THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 12, Issue of March 19, pp. 8224–8230, 1999

Printed in U.S.A.

Characterization of a Novel Rat Brain Glycosylphosphatidylinositol-anchored Protein (Kilon), a Member of the IgLON Cell Adhesion Molecule Family*

(Received for publication, November 30, 1998)

Nobuo Funatsu‡§¶, Seiji Miyata‡, Haruko Kumanogoh‡§, Masaki Shigeta‡, Kazushige Hamada‡, Yasuhisa Endo‡, Yoshihiro Sokawa‡, and Shohei Maekawa‡‖

From the ‡Department of Biotechnology, §Venture Laboratory, Kyoto Institute of Technology, Kyoto 606-8585, Japan

In the central nervous system, many cell adhesion molecules are known to participate in the establishment and remodeling of the neural circuit. Some of the cell adhesion molecules are known to be anchored to the membrane by the glycosylphosphatidylinositol (GPI) inserted to their C termini, and many GPI-anchored proteins are known to be localized in a Triton-insoluble membrane fraction of low density or so-called "raft." In this study, we surveyed the GPI-anchored proteins in the Triton-insoluble low density fraction from 2-week-old rat brain by solubilization with phosphatidylinositol-specific phospholipase C. By Western blotting and partial peptide sequencing after the deglycosylation with peptide *N*-glycosidase F, the presence of Thy-1, F3/contactin, and T-cadherin was shown. In addition, one of the major proteins, having an apparent molecular mass of 36 kDa after the peptide *N*-glycosidase F digestion, was found to be a novel protein. The result of cDNA cloning showed that the protein is an immunoglobulin superfamily member with three C2 domains and has six putative glycosylation sites. Since this protein shows high sequence similarity to IgLON family members including LAMP, OBCAM, neurotrimin, CEPU-1, AvGP50, and GP55, we termed this protein Kilon (a kindred of IgLON). Kilon-specific monoclonal antibodies were produced, and Western blotting analysis showed that expression of Kilon is restricted to brain, and Kilon has an apparent molecular mass of 46 kDa in SDS-polyacrylamide gel electrophoresis in its expressed form. In brain, the expression of Kilon is already detected in E16 stage, and its level gradually increases during development. Kilon immunostaining was observed in the cerebral cortex and hippocampus, in which the strongly stained puncta were observed on dendrites and soma of pyramidal neurons.

Cell adhesion molecules (CAMs)[1] play central roles in the establishment and the remodeling of the central nervous system. CAMs are classified into Ca^{2+}-dependent and Ca^{2+}-independent groups. Cadherins are dependent on Ca^{2+} ions for binding. The cytoplasmic domain of different cadherins is highly conserved, and proteins that bind to the cytoplasmic domain interact with cytoskeletal proteins and signal transduction pathways to regulate cell adhesion (1). Integrins make up a large family of heterodimeric proteins that mediate cell to cell and cell to extracellular matrix-adhesive connections, and the intracellular domain interacts with the actin cytoskeleton through several intermediate proteins such as α-actinin, vinculin, talin, and tensin (2, 3). The largest classes of Ca^{2+}-independent CAMs are members of the Ig superfamily, which contains Thy-1 (Po), MAG, NCAM, L1, F3/F11/contactin, and others. These molecules have a number of Ig motifs and fibronectin-III repeats in their extracellular domain. The number of Ig and fibronectin repeats differ in the various members of the Ig superfamily, but generally they share a similar organization. For many of the molecules, multiple isoforms with distinct functional effects and expression patterns are known. The method of attachment to the cell membrane and the length of the cytoplasmic tail are important structural features with significant functional consequences, and these consequences are under extensive investigation (2–7).

Much attention has been paid to a membranous subdomain that is insoluble in the non-ionic detergent such as Triton X-100 and has a low density (Triton-insoluble low density fraction: TIF, also called "raft", "DIGs" (detergent-insoluble, glycolipid-enriched complexes), or "DRMs" (detergent-resistant membrane domains), "Caveolae-like domains," etc.), since this domain has so many signal-transducing molecules such as trimeric G proteins, protein tyrosine kinases, cytoskeletal proteins, and calmodulin-binding proteins (8–14). The detergent insolubility of this fraction is attributed to the enrichment of cholesterol and sphingomyelin (15, 16). Glycosylphosphatidylinositol (GPI)-anchored proteins, which are extracellular proteins anchored in the lipid bilayer by GPI instead of membrane-spanning peptides, are localized in this region (8, 9, 17–20). Some CAMs are known to be the GPI-anchored proteins, and little is known about their signal-transducing pathways. Since GPI-anchored proteins are enriched in TIF, this region could be a good clue to elucidate the molecular mechanisms of signal transduction through the GPI-anchored cell adhesion molecules. At the first step, we analyzed the GPI-anchored proteins in the TIF from 2-week-old rat brain and identified F3/contactin, T-cadherin, and Thy-1 as major GPI-anchored proteins. In addition, a novel GPI-anchored protein was identified, and the

* This work was supported in part by Grant-in-aid for Scientific Research (B) 08459016 and by Grant-in-aid for Exploratory Research 08878124 from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB017139.

¶ Supported in part by a Sasagawa Scientific Research grant.

‖ To whom correspondence should be addressed. Tel.: 81 75 724 7789; Fax: 81 75 724 7760; E-mail: smaekawa@ipc.kit.ac.jp.

[1] The abbreviations used are: CAMs, cell adhesion molecules; TIF, Triton-insoluble low density fraction; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGF, peptide *N*-glycosidase F; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PCR, polymerase chain reaction; kb, kilobase pair; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; PBS, phosphate-buffered saline.

result of cDNA cloning showed that this protein belongs to the IgLON family, which contains LAMP, OBCAM, neurotrimin, CEPU-1, GP55, and AvGP50 (21–26). We call this protein Kilon, for this protein is a kindred of IgLON.

