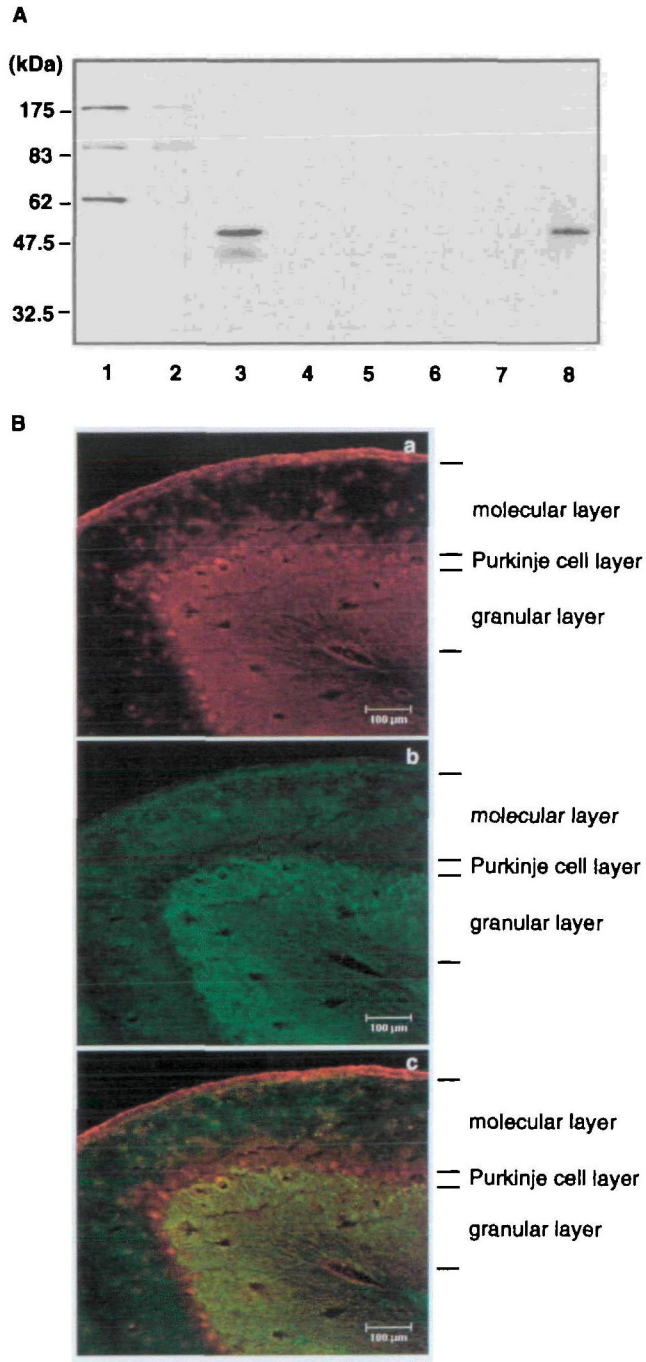

Fig. 4. **Localization of GT1b-binding protein in various organs of adult rat.** (A) Membrane lysates (20 μg/lane) were analyzed by Western blotting using YAK-2 mAb. The three bands, p58, p90, and p160, were detected only in brain (lane 1, cerebellum; lane 2, cerebrum), and not in lung (lane 4), liver (lane 5), spleen (lane 6), or kidney (lane 7). The bands detected in heart (lane 3) and testis (lane 8) were nonspecific binding. (B) A frozen section prepared from adult rat cerebellum was double-stained with mAbs to YAK-2 and GMR5 (anti-GT1b mAb). The YAK-2 mAb (a; G-excitation) stained neuronal regions, such as the Purkinje cell and granular layers, specifically. The GMR5 mAb (b; B-excitation) against GT1b stained the molecular and granular layers, but not the Purkinje cell layer (*see also Ref. 26*). GT1b-binding protein and GT1b were colocalized in granular cell layer (orange colored region; c). Bar, 100 μm.

