The *rgl-1* is a legitimate homologue of *lethal giant larvae* recessive oncogene in rat

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Abstract. We have cloned a rat homologue of the *Drosophila* recessive oncogene *lethal (2) giant larvae* from rat brain by RT-PCR using primers prepared from sequences conserved amongst *lgl* family genes. Sequence analysis predicts that the rat *rgl-1* gene encodes a 1,036 amino acid polypeptide with a molecular weight of approximately 112 kDa, which contains a domain characteristic of WD-40 proteins. Northern blot analysis revealed that the highest expression of *rgl-1* is detected in the testis, with moderate expression in ovary, brain, spleen, and kidney. Since there is a high degree of amino acid similarity among lgl proteins in various species, it is likely that there is an evolutionary relationship among these proteins. The amino acid identity of rgl-1 to *Drosophila* l(2)gl and mouse mgl-1 proteins showed 30.6 and 96.8%, respectively. The rat tomosyn, previously known as a homologue of *Drosophila l(2)gl*, showed much lower amino acid identity to *Drosophila* l(2)gl and mouse mgl-1 proteins (17.8 and 20%, respectively). Functional analysis showed that the expression of a rat *rgl-1* cDNA in *Saccharomyces cerevisiae* missing *sop* genes, the yeast homologues of the *Drosophila l(2)gl*, restored partially the Na^+ tolerance of the cell. Taken together, these results indicate that *rgl-1*, not tomosyn, is the legitimate homologue of *lgl* gene.

Introduction

Tumor suppressor genes have been identified in organisms including *Drosophila* and humans. Genetic alterations in these genes, detected in both invertebrate and vertebrate tumors, drive the cell to proliferate or evade death. A number of known tumor suppressor genes encode cell surface receptors, cell adhesion molecules, cytoplasmic proteins, and nuclear factors. Many of these proteins are involved in signal transduction pathways that participate in the regulation of cell proliferation or death (1,2).

Of more than 50 tumor suppressor genes identified by mutations causing overgrowth of tissues, the l*ethal (2) giant larvae* [*l(2)gl*] gene was the first recessive oncogene described in *Drosophila*. It has been shown that homozygous mutations lead to the development of transplantable neoplasm of the presumptive adult optic centers of the larval brain and of the imaginal discs (3,4). Genetic alterations in several tumor suppressor genes have also been shown to cause overgrowth of imaginal discs. These include *l(2)gl*, *dlg* (*discs large*), and *scrib* (*scribble*) (5,6).

The products of a number of tumor suppressor genes in both *Drosophila* and humans show similar cellular and nuclear localizations, as well as conservation of amino acid sequences suggesting that these proteins may play similar roles in both organisms. The *l(2)gl* homologues have now been identified in organisms from yeast to humans (*sop1, sop2, C. elegans* homologue, *mgl-1, bgl-1, hugl*), suggesting that there is an evolutionary relationship among these genes (7-12). This has lead to their classification as a discrete *lgl* family.

Recent studies on *lgl* gene products have shown that the members of this family function as components of the cytoskeletal complex (13). In *Drosophila*, this complex is comprised of at least ten additional proteins including a non-muscle myosin type II heavy chain (zip) (14), a serine kinase (14), a nucleosome-assembly-protein-1 (NAP-1) (15), and D-abelson (abl) (16). In addition, the *Drosophila* l(2)gl protein has been shown to homo-oligomerize (17) and to be involved in inter-cellular interactions (18). The presence of WD-40-like motifs is a characteristic feature of proteins in this family (11,18). A large number of proteins containing the WD-40 repeat sequence have been identified in eukaryotes. This sequence feature has been shown to be important for a number of cellular functions, including protein-protein interaction. It has been demonstrated that *sop* genes in *Saccharomyces cerevisiae*, homologues of *Drosophila l(2)gl*, are required for cation homeostasis and this can be functionally substituted by the *Drosophila l(2)gl* tumor suppressor gene (11).

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Table I. The primer sequences designed for *rgl-1* cloning.