EXPERIMENTAL PROCEDURES

Preparation of TIF from Rat Brain—TIF was prepared as described previously with slight modification (13). All procedures were carried out on ice or at 4 °C, unless otherwise mentioned. Frozen whole brains from 2-week-old rat were thawed, minced with scissors, and homogenized with a Teflon glass homogenizer in 1 volume (w/v) of TME solution (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.4) containing 2% Triton X-100. Protease inhibitors used were 1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.01 mg/ml leupeptin, and 0.01 mg/ml pepstatin. An aliquot of 2.4 M sucrose solution was added to this homogenate, and the final sucrose concentration was adjusted to be 0.8 M. The sample was then placed in the centrifuge tubes. TME solution was overlaid, and the sample was centrifuged for 6 h at 70,000 × *g* using a swing rotor (Hitachi SW27–2). A membrane fraction was concentrated at the interface of TME solution, and the original sample solution was collected and recovered as a pellet after dilution with 5 volumes of TME solution containing 1% Triton X-100 and centrifugation for 1 h at 100,000 × *g*. This fraction was then homogenized in 5 volumes of TME solution containing 1 M NaCl. After centrifugation for 1 h at 100,000 × *g*, the pellet fraction was recovered and suspended in 5 volumes of 50 mM Tris-HCl, 1 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, and 5% Nonidet-40, pH 9.5, and was kept for 1 h at 0 °C. After centrifugation for 1.5 h at 100,000 × *g*, the resulting pellet was recovered and suspended in 10 mM Tris-HCl, 5 mM EDTA, pH 7.4. TIF, thus obtained, was frozen at −80 °C until use.

Phosphatidylinositol-specific Phospholipase C (PI-PLC) Treatment—GPI-anchored proteins were solubilized from TIF with PI-PLC (Funakoshi, Japan). TIF (20 mg of protein, 1 mg/ml) was incubated with 0.125 units/ml PI-PLC for 12 h at 37 °C in the presence of 0.2% Triton X-100 and the protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, each 0.01 mg/ml pepstatin and leupeptin). After the addition of 4 M NaCl solution to be 0.1 M, the sample was centrifuged for 3 h at 100,000 × *g*, and the supernatant was recovered. The supernatant was then lyophilized after dialysis against 50 mM ammonium acetate solution. The fraction was then suspended in a solution containing 50 mM Tris-HCl, 50 mM EDTA, pH 8.6, applied to a Extracti-Gel D column (0.7 × 5 cm) (Pierce). The flow-through fraction was then recovered as the PI-PLC supernatant.

Peptide N-Glycosidase F (PNGF) Treatment—To remove the *N*-linked sugar chain, PNGF (Boehringer Mannheim) was used. The PI-PLC supernatant was incubated first for 30 min at 56 °C in the presence of 0.1% SDS and 2 mM NaN_3. PNGF was then added at a ratio of 50 units/mg protein and incubated for 24 h at 37 °C.

Partial Amino Acid Sequence—Samples were incubated for 60 min at 60 °C after the addition of Laemmli sample solution. SDS-PAGE was done using a 12.5–15% gradient polyacrylamide gel, and proteins were transferred to a sheet of $PVDF^{SQ}$ membrane (Millipore) using a buffer containing 10 mM CAPS and 10% methanol, pH 11. The membrane was stained with a solution containing 0.1% Ponceau S in 1% acetic acid and destained with distilled water. For peptide sequencing, immobilized protein bands were cut, reduced with dithiothreitol, pyridyletylated with 4-vinylpyridine, and digested with endoproteinase Lys-C (Wako, Japan) for 12 h at 37 °C in 20 mM Tris-HCl, 10% acetonitrile, pH 9.0. The peptide fragments were separated on a C18 reversed-phase high performance liquid chromatography column. For N-terminal sequence, immobilized proteins were directly applied to the sequencer. N-terminal amino acid sequence or internal amino acid sequences were then obtained with an Applied Biosystems 476A protein sequencer.

cDNA Cloning and Recombinant Protein Expression—From the N-terminal peptide sequence (PEP-1, VDFPWAAVDNMLVRK), a sense PCR primer (N-1, gtNgattttccNtgggcNgcNgtNgaYaaYatgYt) was constructed. From one of the internal sequences (PEP-3, DYGNYTCVATNK), an antisense PCR primer (N-3, cttgttKgtNgccacacaKgtatagtt) was constructed (where Y = c + t, R = a + g, and K = g + t). Using 2-week-old rat brain cDNA library (Uni-Zap XR, Stratagene), PCR was carried out 35 cycles at 94 °C for 1 min, 60 °C for 1 min, 74 °C for 1.5 min using 2.5 μM primers per reaction, and a band of 0.79 kb was specifically amplified. This product was blunted and subcloned into the *Sma*I-cutting site of pBluescript $KSII^+$ (Stratagene). The resulting plasmid ($pKS^+0.79$) was transformed into *E. coli* JM109 and was amplified in a large scale. The $pKS^+0.79$ was double-digested with *Apa*I and *Hin*dIII, and the resulting 0.79-kb fragment was purified. Uni-Zap XR library (450,000 plaque-forming units) was screened using the 0.79-kb fragment labeled with a thermostable alkaline phosphatase as described by the AlkPhos Direct system for chemiluminescence protocol (Amersham Pharmacia Biotech), and several cDNA clones were isolated.

Nucleotide sequencing was performed by the dideoxy chain termination method on an Applied Biosystems 373A automated DNA sequencer using the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia Biotech).

To express Kilon in *Escherichia coli* JM 109, the fragment of this clone was subcloned into the *Eco*RI site of pGEX-2T (Amersham Pharmacia Biotech), and the expression of the GST fusion protein was induced with 1 mM isopropyl-1-thio-b-D-galactopyranoside at 37 °C. After 3 h incubation, cells were collected, washed, and disrupted with a sonicator. After centrifugation at 20,000 × *g* for 30 min, the supernatant was recovered and applied to a glutathione-Sepharose column. The unbound proteins were washed with a solution of 10 mM Tris-HCl, 0.15 M NaCl, 0.2 mM EGTA, pH 7.4, and the bound proteins were eluted with the above solution containing 10 mM glutathione.

Monoclonal Antibody Production—Proteins eluted from TIF with PI-PLC were digested with PNGF and used as the antigen. After PNGF treatment, proteins were passed through an Extracti-Gel column to remove detergents in the solution. After dialysis against 50 mM ammonium acetate, the sample was lyophilized. The sample was finally dissolved in PBS and frozen at −80 °C until use. Each 0.04 mg of protein was injected intraperitoneally after mixing with Freund's complete adjuvant. After four times immunizations with the adjuvant, one mouse received four successive intraperitoneal injections (once a day). Two days after the last injection, the spleen was dissected and used for cell fusion with myeloma cells. The fusion of cells and screening of the hybridomas were performed as described previously (27). TIF and the PNGF fraction were used for screening. Two clones (89B3 and 89E4) were found to be specific for Kilon, and 89B3 was used for immunological studies. During this screening, two other monoclonal antibodies (87F2 and 84A3) reactive for the 36-kDa band were obtained. Using these mAbs, Uni-Zap cDNA library was screened, and one clone was obtained. DNA sequencing of this clone showed that this clone contains an insert for OBCAM.

