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Cloning of a novel mouse *Gabarapl2* cDNA and its characterization

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KEY WORDS: $GABA_A$ receptors; human; mice; amino acid sequence homology; molecular models; reverse transcriptase polymerase chain reaction; Northern blotting; DNA

ABSTRACT

AIM: To clone a novel mouse $GABA_A$-receptor-associated protein like 2 (*Gabarapl2*) gene, and to analysis its primary function. **METHODS**: With the aid of computer, the human *GABARAPL2* cDNA was used as information probe to search mouse EST database of GenBank for mouse homolog. A series of overlapping EST were found and assembled into an EST contig using Genetics Computer Group (GCG) ASSEMBLY program. The existence of the gene was then identified by experiment. Northern blotting was performed to hybridize [α-^{32}P]dATP labeled probe with mRNA of 11 different mouse tissues that had been transferred to the nylon membrane. **RESULTS**: The novel gene was deposited in GenBank under Accession No AF190644. Its cDNA contained an intact open reading frame and a canonical polyadenylation signal AATAAA followed by polyA. The deduced protein was completely identical to that of human GABARAPL2, and was termed Gabarapl2 by Mouse Gene Nomenclature Committee. The putative protein of Gabarapl2 has a calculated molecular weight of 13 700 and an isoelectric point of 8.56. It was also predicted to contain two protein kinase C phosphorylation sites and one tyrosine kinase phosphorylation site. Northern hybridization showed that *Gabarapl2* was expressed as a single 1.35 kb transcript, with high levels in brain, thymus, lung, heart, kidney, and liver, and low in pancreas, testis, small intestine, colon, and stomach. **CONCLUSION**: A novel mouse *Gabarapl2* gene was cloned and identified.

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INTRODUCTION

GABA (gamma-aminobutyric acid), which is present in large amounts in the brain, is distributed among distinctly different cellular pools, possibly reflecting its multiple functions as neurotransmitter, neurodifferentiative agent, and metabolite. GABA has various behavioral and physiologic effects such as analgesia, depression, anxiolytic effects, anticonvulsive activity, feeding, and cardiovascular regulation[1]. It acts as a trophic signal that enhances the growth rate of neuronal processes, facilitates synapse formation, promotes synthesis of specific proteins and controls the induction and development of different GABA receptor subtypes[2]. GABA exerts its effects through two ligand-gated ion channels, $GABA_A$ and $GABA_C$ receptors, and a G protein linked receptor, $GABA_B$ receptor. $GABA_A$ receptor, the most prominent one, is a hetero-oligomeric protein composed of several distinct polypeptide types[3,4]. Most of the subunits have multiple subtypes ($\alpha 1-6$, $\beta 1-4$, $\gamma 1-3$, δ, $\rho 1-2$), which exhibit high homology in each subunit family (70 % - 80 % amino acid sequence identity)[5].

Recently, experiments showed that the intracellular loop of the abundant $\gamma 2$ subunit of $GABA_A$ receptor bound to $GABA_A$-receptor-associated protein (GABARAP), which was involved in the postsynaptic localization of ionotropic $GABA_A$ receptor to cytoskeleton[6]. Given the homology among γ subtypes (70 % - 80 % amino acid homology), it is possible that there are other molecules interacting with $\gamma 1$ or $\gamma 3$ to mediate the localization of $GABA_A$ receptor, similar to the case for $\gamma 2$. Here, a novel gene in mouse encoding a homolog of GABARAP (57.3 % identity and 67.5 % similarity) and GABARAPL2 protein (100 % identity) was cloned. It was later termed mouse $GABA_A$-receptor-associated protein like 2 (*Gabarapl2*). To clarify the function of the novel gene, we also performed its homologous comparison and structural domain prediction. Northern hybridization for *Gabarapl2* in multiple mouse tissues was carried out.

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MATERIALS AND METHODS

Searching database To search for the homolog of human *GABARAPL2* in mouse, we used *GABARAPL2* (Accession № AF087848) cDNA as information probe to search mouse EST database of GenBank (http://www.ncbi.nlm.nih.gov/blastn/est) with BLASTN program. A series of overlapping EST fragments, whose homology to probe were higher than 60 % and lower than 98 %, were found and assembled into a 1012 bp EST contig using GCG (Genetics Computer Group, Wisconsin Package Version 9.1) ASSEMBLY program.