	Primer names	Sequences	Origin from mouse *mgl-1*
1	RGLF-1.1	5'-GC**GGATCC**GGGCGCAAGATG-3'	-9-3
2	RGLR-1.1	5'-AAGTCCAGGGTCACCAGT-3'	987-1,004
3	RGLF-1.57	5'-CAACAAGATTCTGTGGCGGAG-3'	870-890
4	RGLR-1.57	5'-CGCCTGGGCCAGGTCCCG-3'	2,422-2,439
5	RGLF-0.8	5'-GTGGCCATTGCTGTGCTG-3'	2,362-2,379
6	RGLR-0.8	5'-ACCCCTAGTTGCCCTGGG-3'	3,133-3,150

The underlined sequence (for RGLF-1.1 primer) was designed for cloning site of *Bam*HI.

In this study, we report the isolation and expression pattern of a rat homologue, *rgl-1*, to the *lgl* tumor suppressor gene family and rat *rgl-1* also can function for cation homeostasis in *Saccharomyces cerevisiae*. Molecular cloning and structural characterization of rat *rgl-1*, as well as studies on its expression, may provide information on the functionally important domains of this molecule and its regulation during development.

Materials and methods

RT-PCR and cloning of rat rgl-1. In order to investigate the expression of *lgl* family members in the Sprague-Dawley rat brain, we performed RT-PCR using three pairs of primers designed with reference to cDNA coding sequence identified as mouse *mgl-1* (GenBank accession number 414350). The nucleotide sequences of these primers are shown in Table I. The following combinations of these primers were used for amplification: RGLF-1.1 and RGLR-1.1; RGLF-1.57 and RGLR-1.57; RGLF-0.8 and RGLR-0.8. Total RNA isolated from rat brain using TRIzol (Gibco BRL) served as the template for reverse transcription. PCR amplified fragments with predicted sizes were subcloned into pGEM-T Easy vector (Promega) and introduced into *Escherichia coli* DH5α. Since the amplification primers were designed to produce three overlapping fragments, the open reading frame (ORF) was assembled and both DNA strands were analyzed, confirming the fidelity of the sequence information.

Northern blot analysis. Expression of the rat *rgl-1* mRNA was studied by Northern blot analysis (Fig. 3A). Total cellular RNA was isolated from various adult rat tissues. Thirty μg of RNA were electrophoresed on denaturing formaldehyde gels and blotted onto nylon membranes (Boehringer Mannheim) by capillary blot. The immobilized nucleic acids were then hybridized with a radiolabeled DNA probe corresponding to nucleotides 1-592 bp of the *rgl-1* clone. The probe used for Northern blot analysis was labeled by random primers with [α-^{32}P]dCTP. Hybridization was carried out in a bag containing 5X SSC, 1X Denhardt's solution, 100 μg/ml of denatured salmon sperm DNA, 0.1% SDS and 50% formamide at 42˚C overnight. After extensive washing at 68˚C in 0.1X SSC and 0.2% SDS, the membrane was exposed to Kodak X-ray film with an intensifying screen for 24 h.

Expression of rat rgl-1 protein in Escherichia coli. In order to investigate the molecular weight of rgl-1 protein, we selected a vector suitable for expression of recombinant *rgl-1* in *E. coli*. The *rgl-1* cDNA was cloned into *Bam*HI- *Eco*RI-digested pGEX-2TK (Promega), downstream of the glutathione-S-transferase (GST) coding element and transformed into *E. coli* MC1061. Fusion protein expression was induced by the addition of IPTG (isopropyl-ß-thiogalactopyranoside) to exponentially growing bacterial cultures. Cells were collected and lysed in lysis buffer [0.01 M phosphate (pH 7.4), 8 M urea, 1% SDS, and 1% ß-mercaptoethanol]. These whole cell lysates were analyzed by immunoblotting with a rabbit anti-GST antiserum, an HRP-conjugated secondary antibody and enhanced chemiluminescence (ECL).

Determination of sodium tolerance. The analysis for the tolerance to salt stress was previously described (16). To investigate the capability of sodium tolerance for rat rgl-1 in *Saccharomyces cerevisiae*, a salt-sensitive mutant strain *sop1*Δ *sop2*Δ (MAT a *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1 ura3-1 sop1*Δ::*LEU2sop2*Δ::*HIS3*) was used. Wild-type strain *W303* (MAT a *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1 ura3-1*) was used as a control. To express the rat *rgl-1* gene in *Sacchromyces cerevisiae*, a construct for the constitutive expression of the *rgl-1* gene was generated by subcloning a 3,111-bp fragment of *rgl-1* cDNA into the polylinker of the pYX212 plasmid (R&D Systems Inc.). The complementation was analyzed in a high salt medium containing 0.3 M NaCl.