Immunocytochemistry—Rats (9 weeks old) were deeply anesthetized with sodium pentobarbital (70 mg/kg) and transcardially perfused with 50 ml of heparinized phosphate-buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brain was postfixed with the same fixative solution for 24 h at 4 °C. The brain blocks including the cerebral cortex and hippocampus were dissected, and coronal sections were cut using a vibratome (DTK-1000, DSK, Kyoto, Japan) at the thickness of 25 μm. Free-floating sections were first pretreated with 0.1% H_2O_2 in PBS for 20 min and then with 0.5% sodium borohydride in PBS for 10 min. They were preincubated with 5% normal horse serum in PBS containing 0.3% Triton X-100 for at least 24 h at 4 °C and then incubated with an mAb 89B3 (dilution 1:3,000) in PBS/Triton X-100 containing 1% normal horse serum for 48 h at 4 °C. The sections were further incubated with biotin-conjugated horse anti-mouse IgG (dilution 1:200, Vector, CA) for 2 h and then incubated with a avidin and peroxidase ABC solution (dilution 1:200, elite ABC kit, Vector) for 2 h. The visualization of the peroxidase was performed with 0.02% 3,3′-diaminobenzidine and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.4, containing 0.05% sulfate nickel. All specific immunoreactivity was absent when the primary antibody against Kilon was omitted in control experiments.

Others—Tissue extracts were prepared from 2-week-old rats as described previously (27). SDS-PAGE, Western blotting using ECL system (Amersham Pharmacia Biotech), and protein determination were performed as described (13). Anti-Thy-1 antibodies were obtained from Cedarlane and Sigma.

RESULTS

Solubilization of Proteins from TIF and Its Treatment with PNGF—GPI-anchored proteins are solubilized from the membrane fractions by PI-PLC. For example, using adult chick brains, F3/F11/contactin and T-cadherin, both of which are GPI-anchored proteins, were released from the detergent-insoluble membrane fractions by PI-PLC (28–31). We also used the PI-PLC to solubilize GPI-anchored proteins from rat brain-derived TIF and recovered the supernatant (PI-PLC supernatant) after centrifugation (Fig. 1). Three major broad protein bands with apparent molecular masses of 110–140, 44–65, and

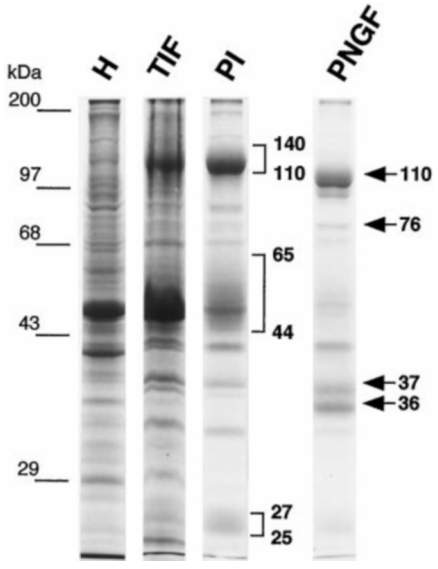

FIG. 1. **Solubilization of GPI-anchored proteins from TIF and their deglycosylation with PNGF.** TIF was prepared from 2-week-old rat brain (*TIF*) and treated with PI-PLC. Solubilized proteins were recovered after centrifugation (PI-PLC supernatant) and then treated with PNGF (*PNGF fraction*). Samples were analyzed with SDS-PAGE using 10% acrylamide gels, and the gels were stained with CBB. *H*, homogenate (20 μg of protein); *TIF*, Triton-insoluble low density fraction (25 μg of protein); *PI*, PI-PLC supernatant (15 μg of protein); *PNGF*, PNGF fraction (15 μg of protein). *Left bars* indicate the positions of molecular mass markers. Calculated molecular masses of the major proteins in the PI-PLC supernatant are shown on the *right*. The N-terminal sequences of the major proteins in the PNGF fraction (*arrows*) were analyzed.

25–27 kDa were observed after SDS-PAGE (Fig. 1). With Western blotting using anti-Thy-1 antibodies, the 25–27-kDa band was identified as Thy-1 (data not shown). Since highly glycosylated proteins are known to show a broad band on SDS-PAGE, we next deglycosylated the PI-PLC supernatant using peptide *N*-glycosidase F (PNGF). After PNGF treatment (PNGF fraction), the apparent molecular masses of major proteins changed to 110, 76, 37, and 36 kDa (Fig. 1).

Protein Sequencing—In order to identify these protein components, N-terminal peptide sequences of the 110-, 76-, 37-, and 36-kDa protein bands were obtained. The 110-kDa protein, of which the N-terminal peptide sequence was EFT, was estimated to be F3/contactin, for this sequence coincided with the reported N-terminal sequence of F3/contactin, the molecular mass of the deglycosylated band matched well with the calculated molecular mass of F3/contactin (32, 33), and the presence of the protein in this biochemical fraction was shown by an immunological method (29). The 76-kDa protein, of which the peptide sequence was SIVVSPILIPENQR, was identified as T-cadherin, for this sequence was found in the deduced amino acid sequence of human T-cadherin (34, 35). The presence of T-cadherin in a Triton-insoluble fraction is also reported (30, 31). The peptide sequence of the 37-kDa protein band was GDATFPKAMD*X*VT, and this sequence was found in the deduced sequences of OBCAM and neurotrimin (23, 36). The N-terminal sequence of the 36-kDa protein band, VDFPWAAVDNMLVRK (PEP-1), however, did not correspond to any identified proteins. Two internal peptide sequences of the 36-kDa band, GDTAVLRCYLEDGASK (PEP-2) and DYGNYTCVATNK (PEP-3), were further obtained after the high performance liquid chromatography separation of the protease digests. The sequence of PEP-2 was not found in the identified proteins. The sequence of PEP-3, on the other hand, was found in the sequence of OBCAM fragment 283–294 (36).