Screening cDNA library In order to clone and identify the mouse EST contig, a pair of primers mA (5′-GTT GTG GTC GCT TCG CCG AAG TC-3′, nt 59 – 81) and mB (5′-CTA GTA GGT CTG TGT GGA GGC TC-3′, nt 568 – 546) were designed based on 5′-UTR and 3′-UTR sequences of the contig. PCR amplification was performed with primers mA and mB, using mouse brain cDNA generated by reverse transcription as templates. The PCR condition was as follows: denaturing at 94 ℃ for 3 min, followed by 35 cycles of denaturing at 93 ℃ for 1 min, annealing at 60 ℃ for 1 min, elongating at 72 ℃ for 1 min, respectively; finally, elongating at 72 ℃ for 10 min. After amplification, the expected 510 bp mouse cDNA fragment mAB was obtained. It was then cloned into pGEM-T vector (Promega) and sequenced with dye-terminator method (ABI autosequencer type 377). The result showed that the sequence of mAB fragment was identical to the corresponding sequence of the above mouse EST contig.

Mouse total RNA membrane preparation Total RNA of 11 mouse tissues was isolated by using TRIZOL Reagent (Life Technology) according to the protocol provided by the manufacturer. Then the acquired RNA (about 20 – 30 μg/lane) was run on denaturing 1.0 % agarose gel containing formaldehyde under constant voltage (80 V). After bromophenol blue run about 7 cm in the lane, the separated RNA on the gel were transferred to a nylon membrane (Amersham). The membrane was dried at 84 ℃ for 2 h for Northern hybridization.

Northern hybridization Fragment mAB was labeled with [α-^{32}P]dATP (Amersham) via PCR amplifications with the reverse transcript cDNA as template. After purification with Sephadex G-50 (Sigma), the labeled probe was used to hybridize with the membrane blotted RNA from various mouse tissues at 42 ℃ in a solution containing 50 % formamide, 5 × SSPE, 10 × Denhardt's reagent, and 0.5 % SDS for 24 h. After hybridization, the membrane was exposed to X-film at −80 ℃ for 4 d. The intensities of the hybridization signals on X-film was determined by scanning and analyzed with GDS 8000 Complete Gel Documentation & Analysis System (Bio-Rad).

RESULTS

Computational assembly of the novel gene With BLASTN program, the human *GABARAPL2* cDNA was used as information probe to search mouse EST database. A series of overlapping EST were obtained and then assembled into an EST-contig (Fig 1). The contig contain 1012 nucleic acid.

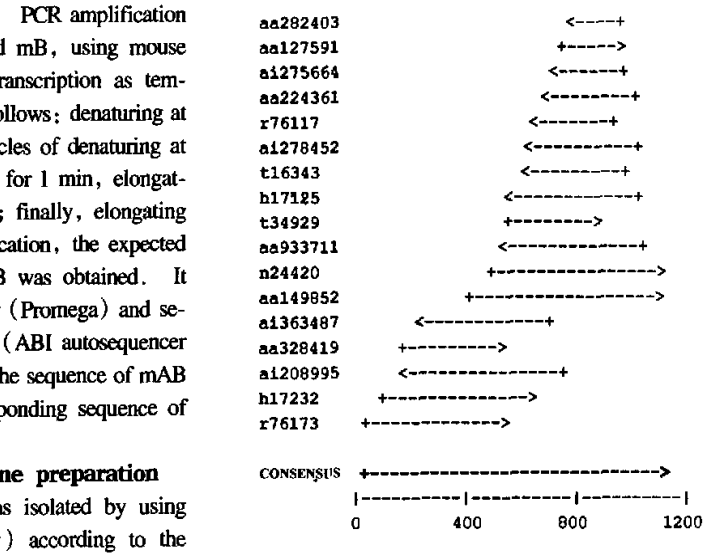

Fig 1. Mouse *Gabarapl2* EST-contig (only part of the typical EST fragments were shown).

Cloning and identification of mouse *Gabarapl2* PCR amplification was performed on mouse brain reverse transcript cDNA and the obtained fragment was sequenced. Thus the natural existence of the gene was confirmed and a cDNA of 1012 bp in length was cloned. It contains an intact open reading frame (nt 116 – 469) coding for 117 amino acids. The canonical polyadenylation signal AATAAA (nt 957 – 962) followed by polyA tail was also present (Fig 2). By searching database, it was found that the gene was a novel one. The sequence was then sent to GenBank and was deposited under Accession № AF190644. Since the deduced amino acid se-

quence of the novel gene shares 57.3 % identity and 67.5 % similarity with that of human *GABARAP*, and is completely identical to that of human *GABARAPL2*, it was later termed *Gabarapl2* by Mouse Gene Nomenclature Committee.