Results

Cloning of rgl-1 cDNA in the rat brain. A potentially significant gene, *rgl-1*, was identified in the rat brain using RT-PCR. For reverse transcription, total RNA isolated from rat brain was used as a template. And three pairs of primers used were designed with reference to cDNA coding sequence of mouse *mgl-1* (GenBank accession number 414350). Amplified fragments with predicted sizes were subcloned into pGEM-T Easy vectors, and the nucleotide sequences were determined by automated sequencing. Since the primers for RT-PCR were designed to produce three overlapping fragments, the open reading frame (ORF) was determined by combining these sequences. We found that the full-length cDNA for *rgl-1* consists of 3,111 nucleotides in open reading frame and the deduced amino acid sequence of 1,036 amino acids (Fig. 1). The predicted structure of the rgl-1 protein is similar to other members of lgl family (Fig. 2). The lgl family

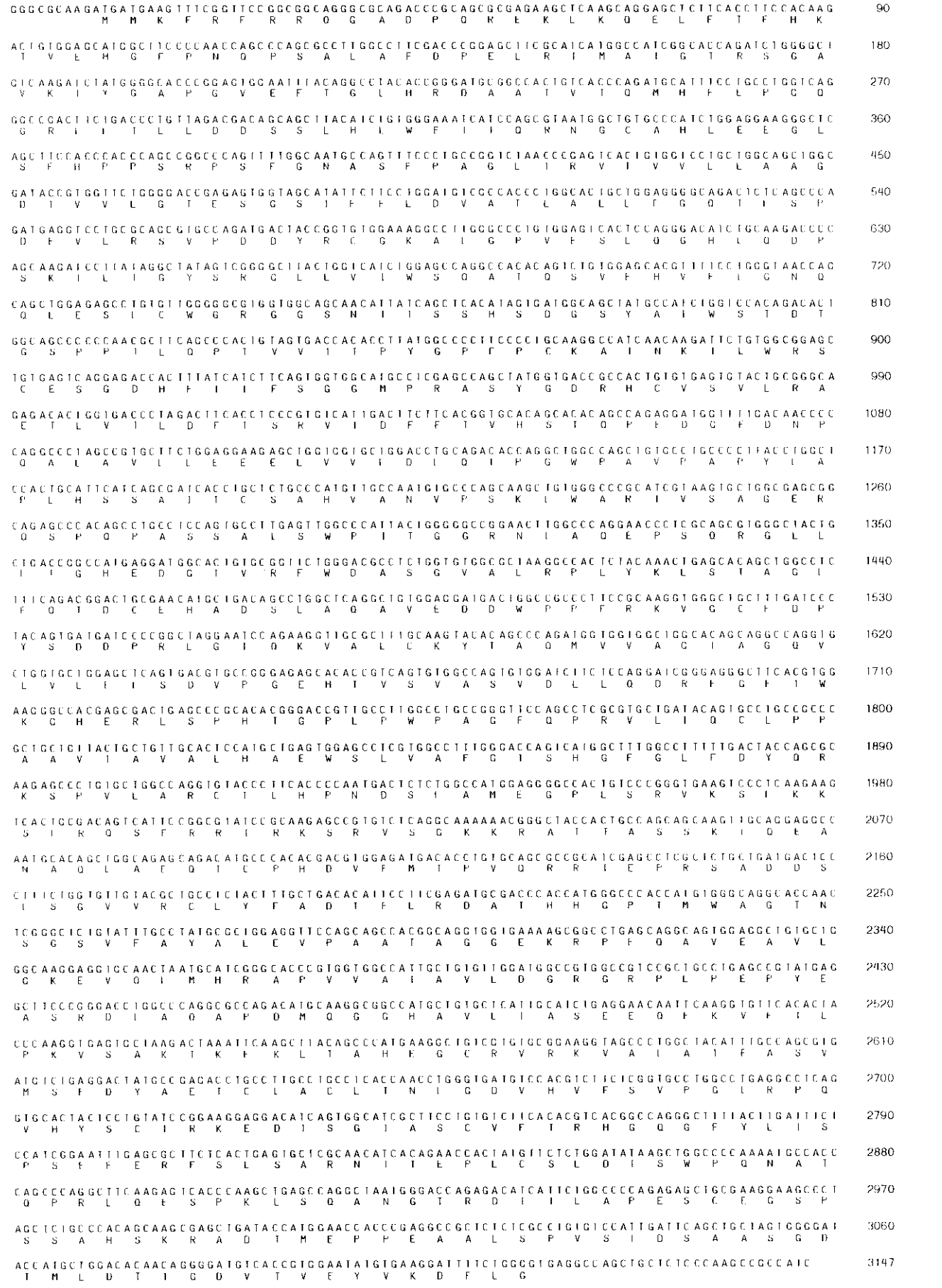

Figure 1. Nucleotide sequence of the rat *rgl-1* cDNA and the predicted amino acid sequence. The numbers indicate the positions of nucleotides from the initiation codon. The stop codon is indicated by a dot.