cDNA Cloning—From the N-terminal (PEP-1) and an internal amino acid sequence (PEP-3), two degenerative primers for PCR (N-1 and N-3) were designed and produced. A PCR experiment using a rat brain cDNA library resulted in the amplification of a single 0.79-kb band. Since the direct sequencing of this band showed no identity with known genes, this product was used as a probe to screen a full-length cDNA from the same cDNA library. After screening 450,000 clones, 20 positive clones were isolated. The clone having the longest insert was selected. The insert was subcloned into pBluescript KSII$^+$, and its complete sequence was determined (Fig. 2*A*). The insert contained 1827 base pairs, including an open reading frame of 1044 base pairs coding for 348 amino acids. The nucleotide sequence at the deduced start methionine matches the consensus initiation (37). Sequences in the 0.79-kb PCR product was found in the insert. The deduced amino acid sequence contained two of the peptide sequences determined from the direct sequence analysis of the protein (PEP-1 and PEP-2 in Fig. 2*A*). PEP-3 sequence, a sequence used for the design of the PCR primer, was not found in the sequence. We found, however, that one of the sequence patterns of the degenerative primer (N-3) matches with the nucleotide sequence from 949 to 975 (a *dashed line* in Fig. 2*A*). As described below, this region has a considerable homology within LAMP, OBCAM, neurotrimin, and Kilon. Since the sequence of Pep-3 is observed in the sequences of OBCAM, and OBCAM-specific monoclonal antibodies react with the 36-kDa protein band (data not shown), the 36-kDa band in SDS-PAGE was judged to be a mixture of Kilon and OBCAM.

From the N-terminal sequence of the protein, the original N-terminal 31-amino acid sequence of Kilon is thought to be cleaved as a signal sequence (*double underlined* in Fig. 2*A*) (38). The presence of a hydrophobic core surrounded by two hydrophilic sequences in this region coincides well with the present concept of the signal peptide (Fig. 2, *A* and *B*). This hydropathy plot also showed the presence of another hydrophobic region at the C terminus. In case of GPI-anchored proteins, the addition of the GPI anchor is known to occur after the cleavage of the C-terminal hydrophobic region (40–42). Although a putative GPI anchor attachment site was found (Gly-318 in Fig. 2*A*), there is no datum to assign the C-terminal amino acid of this protein at present. A homology search of the coding sequence of this protein with SWISS-PROT data bases showed that this protein is a member of the immunoglobulin superfamily having three C2 domains and six putative glycosylated sites and has high similarities to rat LAMP (56%), rat OBCAM (49%), rat neurotrimin (48%), chick GP55A (51%), and chick CEPU-1 (49%) (Table I) (21, 23–25, 43, 44). Like the IgLON family members, this protein has three sets of cysteines that are likely to form intradomain disulfide linkages in each of its immunoglobulin-like domains (Fig. 2*C*). A sequence comparison of these proteins is shown in Fig. 3. The regions of highest homology to most of these proteins occur in the conserved sequences surrounding the cysteines involved in the intradomain disulfide bonding (shown by *dots*). Since these proteins are recently classified into a subfamily of the immunoglobulin superfamily (IgLONs, immunoglobulin superfamily containing LAMP, OBCAM, and neurotrimin), the name, Kilon (kindred of LON) was coined for this novel protein.

Immunological Analysis of Kilon—For the further characterization of Kilon, monoclonal antibody production was attempted using the PNGF fraction as the antigen. One of the mAbs (89B3) reacted with the 36-kDa protein of the PNGF fraction (Fig. 4*A*) and also reacted specifically with the bacterially expressed GST-Kilon fusion protein (Fig. 4*B*) but did not react with the bacterially expressed GST alone (data not shown). This antibody reacted with one broad band with an

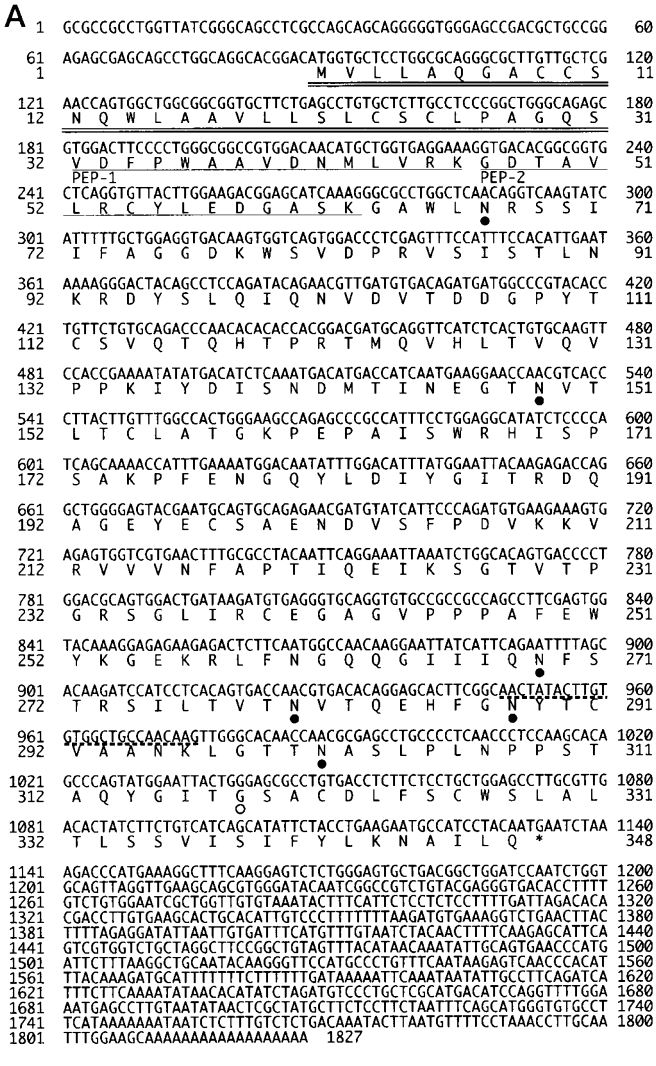

FIG. 2. **Structure of Kilon.** *A*, nucleotide sequence and alignment of deduced amino acid sequence of Kilon coding clone. One obtained amino acid sequence in the Lys-C endopeptidase digest (PEP-2) and the N-terminal sequence (PEP-1) were found in the deduced sequence (*underlined*). The nucleotide sequence, estimated to correspond to N-3, was indicated by a *dashed line*. The N-terminal signal sequence was *double underlined*. A putative GPI anchor attachment site was indicated by the ○. Six potential *N*-glycosylation sites were designated by ●. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence data bases with the accession number AB017139. *B*, a hydropathy plot of Kilon. Hydropathy was calculated according to Kyte and Doolittle (39). Positive values on the *y* axis indicate hydrophobic regions. *C*, a domain model of Kilon. Ig-related domains were drawn as *loops* that are closed by disulfide bridges, and six putative *N*-linked glycosylation sites were shown as *lines ending* with *dots*.