Homologous comparison and domain analysis The putative protein of Gabarapl2 has a calculated molecular weight of 13 700 and an isoelectric point of 8.56 determined by PCGENE program. It was predicated by PROSITE program (http://www.expasy.ch/prosite) to contain two protein kinase C phosphorylation sites ($_{18}$SAK$_{20}$, $_{72}$SEK$_{74}$) and one tyrosine kinase phosphorylation site ($_{99}$KDEDGFLY$_{106}$) (Fig 2). It was also found that the *N*-terminal region was rich in basic amino acids. By BLASTN program of NCBI, it was found that there existed a family of GABA receptor-associated proteins in various animals such as mouse, rat, and bovine, except human, sharing high homology. The alignment of them clearly illustrates their relationship (Fig 3).

Tissue expression of mouse *Gabarapl2* Northern hybridization of mouse *Gabarapl2* was carried out in 11 mouse tissues. The results showed that it was expressed as a single transcript, with high levels in brain, thymus, lung, heart, kidney, and liver, and low in pancreas, testis, small intestine, colon, and stomach (Fig 4). The size of transcripts in all the tissues expressed was 1.35 kb.

DISCUSSION

The neurons anchor various receptors with high concentrations at postsynaptic sites, matching specifically the receptors with neurotransmitter released from the presynaptic terminal. Recently, several studies suggest that

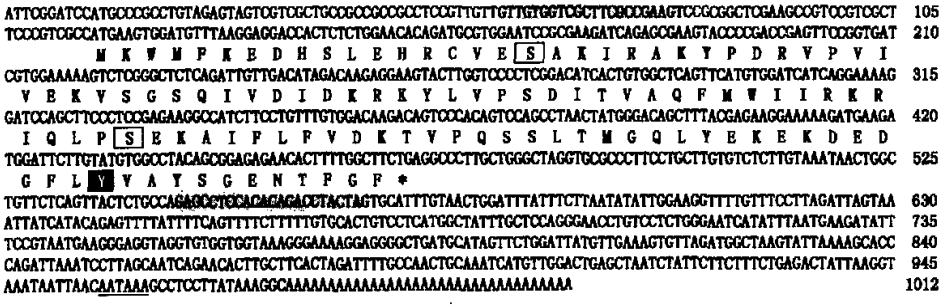

Fig 2. Mouse *Gabarapl2* cDNA and its deduced amino acid sequence. The canonical polyadenylation signal AATAAA is underlined. Tyrosine kinase phosphorylation site is highlighted black. Protein kinase C phosphorylation site is boxed. Primers mA and mB to identify the gene are shaded in gray.

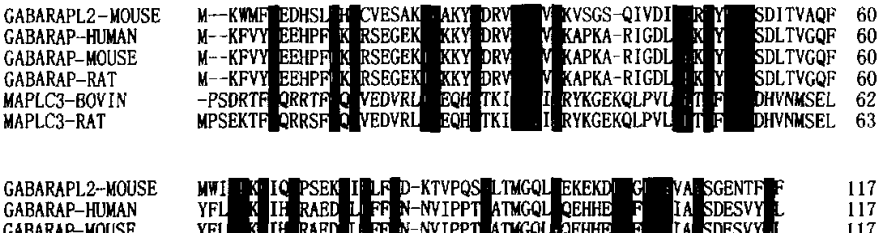

Fig 3. The multiple alignment of the deduced amino acid sequence of homologs of mouse Gabarapl2 (AF190644) in different animals: GABARAP-HUMAN (AF161586), GABARAP-MOUSE (AF161587), GABARAP-RAT (AF161588), MAPLC3-BOVIN (041515), MAPLC3-RAT (U05784). The conserved amino acid residues are highlighted.