of 1,737 bp, completely matched that of the rBNPI cDNA cloned by Ni *et al.* (*28*). However, clone #2-1 showed 10 and 277 bp deletions at the 5′ and 3′ termini, respectively, of the rBNPI cDNA. In addition, when the transcript of #2-1 was analyzed by Northern hybridization, it was detected only in brain with a length of 2.8 kb (not shown), which is consistent with the data for the rBNPI transcript (*28*). The sequences of two clones, #1-4 (1,288 bp) and #3-8 (1,756 bp), also completely matched with cDNA sequences of rat calmodulin (*36*) and rat ADP-ribosylation factor type 1 (*37*), respectively. The other two clones, #3-3 (1,449 bp) and #3-5 (1,128 bp), were not found to have any homologous sequence with sequence data bases using the BLAST program.

To ascertain whether proteins encoded by the selected cDNA clones are able to bind GT1b micelles, bacterially expressed proteins (as β-galactosidase-fusion proteins) of the five putative clones (prepared by a Y1089 *E. coli* expression system) were subjected to Western blotting with YAK-2 mAb and to micelle binding (Fig. 5). The recombi-

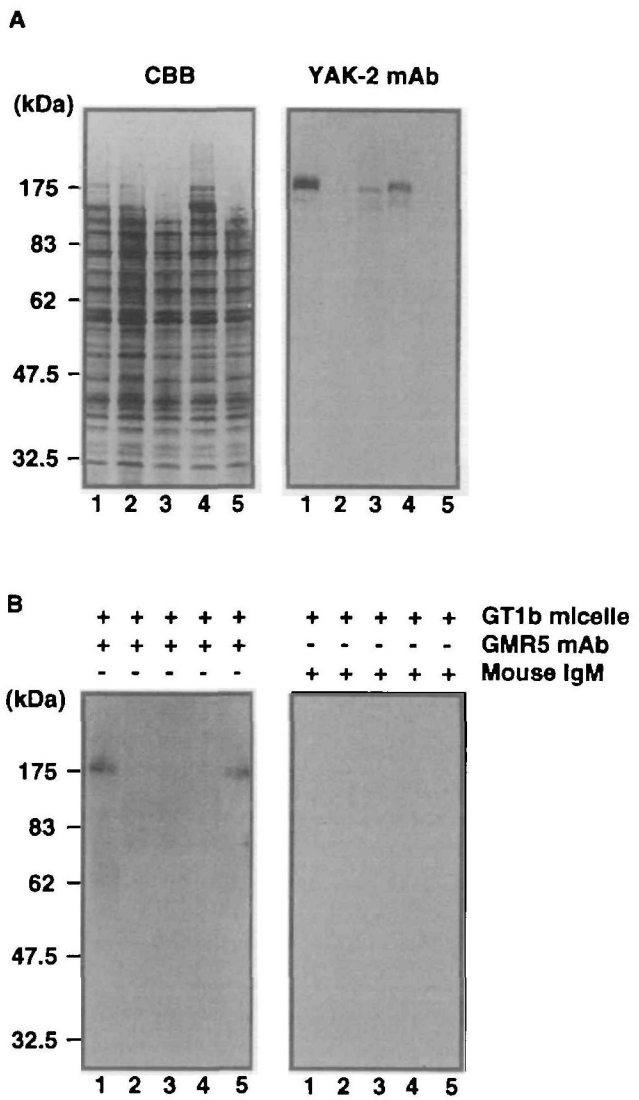

Fig. 5. **Western blot and micelle binding analyses of bacterially expressed rBNPI/p58.** The recombinant proteins (10 μg/lane) pretreated under nonreducing conditions were separated by SDS-PAGE and electroblotted onto PVDF membranes. (A) The blots were stained with Coomassie Brilliant Blue (CBB) or YAK-2 mAb. A 180 kDa band (fusion protein with β-galactosidase) was detected in clones #2-1 (lane 1), #3-3 (lane 3), and #3-5 (lane 4). (B) When the blots were probed with GT1b micelles, the band at 180 kDa was detected in clones #2-1 (lane 1) and #1-4 (lane 5).