TABLE I

Homology of Kilon to other members of the immunoglobulin superfamily molecules

A search of the Swissprot database with the full-length peptide sequence of this protein using the FASTA program revealed significant similarity to members of the IgLON family. Values indicate percent identity; values in parentheses refer to the number of overlapping amino acids.

	LAMP	OBCAM	Neurotrimin	GP55A	CEPU-1
Kilon	56 (330)	49 (324)	48 (335)	51 (267)	49 (339)
LAMP		56 (322)	55 (332)	57 (270)	54 (325)
OBCAM			77 (345)	84 (277)	68 (346)
Neurotrimin				74 (276)	78 (345)
GP55A					70 (287)

apparent molecular mass of 46 kDa in TIF and PI-PLC fractions (Fig. 4*A*). Kilon was highly enriched in the TIF, for no detectable reaction was observed in the homogenate fraction compared with TIF under this reaction condition.

The tissue distribution of this protein was examined, and high expression in brain was shown (Fig. 5*A*). No expression was detected in other tissues such as kidney, liver, lung, skeletal muscle, spleen, and testis. Although a broad band of about 38 kDa also reacted weakly in the skeletal muscle extract, nothing is known about the nature of this protein at present. Within brain, expression of Kilon was observed in cerebrum, brain stem, and hippocampus, and much less expression was detected in cerebellum (Fig. 5*B*). A developmental change of the expression of this protein in whole brain showed that this protein is already expressed in E16 stage, and its level gradually increases during development (Fig. 5*C*).

Immunocytochemical studies using the mAb (89B3) showed specific staining in the rat cerebral cortex (Fig. 6, *A* and *B*) and hippocampus (Fig. 6, *C* and *D*). In the cerebral cortex, numerous puncta of Kilon immunoreactivity were visible in all regions and were most densely distributed in large neurons of layer V (Fig. 6*A*). These strongly stained neurons were identified as pyramidal neurons, because of their soma location in layer V, large soma size, and extension of their apical dendrite to layer I. Both soma and dendrite of pyramidal neurons were lightly stained, whereas strongly stained puncta were found on pyramidal neurons (Fig. 6*B*). The punctate staining appeared to be seen more frequently on dendrites than on soma of the neurons. The pattern of Kilon immunoreactivity in the hippocampus was basically similar to that in the cerebral cortex (Fig. 6, *C* and *D*). The strongly stained puncta were observed on dendrites and soma of the pyramidal neurons. The same pattern of Kilon immunoreactivity was also observed in Purkinje cell dendrites and soma of the adult rat cerebellum (data not shown).

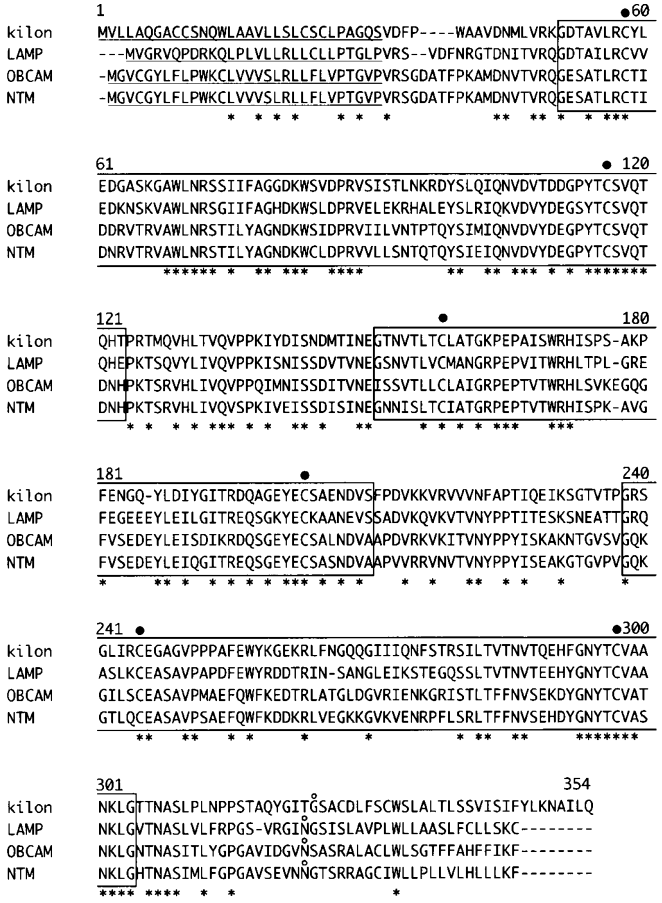

FIG. 3. **Comparison of the peptide sequences of Kilon and other IgLON family members.** Amino acids identical to four proteins are indicated by the *. The *boxed regions* indicate the C2 domains. Putative N-terminal signal peptide sequences were *underlined*. The six cysteine residues common to all proteins were designated by the ●. Putative GPI anchor attachment sites were indicated by the *symbol* ○. This analysis was done using the ClustalW (1.7) Multiple Sequence Alignment Program (45).