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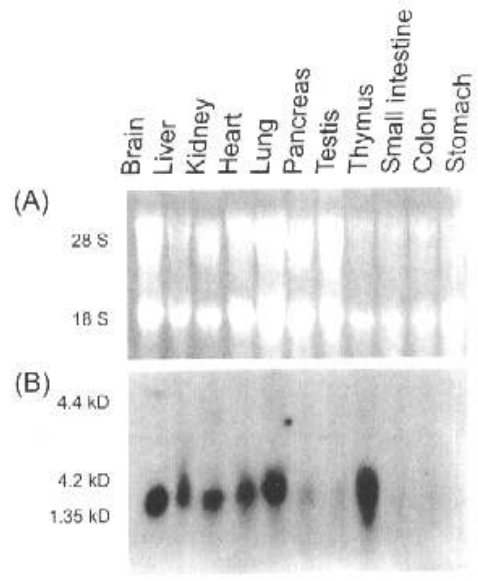

Fig 4. (A) The electrophoresis pattern of RNA on denaturing agarose gel. Each lane was loaded with 20 – 30 μg total RNA from 11 different mouse tissues. (B) Northern blot result of mouse *Gabarapl2* on nylon membrane blotted with 11 mouse tissue RNA.

receptor-associated proteins are involved in forming postsynaptic specializations of receptors, possibly by linking the receptors to the postsynaptic cytoskeleton. Wang *et al* found that GABARAP could bind to both the intracellular loop of the γ2 subunit of $GABA_A$ receptors and microtubules[6]. Hanley *et al* have revealed that microtubule-associated protein 1B (MAP1B) could bind to ρ1 subunit of $GABA_C$ receptors, and the expression of MAP1B in COS cells lead to the intracellular aggregation of ρ1 molecules, suggesting that ρ1's interaction with MAP1B had a "clustering" function[7]. Besides for $GABA_A$ and $GABA_C$ receptors, the receptor of glycine, another inhibitory neurotransmitter, is similarly mediated by a microtubule-binding protein, gephyrin, to attach to the cytoskeleton[8]. Although no direct interactions between $GABA_A$ receptor and gephyrin could be demonstrated by experiments, inhibiting gephyrin expression can cause loss of $GABA_A$ receptor clusters in cultured cortical neurons[9]. It showed that gephyrin appeared to be involved in the postsynaptic organization of $GABA_A$ receptors. Many experiments showed that the functional properties of recombinant $GABA_A$ receptors composed of different kinds of subunits and subtypes were not identical[10–12]. It is reminiscent of the existence of various proteins linking receptors to cytoskeleton.

The putative *Gabarapl2* reported here might be such a kind of protein. It has an overall positive charge. Its *N*-terminal region is rich in basic amino acids, which might bind to the acidic residues of the binding domain of tubulin. Furthermore, according to GOR method[14], the 22 residues in its *N*-terminus were predicted to fold into an α-helix, with basic amino acids (K_2, K_6, H_9, H_{13}, K_{20}) aligning on one side of the helix. Although no tubulin-binding motif (KKEE, KKEI/V)[15] corresponding to that of microtubule associated protein is present in Gabarapl2, Gabarapl2 might mediate $GABA_A$ receptors to microtubules by the positively charged α-helix of *N*-terminus which confered most of the tubulin-binding activity to Gabarapl2.

Several phosphorylation sites were predicted from Gabarapl2, such as protein kinase C phosphorylation site and tyrosine kinase phosphorylation site. Gabarapl2 exhibits 57.3 % identity and 67.5 % similarity to GABARAP, whose sequence is similar to light chain 3 (LC3) subunit of microtubule associated protein 1A and 1B (MAP1A and 1B). Since phosphorylation of certain MAP regulated the microtubule dynamics and contributed to the organization of microtubule cytoskeleton[16–18], phosphorylated Gabarapl2 might be involved in the cytoskeleton changes and consequent modulation of the function of the $GABA_A$ receptors. Gabarapl2 seems to be an intermediate molecule in the signal transduction pathway of gamma-aminobutyric acid.

Since mouse is a suitable model animal, cloning mouse *Gabarapl2* is beneficial to the studies of the functions of both mouse and human homologs. The deduced protein encoded by mouse *Gabarapl2* was shown to be completely identical to that of human *GABARAPL2*, revealing the highly conservation of the gene between human and mouse. Although the gene is expressed highly in brain as expected, shown by Northern blot analysis, the mRNA concentrations in thymus and lung were also high. The ubiquitous expression pattern implies Gabarapl2 was involved in many different biological events, besides its participation in GABA receptor localization.

In conclusion, a novel gene coding for mouse $GABA_A$-receptor-associated protein like 2 was cloned and identified in this study.

REFERENCES

1 Matsumoto RR. GABA receptors: are cellular differences reflected in function? Brain Res Brain Res Rev 1989; 14: 203–25.