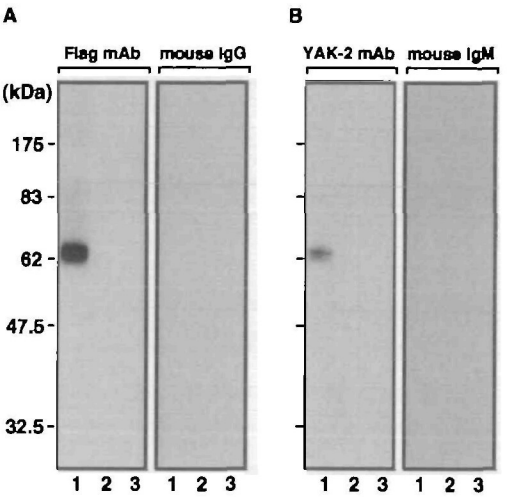

Fig. 6. **Immunoprecipitation of rBNPI/p58 transiently expressed in COS7 cells.** The membrane lysates from COS7 cells transfected with rBNPI/p58 cDNA (lane 1), COS7 cells transfected with vector only (lane 2), or parental COS7 cells (lane 3) were immunoprecipitated with rabbit anti-Flag antibody. The precipitates were analyzed with anti-Flag (A) and YAK-2 (B) mAbs. Only the lysate from COS7 cells transfected with rBNPI/p58 cDNA (lane 1) was detected as one band at 64 kDa (a fusion protein with Flag) with both probes.

nant protein of #2-1 was not only detected with the YAK-2 mAb, but also with GT1b micelles (Fig. 5). The recombinant protein of #1-4 was only detected by GT1b micelle binding, while those of #3-3 and #3-5 were only detectable with YAK-2 mAb (Fig. 5). Both probes failed to detect any recombinant protein of #3-8 (Fig. 5). Based on detection of the recombinant proteins with the YAK-2 mAb and GT1b micelles, it appears possible that the rBNPI encoded by clone #2-1 is a GT1b-binding protein.

To obtain direct evidence that the rBNPI encoded by #2-1* (including the open reading frame of rBNPI; see "EXPERIMENTAL PROCEDURES") is p58, we performed the transient expression of rBNPI as a Flag-fusion protein in COS7 cells. As shown in Fig. 6, when membrane lysate from cells transfected with C-Flag-CMV5a-2-1* was immunoprecipitated, anti-Flag mAb detected a band at 64 kDa, and this band was also detected by YAK-2 mAb. The molecular mass of 64 kDa agrees well with that expected for a 588 amino acid Flag-fusion rBNPI. Consistently, the membrane lysate from COS7 cells transfected with vector alone gave no immunoprecipitated band (Fig. 6). These results clearly indicate that rBNPI and p58 are the same molecule.

If p58 is rBNPI, then COS7 cells transfected with C-Flag-CMV5a-2-1* should express p58 on the cell surface. This was examined by indirect immunofluorescence microscopy. As shown in Fig. 7, when transfected COS7 cells were fixed with 4% paraformaldehyde and then immunostained, the cells were stained by both the YAK-2 and anti-Flag mAbs. However, when intact cells were used, only the YAK-2 mAb gave fluorescence on the cell surface. No fluorescence was obtained by staining with the anti-Flag mAb, because the extracellularly administered anti-Flag mAb was not able to gain access to the Flag tag orienting intracellularly at the C-terminus of rBNPI. The parental cells and cells transfected with vector alone were not stained with YAK-2 mAb (not shown). These results indicate that rBNPI is expressed on the plasma membrane of COS7 cells.