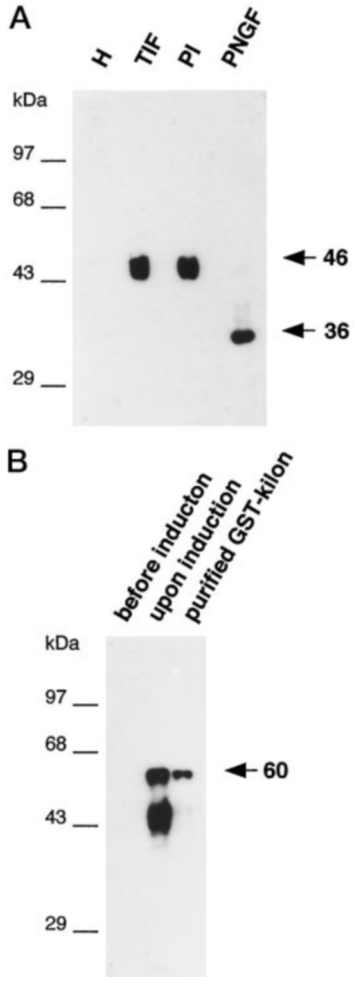

FIG. 4. **Western blot analysis with a monoclonal antibody against Kilon (89B3).** *A*, enrichment of Kilon in TIF. Hybridomas, which produce antibodies reactive with the 36-kDa protein in PNGF fraction, were screened as described under "Experimental Procedures." The mAb (89B3) recognizes the 36-kDa band in the PNGF fraction and a 46-kDa band in TIF and in PI-PLC supernatant (shown by *arrows*). Poor staining in the homogenate shows the enrichment of Kilon in TIF. *H*, homogenate; *PI*, PI-PLC supernatant; *PNGF*, PNGF fraction. Equal amounts of protein (3 μg of protein) were electrophoresed and processed for Western blotting using the mAb (89B3). *Left bars* indicate the positions of molecular mass markers. *B*, reactivity of the mAb (89B3) with a bacterially expressed Kilon. The GST-Kilon fusion protein (60 kDa) was induced in *E. coli* JM 109 with 1 mM isopropyl-1-thio-b-D-galactopyranoside for 3 h at 37 °C (*upon induction*). After disruption of cells, the GST-Kilon fusion protein was purified with a glutathione-Sepharose column (*purified GST-Kilon*). Cell extracts (2.5 μg of protein) and the purified protein (0.25 μg of protein) were electrophoresed and processed for Western blotting using the mAb (89B3). The location of the GST-Kilon fusion protein (60 kDa) is indicated by the *arrow*. *Left bars* indicate the positions of molecular mass markers.

DISCUSSION

A novel GPI-anchored protein that belongs to the Ig superfamily was detected in the Triton-insoluble low density fraction of rat brain, and its amino acid sequence was deduced from the cDNA cloning. This protein, Kilon, has three C2 domains and has a homology to the IgLON family. IgLON family is a recently recognized protein family named from its member proteins, LAMP, OBCAM, and neurotrimin, and also contains CEPU-1, GP-55, and AvGP50 (21–26). From the data of cDNA sequences, all these proteins without the N-terminal and C-terminal signal sequences have their calculated molecular masses of about 32 kDa. The molecular masses of the expressed form of these proteins obtained from SDS-PAGE are much larger and are different each other as follows: LAMP, 68 kDa; OBCAM, 58 and 51 kDa; neurotrimin, 65 kDa; and CEPU-1, 51 kDa. The expressed form of Kilon showed a molecular mass of 46 kDa, also much larger than the deduced value from the amino acid sequences. The difference between the molecular mass obtained from SDS-PAGE and the molecular mass deduced from cDNA sequencing is attributed to the post-translational modification of the protein. In this case, the nascent polypeptide chain receives the cleavage of the N-terminal signal sequence, the cleavage of the retention signal, the addition of GPI at the C-terminal, and the addition of *N*-linked carbohydrate chains. The smaller molecular mass of this protein suggests the lower level of the glycosylation than other proteins.

Since the 36-kDa protein band obtained after PNGF treatment contains at least two proteins (Kilon and OBCAM), and these proteins are very homologous, it is very important to obtain specific antibodies for immunological studies. Two independent groups reported that OBCAM in rat brain has a broad band of more than 50 kDa (46, 47). Our antibodies against OBCAM reacted with the 36-kDa band in the PNGF fraction and a broad band around 46 and 51 kDa in the rat brain fraction but did not react with bacterially expressed Kilon. In contrast, 89B3 antibody recognized a band of 46 kDa in the brain fraction, reacted with bacterially expressed Kilon, but did not react with bacterially expressed OBCAM. Since the expressed form of other IgLON family proteins are much larger than Kilon, we judged that the mAb (89B3) reacts with a

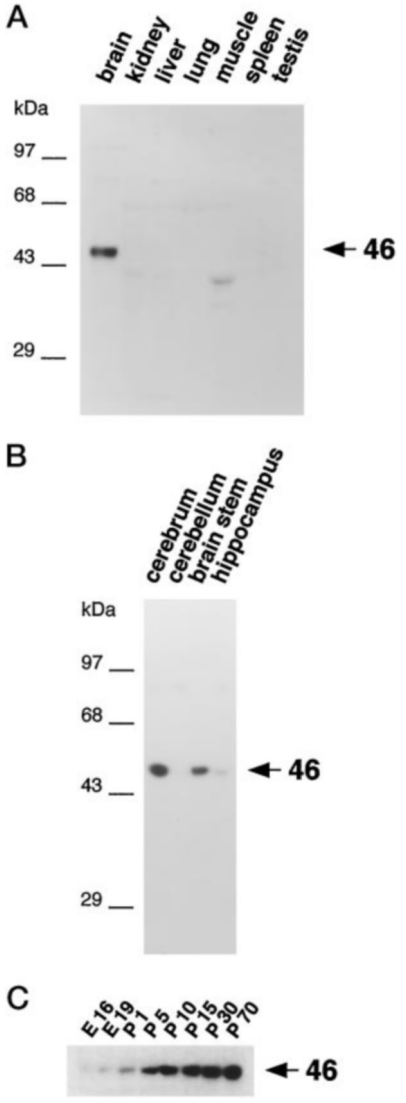

FIG. 5. **Expression of Kilon.** *A*, tissue distribution. Equal amounts of tissue extracts (20 μg of protein) from a 2-week-old rat were electrophoresed and processed for Western blotting using the mAb (89B3). The location of Kilon (46 kDa) is indicated by the *arrow*. *Left bars* indicate the positions of molecular mass markers. *B*, distribution in brain. From 2-week-old rat brains, cerebrum, cerebellum, brain stem, and hippocampus were dissected and treated for Western blotting using the mAb (89B3). Equal amounts of protein (5 μg of protein) were loaded. The location of Kilon (46 kDa) is indicated by an *arrow*. *Left bars* indicate the positions of molecular mass markers. *C*, developmental change. Whole brain extracts were prepared from rats of different embryonic (*E*) and postnatal (*P*) ages. Equal amounts of protein (5 μg of protein) were electrophoresed and processed for Western blotting using the mAb (89B3). The location of Kilon (46 kDa) is indicated by the *arrow*. *Left bars* indicate the positions of molecular mass markers.