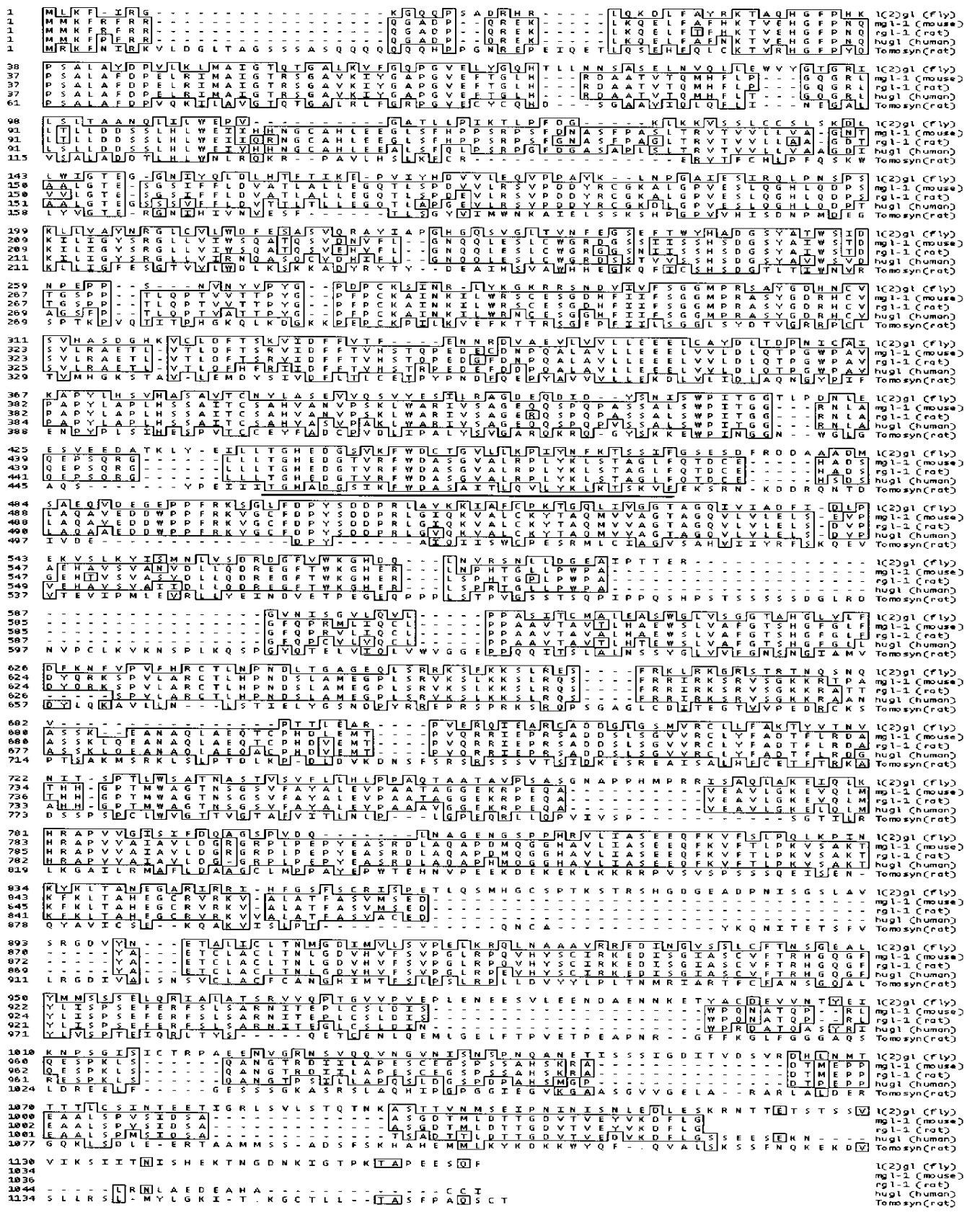

Figure 2. Alignment of amino acid sequences derived from GenBank accession numbers, fly l(2)gl (M17022), mouse mgl-1 (414350), rat rgl-1 (AF356187), human hugl (784996), and rat tomosyn (U92072), using MegAlign software (clustal method) from DNAStar (LaserGene). Identical amino acids are blocked in all five sequences. WD-40 repeat sequences are underlined.

members are usually expressed in various organs including the brains (8,20) and are known to be associated with other cytoskeletal proteins including a non-muscle myosin type II heavy chain and a serine kinase (10,14).