rBNPI Binding to GT1b Micelles—To gain direct evidence that rBNPI/p58 is able to bind GT1b micelles, we tested the binding activity of GT1b micelles to membrane lysates from COS7 cells transfected with C-Flag-CMV5a-2-1*. The lysate was only reactive with GT1b micelles, and not with micelles of other gangliosides, including GM1, GD1a, GD1b, and GQ1b (Fig. 8). On the other hand, the membrane lysate from COS7 cells transfected with vector alone were unreactive with all the ganglioside micelles

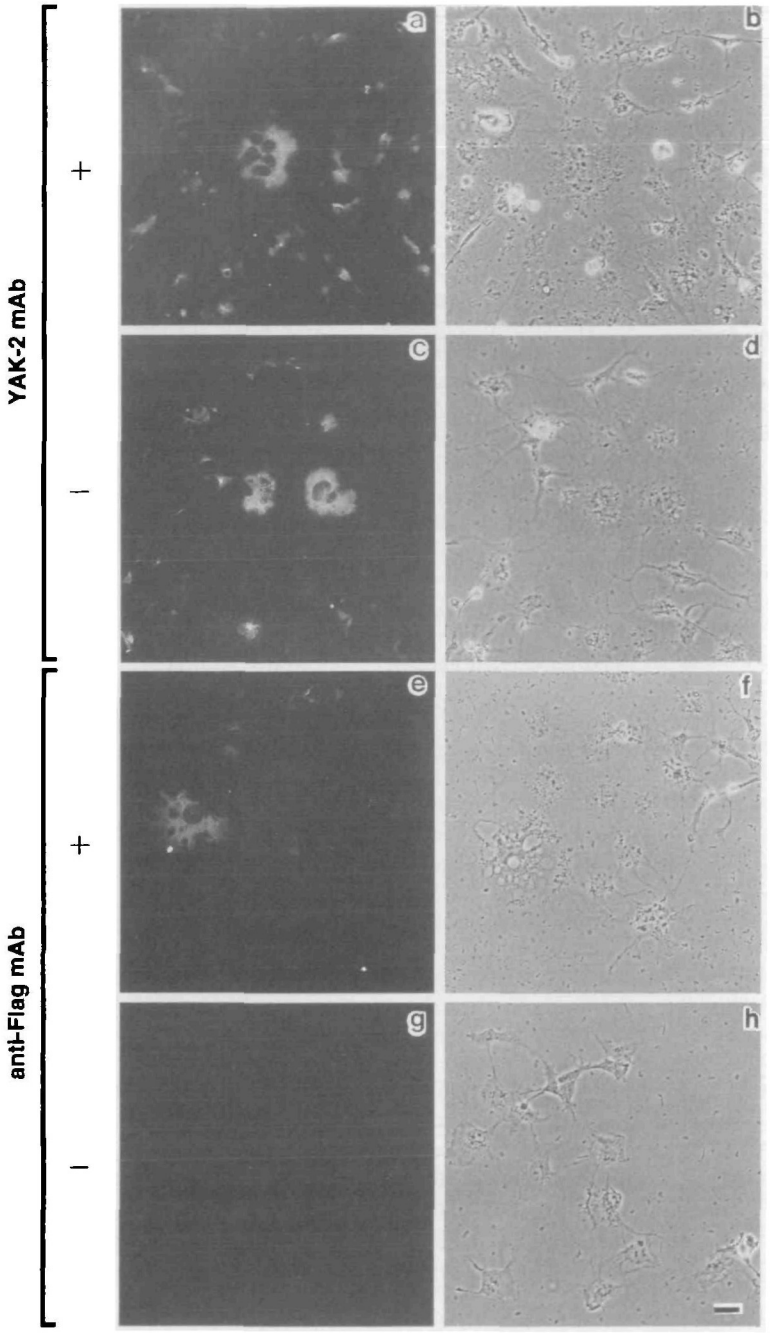

Fig. 7. **Localization of rBNPI/p58 transiently expressed in COS7 cells.** The COS7 cells transfected with rBNPI/p58 cDNA were treated with (+) or without (−) fixative, followed by indirect immunofluorescence staining with YAK-2 (a and c) or anti-Flag (e and g) mAbs. In intact cells, YAK-2 mAb gave fluorescence on the cell surface (c), but the anti-Flag mAb gave no fluorescence (g) because the intracellularly oriented Flag tag at the C-terminus of rBNPI was inaccessible to the extracellularly administered anti-Flag mAb. Photographs b, d, f, and h are phase contrast images of a, c, e, and g, respectively. Bar, 10 μm.