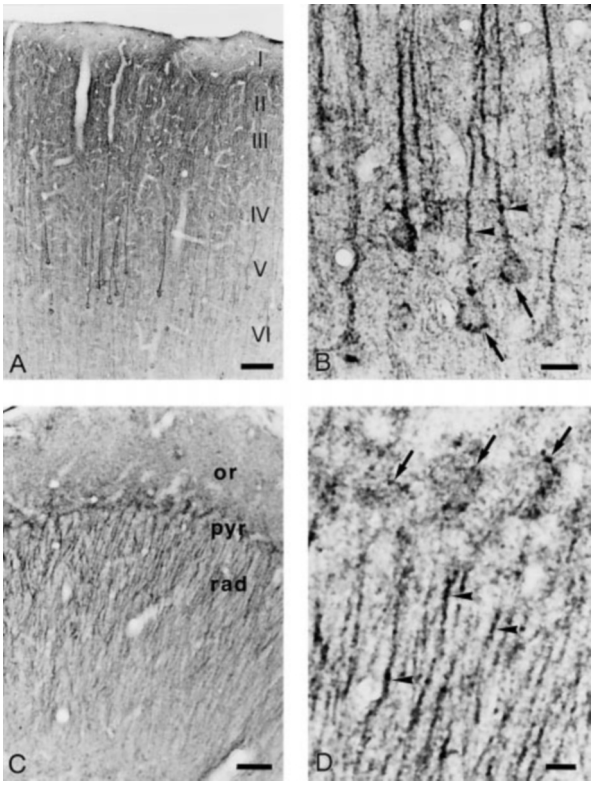

FIG. 6. **Immunocytochemical localization of Kilon in the adult rat brain.** *A*, low magnification photograph of the rat cerebral cortex (pial surface is at *top*). Note strongly stained pyramidal neurons in layer *V*. *Bar,* 10 μm. *B*, higher magnification of pyramidal neurons in the fields of layer *V* shown in *A*. Strongly stained puncta of Kilon immunoreactivity are seen on apical dendrites (*arrowheads*) and soma (*arrows*) of pyramidal neurons. *Bar,* 2 μm. *C*, Low magnification photograph of the CA1 region of the rat hippocampus. Note strongly stained pyramidal neurons. *Bar,* 5 μm. *D*, higher magnification of pyramidal neurons shown in *C*. Strongly stained puncta of Kilon immunoreactivity are seen on dendrites (*arrowheads*) and soma (*arrows*) of pyramidal neurons. *Bar,* 1 μm. *I*, layer I; *II*, layer II; *III*, layer III; *IV*, layer IV; *V*, layer V; *VI*, layer VI; *or*, stratum oriens; *pyr*, stratum pyramidal; *rad*, stratum radiatum.

Kilon-specific sequence, although we have not yet identified the sequence recognized by the antibody.

The expression of Kilon was evident on the neuronal processes in the cerebral cortex and hippocampus at adult rat brain. The distribution of Kilon on dendrites and soma of the pyramidal neurons is similar to that of LAMP (48). The role of LAMP as a cell recognition molecule is fairly well recognized through the works of Levitt and colleagues (21, 49). They showed that native, immunoaffinity purified LAMP exhibits homophilic binding. They also showed that LAMP-transfected cells selectively facilitated neurite outgrowth of primary limbic neurons, and the administration of anti-LAMP *in vivo* resulted in the abnormal growth of the mossy fiber projection from developing granule neurons. Chick brain-derived GP-55 is known to block the neurite outgrowth of dorsal ganglion cells (50). The expression of GP55 reaches highest levels around post-hatched day 5, after which it reaches a plateau or may decrease a little (25). GP55 is highly expressed in the central nervous system, and no expression was observed in non-neural tissues. The expression of neurotrimin (65 kDa in SDS-PAGE) was recognized as early as E15, increasing through early postnatal ages and declining in the adult (23). CEPU-1 is a 51-kDa glycoprotein strongly expressed on cerebellar Purkinje cells, and its expression on the cells coincides with the growth of the dendritic tree (24). Recently, identification of another GPI-anchored protein, termed Neurin-1, involved in the neuron-glia interaction, was reported. Neurin-1 (68-kDa protein in SDS-PAGE) was found on the surface of the axon and growth cone, and its N-terminal sequences were reported to be TPEGVPG. This sequence is not found in other proteins (51), although the cDNA cloning of this protein is not yet done. The spatial and temporal expression pattern of Kilon is fairly similar to some of these proteins, although a precise comparison is not done at present. Further biochemical and immunological characterization of these proteins will be useful in elucidating the molecular mechanism of the construction and remodeling of the nervous system.

Since the GPI anchor proteins do not have the transmembrane or the intracellular domains, they may mediate their biological responses by interaction with other membrane recep-

tors that are able to recruit and activate intracellular signaling molecules (52). In the chick brain membrane fraction, association of c-Fyn and $G_i\alpha$ protein in the activation of signaling pathways through Thy-1 and AvGP50 is shown (53). In the case of F3/contactin, receptor tyrosine phosphatase β and a novel transmembrane receptor (Caspr) were discovered as the interacting proteins and characterized (54, 55). Future studies on the characterization of interacting molecules with GPI-anchored proteins will elucidate the signal transduction pathways through the cell membrane.