Table II. Nucleotide and the amino acid similarity between rat rgl-1 and other lgl family members.

lgl family	Nucleotide sequence identity[a] (%)	Amino acid sequence identity[a] (%)
Human	81.6	87.4
Mouse	94.5	96.8
Fly	27.7	30.6

[a]The sequence of human, mouse, and fly *lgl* were obtained from GenBank with accession number human *hugl* 784996, mouse *mgl-1* 414350, and fly *l(2)gl* M17022, respectively.

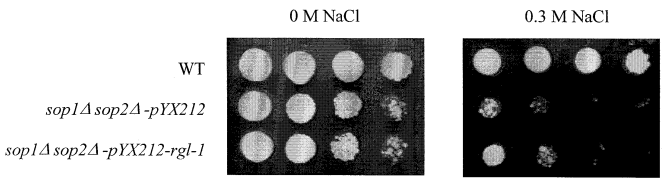

Figure 4. Complementation for the salt sensitivity of the *sop1*Δ *sop2*Δ double mutant. The wild-type strain and the *sop1*Δ *sop2*Δ mutant strain transformed with the rat *rgl-1* cDNA inserted in the multicopy pYX212 plasmid were used for the complementation analysis. Cells were grown overnight in YEPD medium, adjusted to OD_{610} = 1, and serial 10-fold dilutions spotted onto YEPD plates supplemented with 0.3 M NaCl or without NaCl. Plates were incubated for 2 days at 30˚C prior to photograghy.

Sequence comparison. After the isolation of the rat *rgl-1* cDNA, we found that the predicted protein exhibits the structural characteristics of lgl family. The amino acid sequences of lgl family members are derived from the GenBank and they are aligned using the Clustal method with DNAStar program from LaserGene (Fig. 2). The nucleotide sequence of the ORF for *rgl-1* gene showed a homology of 81.6 and 94.5% identity to that of human and mouse, respectively (Table II). It has been previously suggested that rat tomosyn, a syntaxin-binding protein, is the homologue of *Drosophila* l(2)gl and mouse mgl-1 (21). Tomosyn shows overall amino acid identities of 17.8 and 20%, respectively, to these proteins and also contains WD-40 repeat sequences (11,18). However, based on the fact that the rgl-1 protein also contains the WD-40 repeat and shows much higher amino acid identity to the *Drosophila* l(2)gl (30.6%) and mouse mgl-1 (96.8%) proteins (Fig. 2 and Table II), we believe it to be more likely that *rgl-1* is the legitimate rat homologue of *lgl* gene.

Expression analysis of rgl-l. Expression of the rat *rgl-1* mRNA was studied by Northern blot analysis (Fig. 3A). It revealed that all tissues expressed a 4 kb *rgl-1* transcript. The highest expression of *rgl-1* was detected in the testis, with moderate expression in ovary, brain, spleen, and kidney. 18S/28S ribosomal RNA served as convenient loading control for these experiments (Fig. 3A). The molecular weight of rgl-1 protein was determined by Western blot analysis (Fig. 3B). The *rgl-1* cDNA was subcloned into an expression vector (pGEX-2TK) and was expressed as a fusion protein with the glutathione-S-transferase (GST). The expressed protein is a molecular weight of approximately 112 kDa (GST = 26 kDa), largely as predicted.