tested. These results indicate that rBNPI/p58 has GT1b-binding capability.

DISCUSSION

Gangliosides are thought to regulate cellular functions such as neuronal cell adhesion, neuronal cell growth, and neurite outgrowth (*17*), but the molecular mechanisms by which gangliosides affect diverse cellular functions are not well understood. In our previous study (*26, 27*), exclusively localized gangliosides (GD1a, GT1b, and GQ1b) on rat brain synapse-related regions provided a clue as to their functional roles in neuronal cell interaction. This is substantiated in this study by biochemical, molecular biological, and immunohistochemical evidence that GT1b behaves *in vitro* as a specific ligand for rBNPI, a rat brain neuronal cell–specific Na^+-dependent inorganic phosphate cotransporter (*28*).

Among investigations suggesting the presence of ganglioside binding proteins (*24, 38–40*), the report by Yang *et al.* (*24*) deserves mentioning for showing that GD1a and GT1b are physiological ligands for MAG, a 100 kDa membrane protein in mammalian brain. In the present study, we used a ganglioside micelle binding assay to reveal a novel protein-ganglioside recognition system *via* p58 and its oligomers with GT1b in adult rat brain (Fig. 1). This molecule is thought to be quite different from previously identified carbohydrate recognition molecules such as selectins and sia-

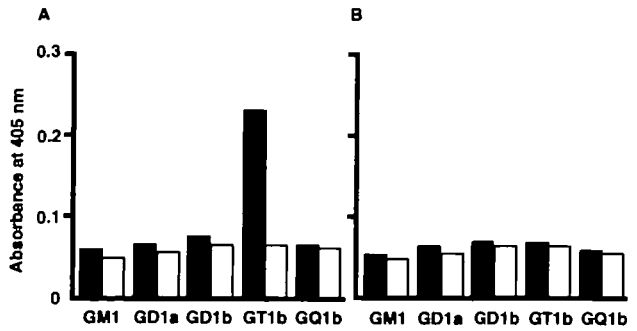

Fig. 8. **Binding activity of membrane lysate from COS7 transfectants to GT1b micelles.** The membrane lysates from COS7 cells transfected with rBNPI/p58 cDNA (A) or vector only (B) were added to microtiter plates. Each well was incubated with (closed column) or without (open column) the indicated ganglioside micelles, and then with the corresponding anti-ganglioside mAb. The bound anti-ganglioside mAbs were visualized by absorbance at 405 nm.

loadhesin (*18, 23*), because the binding of the protein to GT1b micelles occurs in a Ca^{2+}-independent manner (not shown). Additionally, molecular cloning of the GT1b-binding protein indicated that the protein has no consensus sequence of C-type lectins or galectins.