2 Belhage B, Hansen GH, Elster L, Schousboe A. Effects of gamma-aminobutyric acid (GABA) on synaptogenesis and synaptic function. Perspect Dev Neurobiol 1998; 5: 235 – 46.
3 Whiting PJ, Bonnert TP, McKernan RM, Farrar S, Le Bourdelles B, Heaven RP, *et al*. Molecular and functional diversity of the expanding GABA-A receptor gene family. Ann NY Acad Sci 1999; 868: 645 – 53.
4 Olsen RW, Tobin AJ. Molecular biology of $GABA_A$ receptors. FASEB J 1990; 4: 1469 – 80.
5 Macdonald RL, Olsen RW. $GABA_A$ receptor channels. Annu Rev Neurosci 1994; 17: 569 – 602.
6 Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW. GABA (A)-receptor-associated protein links GABA(A) receptors and the cytoskeleton. Nature 1999; 397: 69 – 72.
7 Hanley JG, Koulen P, Bedford F, Gordon-Weeks PR, Moss SJ. The protein MAP-1B links GABA (C) receptors to the cytoskeleton at retinal synapses. Nature 1999; 397: 66 – 9.
8 Froehner SC. Gathering glycine receptors at synapses. Science 1998; 282: 1277 – 9.
9 Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B. *Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the gamma subunit and gephyrin*. Nat Neurosci 1998; 1: 563 – 71.
10 Sieghart W, Fuchs K, Tretter V, Ebert V, Jechlinger M, Hoger H, *et al*. Structure and subunit composition of GABA (A) receptors. Neurochem Int 1999; 34: 379 – 85.
11 Fisher JL, Macdonald RL. Single channel properties of recombinant $GABA_A$ receptors containing gamma 2 or delta subtypes expressed with alpha 1 and beta 3 subtypes in mouse L929 cells. J Physiol (Lond) 1997; 505: 283 – 97.
12 Neelands TR, Fisher JL, Bianchi M, Macdonald RL. Spontaneous and gamma-aminobutyric acid (GABA)-activated GABA (A) receptor channels formed by epsilon subunit-containing isoforms. Mol Pharmacol 1999; 55: 168 – 78.
13 Nusser Z, Sieghart W, Somogyi P. Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 1998; 18: 1693 – 703.
14 Garnier J, Osguthorpe DJ, Robson B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 1978; 120: 97 – 120.
15 Noble M, Lewis SA, Cowan NJ. The microtubule binding domain of microtubule-associated protein MAP1B contains a repeated sequence motif unrelated to that of MAP2 and tau. J Cell Biol 1989; 109: 3367 – 76.
16 Mann SS, Hammarback JA. Molecular characterization of light chain 3. A microtubule binding subunit of MAP1A and MAP1B. J Biol Chem 1994; 269: 11492 – 7.
17 Mann SS, Hammarback JA. Gene localization and developmental expression of light chain 3: a common subunit of microtubule-associated protein 1A (MAP1A) and MAP1B. J Neurosci Res 1996; 43: 535 – 44.
18 Avila J, Dominguez J, Diaz-Nido J. Regulation of microtubule dynamics by microtubule-associated protein expression and phosphorylation during neuronal development. Int J Dev Biol 1994; 38: 13 – 25.

一种新的小鼠 *Gabarapl2* cDNA 的克隆和鉴定

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关键词: $GABA_A$ 受体; 人类; 小鼠; 氨基酸序列同源性; 分子模型; 逆转录聚合酶链反应; RNA 印迹法; DNA

目的: 克隆一个新的小鼠 $GABA_A$ 受体相关蛋白相似蛋白 2 基因(*Gabarapl2*), 并对其功能进行初步分析. **方法**: 将已知的人 *GABARAPL2* cDNA 序列为信息探针筛选 GenBank 小鼠 EST 数据库, 将获得的一系列互相重叠的同源度较高的 EST 序列进行拼接. 经过实验验证, 在小鼠中分离和鉴定了新基因. 自行制备小鼠 RNA 印迹膜, 用杂交方法分析该基因在不同组织中的表达情况. **结果**: 新基因在 GenBank 注册, 登录号 AF190644. 同源比较显示, 该推导蛋白与人 *GABARAPL2* 基因编码的蛋白完全相同, 被基因命名委员会命名为小鼠 *Gabarapl2*. 该基因的推导蛋白含多个蛋白激酶磷酸化位点. 杂交显示, 该基因在脑、胸腺等组织表达较高, 转录本大小约 1.35 kb. **结论**: 克隆到一个新的小鼠 *Gabarapl2* 基因.

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