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)

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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 cerebrum cortex and hippocampus, in which the strongly stained puncta were observed on dendrites and soma of pyramidal neurons.

Cell adhesion molecules (CAMs) play central roles in the establishment and the remodeling of the central nervous system. CAMs are classified into Ca2+-dependent and Ca2+-independent groups. Cadherins are dependent on Ca2+ 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 Ca2+-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.). This domain has so many signal-transducing molecules such as trimeric G proteins, protein tyrosine kinases, cytoskeletal proteins, and cell adhesion molecules (8–14). The detergent insolubility of this fraction is attributed to the enrichment of choleseterol and sphingomyelin (15, 16). Glycospiphosphatidylinositol (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
result of cDNA cloning showed that this protein belongs to the IgLON family, which contains LAMP, OBCAM, neurotrinin, 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 rats were thawed, minced with scissors, and homogenized with a Teflon glass homogenizer in 1 vol of TME solution (10 mM Tris-HCl, 1 mM MgCl₂, 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 mM sucrose solution was added to this homogenate, and the final sucrose concentration was adjusted to be 0.8 M. The sample of 4M NaCl solution to be 0.1 M, the sample was centrifuged for 3 h at 90,000 × g at 4 °C. PI-PLC for 12 h at 37 °C in the presence of 0.2% Triton X-100 was added to the PI-PLC supernatant.

### Phosphatidylinositol-specific Phospholipase C (PI-PLC) Treatment—

GIPI-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 3 mM 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.

### 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 PVDFSQ membrane (Millipore) using a buffer consisting (where Y₁₅₀, G₁₅₀, and K₁₅₀ are used 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 out and then cut using a vibrotome (DTKT-1000, DSK, Kyoto, Japan) at the thickness of 35 μm. Free-floating sections were first pretreated with 0.1% H₂O₂ in PBS for 20 min and then with 0.5% sodium borohydride in PBS for 10 min. They 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 0.5% sulfite. All specific immunoactivity 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—**GIPI-anchored proteins are solubilized from the membrane fractions by PI-PLC. For example, using adult chick brains, F9/P11/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 24–28 kDa 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.

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The sequence of PEP-3, on the other hand, was found in the identified proteins. The sequence of PEP-2 was not found in the identified proteins. VATNK (PEP-3), were further obtained after the high performance liquid chromatography separation of the protease digests. Two internal peptide sequences of the 36-kDa protein band, VDFP and GDATFPKAMD, were obtained. The sequence of PEP-3 is observed in 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. 2A). 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. 2A). 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 SDSPAGE 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. 2A) (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 hydrophathy 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 (Gly318 in Fig. 2A), 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. 2C). 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. 4A) and also reacted specifically with the bacterially expressed GST-Kilon fusion protein (Fig. 4B) but did not react with the bacterially expressed GST alone (data not shown). This antibody reacted with one broad band with an

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**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 GDATFPKAMDXYVT, 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, VDFP, WAAVDNMVYRK (PEP-1), however, did not correspond to any identified proteins. Two internal peptide sequences of the 36-kDa band, GDVAVLRCYLEDGK (PEP-2) and DGYNTC-VATNK (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 inter-
apparent molecular mass of 46 kDa in TIF and PI-PLC fractions (Fig. 4A). 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. 5A). 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. 5B). 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. 5C).

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. 6A). 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. 6B). 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 same pattern of Kilon immunoreactivity was also observed in Purkinje cell dendrites and soma of the adult rat cerebellum (data not shown).

Table I  Homology of Kilon to other members of the immunoglobulin superfamily molecules

|          | LAMP     | OBCAM   | Neurotrimin | GP55A | CEPU-1 |
|----------|----------|---------|-------------|-------|--------|
| Kilon    | 56 (330) | 49 (324)| 48 (335)    | 51 (267) | 49 (339) |
| LAMP     | 56       | 55      | 57          | 54    |        |
| OBCAM    |          | 77      | 84          | 68    |        |
| Neurotrimin |        | 74      | 78          |       |        |
| GP55A    | 70       |        | 7 (345)     |       |        |

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Kilon, a Novel Neuronal Cell Adhesion Molecule

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.

![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 O. 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. |
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...
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-

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 pyramidale; rad, stratum radiatum.
tors that are able to recruit and activate intracellular signaling molecules (52). In the chick brain membrane fraction, association of c-Fyn and G\(_\alpha\) protein in the activation of signaling pathways through Thy-1 and AvGP50 is shown (53). In the case of F3/contactin, receptor tyrosine phosphatase \(\beta\) 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.

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