We established a hybridoma clone producing a mAb (YAK-2) specific for the GT1b-binding protein (Figs. 2 and 3), and a clone (#2-1) encoding p58 was isolated by expression cloning using YAK-2 mAb. The nucleotide sequence completely matched that of rBNPI, a membrane protein with six putative membrane-spanning segments and an apparent molecular mass of 61 kDa (*28*). In addition to the comparison of the sequence data, the following experimental results led us to conclude that the GT1b-binding protein p58 and rBNPI are the same molecule: (i) the apparent molecular mass of p58 is in good agreement with that of rBNPI (61 kDa); (ii) the expressions of rBNPI and p58 are restricted to brain (Fig. 4); (iii) the bacterially expressed rBNPI/p58 protein binds specifically to GT1b micelles (Fig. 5); and (iv) rBNPI/p58, detectable with YAK-2 mAb as a 64 kDa protein on COS7 cells transfected with a construct including the cDNA, specifically binds to GT1b micelles (Figs. 6 and 8). The polypeptide moiety of rBNPI/p58 is responsible for binding to the carbohydrate portion of GT1b, because bacterially expressed p58 retains its binding properties for GT1b micelles (Fig. 4), and because the binding of p58 to gangliosides is GT1b-specific as analyzed with a series of structurally related gangliosides (Figs. 1, 3, and 8). However, the possibility that the rBNPI/p58 polypeptide recognizes both the carbohydrate and ceramide moieties of GT1b simultaneously cannot be ruled out.

Although rBNPI was identified as a Na^+-dependent P_i cotransporter in rat brain, the results of this study indicate it also possesses a GT1b-binding property. This is not surprising, however, because some proteins are known to behave multifunctionally. For example, fucosidase, an enzyme that resides on the sperm surface of the ascidian, *Ciona intestinalis*, functions as a binding protein to the vitelline coat of eggs (*41*); and a sperm surface galactosyltransferase in mouse is reported to be directly involved in ZP3-induced acrosome reaction (*42*).

In the present study, YAK-2 mAb was used to demonstrate the presence of oligomeric forms of rBNPI (p90 and p160), as well as monomeric p58, as evidenced by Western blotting of cerebellum cell membranes pretreated under nonreducing conditions for SDS-PAGE (Fig. 2B), although for unknown reasons the disappearance of p90 under reducing conditions was not complete. Until now, the native form of rBNPI has been unknown. In the case of the membrane lysate of COS7 cells transfected with the construct including rBNPI/p58 cDNA, however, the only band expressed was a monomeric 64 kDa protein with a Flag tag at the C-terminus, despite pretreatment of the membrane lysate under nonreducing conditions for SDS-PAGE (Fig. 6). The cotransportation activity of the transfectants awaits further examination. It also remains to be elucidated why the oligomeric forms were not detected in transfected COS7 cells. In this connection, it may be reasonable to assume the presence of brain-specific posttranslational modification systems for the oligomerization of rBNPI. If this is the case, transfection of the construct into cell lines derived from neuronal cells will provide useful information about the oligomerization of rBNPI.

rBNPI is known to play a vital role in the intracellular energy metabolism of neuronal cells (*43–46*). The intracellular P_i concentration incorporated *via* rBNPI reportedly correlates with cytosolic ATP and NADPH stores produced *via* glycolysis and the pentose phosphate pathway, respectively, in cultured rat cortical neurons (*43*). Glinn *et al.* (*44, 45*) also reported that the maintenance of intracellular P_i in cultured neuronal cells is an essential event for neuronal survival, and that the intracellular free P_i concentration in the brain is dependent on the extracellular P_i concentration. On the other hand, the Na^+-dependent P_i cotransporter system in the kidney, which exerts cotransportation activity with a molecule other than rBNPI, has been shown to be regulated by both the extracellular P_i concentration and by a variety of hormones and metabolic factors (*47*). We have obtained no data suggesting the possibility that GT1b is one of the regulatory factors of rBNPI activity, an intriguing notion because there are some reports that gangliosides play roles as modulators and/or regulators of functional molecules (*17*). Higashi *et al.* (*48, 49*) reported that the activity of a calmodulin-dependent enzyme, cyclic nucleotide phosphodiesterase, is regulated by gangliosides; and Kasahara *et al.* (*50*) reported that GD3 associates with a src family tyrosine kinase Lyn and may regulate Lyn activity in neuronal cells.

In the present study, we demonstrated both functionally and spatially that GT1b is a potential specific ligand for rBNPI/p58 on neuronal cells in adult rat brain. The next point for examination is when the ganglioside-protein interaction is established during the embryonic and/or postnatal stages.

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