REFERENCES

1. Takeichi, M. (1995) *Curr. Opin. Cell Biol.* **7,** 619–627
2. Fields, R. D., and Itoh, K. (1996) *Trends Neurosci.* **19,** 473–480
3. Hortsch, M. (1996) *Neuron* **17,** 587–593
4. Vaughn, D. E., and Bjorkman, P. J. (1996) *Neuron* **16,** 261–273
5. Luthi, A., Laurent, J.-P., Figurov, A., Muller, D., and Schachner, M. (1994) *Nature* **372,** 777–779
6. Nosten-Bertrand, M., Errington, M. L., Murphy, K. P. S. J., Tokugawa, Y., Barboni, E., Kozlova, E., Michalovich, D., Morris, R. G. M., Silver, J., Stewart, C. L., Bliss, T. V. P., and Morris, R. J. (1996) *Nature* **379,** 826–829
7. Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. (1994) *Neuron* **13,** 583–594
8. Simons, K., and Ikonen, E. (1997) *Nature* **387,** 569–572
9. Brown, D. A., and Rose, J. K. (1992) *Cell* **68,** 533–544
10. Brown, D. A., and London, E. (1997) *Biochem. Biophys. Res. Commun.* **240,** 1–7
11. Lisanti, M. P., Scherer, P. E., Tang, Z., and Sargiacomo, M. (1994) *Trends Cell Biol.* **4,** 231–235
12. Harder, T., and Simons, K. (1997) *Curr. Opin. Cell Biol.* **9,** 534–542
13. Maekawa, S., Kumanogoh, H., Funatsu, N., Takei, N., Inoue, K., Endo, Y., Hamada, K., and Sokawa, Y. (1997) *Biochim. Biophys. Acta* **1323,** 1–5
14. Wu, C., Butz, S., Ying, Y., and Anderson, R. G. W. (1997) *J. Biol. Chem.* **272,** 3554–3559
15. Schroeder, R. J., Ahmed, S. N., Zhu, Y., London, E., and Brown, D. A. (1998) *J. Biol. Chem.* **273,** 1150–1157
16. Hanada, K., Nishijima, M., Akamatsu, Y., and Pagano, R. E. (1995) *J. Biol. Chem.* **270,** 6254–6260
17. Gorodinsky, A., and Harris, D. A. (1995) *J. Cell Biol.* **129,** 619–627
18. Varma, R., and Mayor, S. (1998) *Nature* **394,** 798–801
19. Friedrichson, T., and Kurzchalia, T. V. (1998) *Nature* **394,** 802–805
20. Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1998) *J. Cell Biol.* **141,** 929–942
21. Pimenta, A. F., Zhukareva, V., Barbe, M. F., Reinoso, B. S., Grimley, C., Henzel, W., Fisher, I., and Levitt, P. (1995) *Neuron* **15,** 287–297
22. Schofield, P. R., McFarland, K. C., Hayflick, J. S., Wilcox, J. N., Cho, T. M., Roy, S., Lee, N. M., Loh, H. H., and Seeburg, P. H. (1989) *EMBO J.* **8,** 489–495
23. Struyk, A. F., Canoll, P. D., Wolfgang, M. J., Rosen, C. L., D'Eustachio, P., and Salzer, J. L. (1995) *J. Neurosci.* **15,** 2141–2156
24. Spaltmann, F., and Brummendorf, T. (1996) *J. Neurosci.* **16,** 1770–1779
25. Wilson, D. J. A., Kim, D.-S., Clarke, G. A., Marshall-Clarke, S., and Moss, D. J. (1996) *J. Cell Sci.* **109,** 3129–3138
26. Hancox, K. A., Gooley, A. A., and Jeffrey, P. L. (1997) *Mol. Brain Res.* **44,** 273–285
27. Maekawa, S., Maekawa, M., Hattori, S., and Nakamura, S. (1993) *J. Biol. Chem.* **268,** 13703–13709
28. Stefanova, I., Horejsi, V., Ansotegui, I. J., Knapp, W., and Stockinger, H. (1991) *Science* **254,** 1016–1019
29. Olive, S., Dubois, C., Schachner, M., and Rougon, G. (1995) *J. Neurochem.* **65,** 2307–2317
30. Ranscht, B., and Dours-Zimmermann, M. T. (1991) *Neuron* **7,** 391–402
31. Doyle, D. D., Goings, G. E., Upshaw-Earley, J., Page, E., Ranscht, B., and Palfrey, H. C. (1998) *J. Biol. Chem.* **273,** 6937–6943
32. Hosoya, H., Shimazaki, K., Kobayashi, S., Takahashi, H., Shirasawa, T., Takenawa, T., and Watanabe, K. (1995) *Neurosci. Lett.* **186,** 83–86
33. Peles, E., Nativ, M., Campbell, P. L., Sakurai, T., Martinez, R., Lev, S., Clary, D. O., Schilling, J., Barnea, G., Plowman, G. D., Grumet, M., and Schlessinger, J. (1995) *Cell* **82,** 251–260
34. Lee, S. W. (1996) *Nat. Med.* **2,** 776–782
35. Tanihara, H., Sano, K., Heimark, R. L., St John, T., and Suzuki, S. (1994) *Cell Adhes. Commun.***2,** 15–26
36. Lippman, D. A., Lee, N. M., and Loh, H. H. (1992) *Gene* (*Amst.*)**117,** 249–254
37. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8148
38. Heijne, G. V. (1986) *Nucleic Acids Res.* **14,** 4683–4691
39. Kyte, T., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132
40. Cross, G. A. M. (1990) *Annu. Rev. Cell Biol.* **6,** 1–39
41. Englund, P. T. (1993) *Annu. Rev. Biochem.* **62,** 121–138
42. Udenfriend, S., and Kodukula, K. (1995) *Annu. Rev. Biochem.* **64,** 563–591
43. Williams, A. F., and Barclay, A. N. (1988) *Annu. Rev. Immunol.* **6,** 381–405
44. Chothia, C., and Jones, E. Y. (1997) *Annu. Rev. Biochem.* **66,** 823–862
45. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680
46. Hachisuka, A., Yamazaki, T., Sawada, J., and Terao, T. (1996) *Neurochem. Int.* **28,** 373–379
47. Wick, M. J., Fan, G., and Loh, H. H. (1996) *Mol. Brain Res.* **36,** 322–328
48. Zacco, A., Cooper, V., Chantler, P. D., Fisher-Hyland, S., Horton, H. L., and Levitt, P. (1990) *J. Neurosci.* **10,** 73–90
49. Zhukareva, V., and Levitt, P. (1995) *Development* **121,** 1161–1172
50. Clarke, G. A., and Moss, D. J. (1994) *J. Cell Sci.* **107,** 3393–3402
51. Asou, H., Ono, K., Uemura, I., Sugawa, M., and Uyemura, K. (1996) *J. Neurosci. Res.* **45,** 571–587
52. Rosen, C. L., Lisanti, M. P., and Salzer, J. L. (1992) *J. Cell Biol.* **117,** 617–627
53. Henke, R. C., Seeto, G. S., and Jeffrey, P. L. (1997) *J. Neurosci. Res.* **49,** 655–670
54. Peles, E., Nativ, M., Lustig, M., Grumet, M., Schilling, J., Martinez, R., Plowman, G. D., and Schlessinger, J. (1997) *EMBO J.* **16,** 978–988
55. Einheber, S., Zanazzi, G., Ching, W., Scherer, S., Milner, T. A., Peles, E., and Salzer, J. L. (1997) *J. Cell Biol.* **139,** 1495–1506