Complementation of a salt-sensitive yeast mutant by rat rgl-1 gene. The salt sensitivity of yeast mutant strains has been provided an assay to investigate the functional relationship between the *sop* gene products and the *Drosophila l(2)gl* gene product (11). It has been demonstrated that *Drosophila* lgl protein can substitute the function of SOP proteins in yeast, indicating that there is functional conservation of this family of proteins (11). In order to investigate whether the *rgl-1* gene product can restore the salt sensitivity in the absence of SOP function in yeast, we introduced *rgl-1* cDNA into the multicopy pYX212 plasmid under the control of constitutive yeast TPI promoter and determined whether the rat *rgl-1* cDNA would complement. The analysis of salt sensitivity showed that the rgl-1 decreased the Na^+ sensitivity of the *sop1*Δ *sop2*Δ cells to some extent (Fig. 4), indicating a functional relationship between the rgl-1 protein and the SOP proteins.

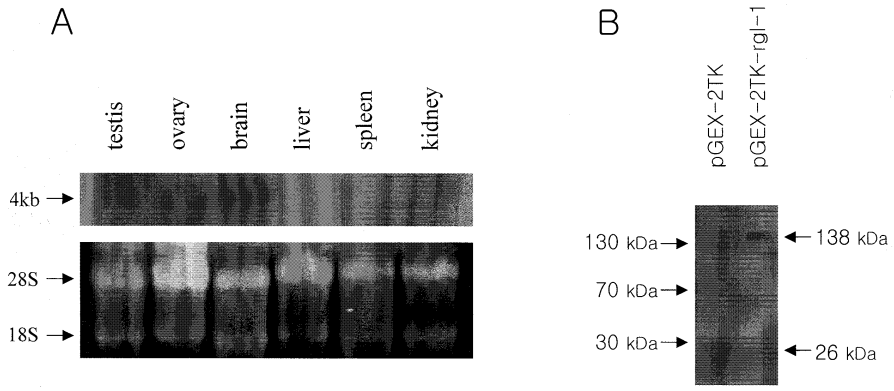

Figure 3. (A), Expression of *rgl-1* mRNA in rat organs. Total RNA was prepared from rat testis, ovary, brain, liver, kidney, and spleen for Nothern blot analysis. (B), Expression of rgl-1 protein *in vitro*. The molecular weight of rgl-1 protein is approximately 112 kDa (138 kDa minus GST molecular weight 26 kDa).

Discussion

It has been demonstrated that homozygous mutations at the *l(2)gl* locus in *Drosophila* result in the development of transplantable neoplasms of the presumptive adult optic centers of the larval brain and of the imaginal discs (3,4). Previously, we isolated a temperature-sensitive mutant allele of ($l(2)gl^{ts3}$) by ethylmethane sulfonate (EMS) screening (22). The temperature sensitivity derives from the substitution of a serine residue with a phenylalanine residue at position 311 (23). Using this mutant allele, it has been shown that the l(2)gl protein is required for organization of the follicle cells during oogenesis (23). Since it has been proposed that this protein is homologous to vertebrate cadherin cell adhesion molecules such as chicken L-CAM and mouse P-cadherin at the amino acid level (24,25), one interpretation of this finding is that lgl family members may play a role in cell-cell communication. Biochemical and genetic analyses revealed that *Drosophila* l(2)gl protein has been found in a cytoskeletal network, which has at least ten proteins including a non-muscle myosin type II heavy chain (zip) (14), a nucleosome-assembly-protein-1 (NAP-1) (15), a serine kinase (14), and D-abelson (abl) (16). This suggests that lgl protein is involved in protein-protein interaction in the cytoskeletal network. In addition, SOP proteins in yeast, known as homologues of lgl family members, function to regulate cation homeostasis and can be functionally substituted by the *Drosophila lethal (2) giant larvae* tumor suppressor gene (11). However, the detailed functions of these proteins have yet to be elucidated.

The *l(2)gl* homologues in other organisms have been identified (7-12) and the amino acid similarity among these genes is very high (Table I). Therefore, members in this gene family seem to be conserved throughout evolution and more investigations should be done to better understand the structural and functional roles during development in vertebrates and invertebrates. The *rgl-1* cDNA we isolated from the rat brain is very homologous to *lgl* family members and can also substitute the function of SOP proteins in yeast, indicating that the rat rgl-1 protein can regulate cation homeostasis. This provides evidence for functional conservation between the rat rgl-1 protein and its yeast homologues as shown between the *Drosophila* l(2)gl protein and yeast homologues (11). In addition, this indicates that *rgl-1*, not tomosyn, is the legitimate homologue of *lgl* gene family in rat. We hope that the cloning of the *rgl-1* cDNA in the rat brain should facilitate investigations of cellular functions for these family members in cell-cell interactions and of cation homeostasis during development in a convenient mammalian model organism.

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