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The *GARP* Gene Encodes a New Member of the Family of Leucine-rich Repeat-containing Proteins[1]

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Abstract

We have characterized a new human gene, named *GARP*, localized in the 11q14 chromosomal region. *GARP* comprises two coding exons, is expressed as two major transcripts of 4.4 and 2.8 kilobases, respectively, and encodes a putative transmembrane protein of 662 amino acids, the extracellular portion of which is almost entirely made of leucine-rich repeats. The molecular weight of the protein immunoprecipitated from transfected cells is 80,000. The GARP protein has structural similarities with the human GP Ibα and GP V platelet proteins, and with the Chaoptin, Toll, and Connectin adhesion molecules of *Drosophila*.

Introduction

As more proteins are being identified, their sequences show that many of them are members of larger families which share one or more common structural motifs. These motifs can be considered a perfect functional fit obtained long ago in evolution, and they constitute a repertoire from which molecules with different functions can be assembled. For example, motifs such as immunoglobulin-like loops, cysteine-rich repeats, fibronectin type III domains, tyrosine kinase domains, and many others, are often found in cell surface molecules. When studying the human 11q13-q14 chromosomal region, which is amplified in breast carcinomas (see Ref. 1 for a review; 2), we isolated a new gene which we named *GARP* (3). The mouse homologue gene is located on chromosome 7, in a region conserved between humans and mice. We show here that this gene encodes a putative transmembrane protein that is composed mostly of a particular type of motifs, named LRR.[3]

LRR have been identified in a wide variety of proteins from species as distantly related as yeast and humans. The average length of a leucine-rich unit is 24 amino acids. It is characterized by a periodic distribution of hydrophobic amino acids, especially leucine residues, and folds into an amphipathic β structure (4). The number of units varies from 1 to over 40.

Proteins with LRR appear to be involved in a variety of functions. They are often membrane bound but can also either be secreted (5) or exhibit a cytoplasmic (6) or nuclear (7) localization. LRR are found in molecules as diverse as proteoglycans, adhesion molecules, enzymes, tyrosine kinase receptors, or G-coupled hormone receptors. The LRR domain seems to be involved in protein-protein interactions. A group of several LRR proteins has been identified in *Drosophila* (8). The Toll, Chaoptin, and Connectin adhesion molecules and the Slit secreted protein seem to play important roles in cell differentiation, morphogenetic events, and migration of cells and axons (5, 9–12). In humans, several members of the LRR family are known. The proteoglycans decorin (13) and biglycan (14), the platelet GP Ibα receptor for the von Willebrand factor (see Ref. 15 for a review), and a number of other molecules (16–23) all contain LRR. These motifs appear to be necessary for the activity of the protein (24–27).

Results

Cloning of the Human *GARP* Gene and cDNA. The initial cloning of *GARP* genomic sequences has been reported (3). These sequences were derived from clones cos9 and cos105 (Fig. 1*A*), isolated from cosmid libraries that were made from DNAs extracted from normal blood cells and the MDA-MB-134 breast carcinoma cell line, respectively. This cell line has an amplified 11q13-q14 region (28, 29).

Several cDNA clones were then obtained by screening a human cDNA placenta library with genomic subclones of fragments PR98 and D, identified as carrying exonic sequences (3) (Fig. 1*A*). Overlapping cDNA clones were obtained and characterized (Fig. 1*D*). Two types of cDNA were obtained (Fig. 1*C*). Accordingly, Northern blot hybridization of human placenta poly(A)$^+$ RNA showed that *GARP* was expressed as two major transcripts of 4.4 and 2.8 kb, respectively (3). Fig. 1*B* shows a tentative map of the *GARP* locus. The *GARP* gene seems to comprise only two coding exons, the first one containing the signal peptide and nine amino acid residues, and the second one containing the rest of the coding sequence and the 3′ untranslated region (see below).

Nucleotide and Deduced Amino Acid Sequence of the Human *GARP* cDNA. The complete nucleotide sequence was determined for the longest cDNA overlap of 4.1 kb (long form). The largest open reading frame is able to code for a protein of 662 residues, starting with a methionine residue located at the beginning of a stretch of hydrophobic amino acids, which putatively constitutes a signal peptide. This domain is followed by a putative extracellular region which constitutes most of the molecule. Twenty LRR are present in the extracellular region. A second hydrophobic stretch of amino acids may correspond to a transmembrane domain. Finally, a short putative intracellular region of 15 residues

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[3] The abbreviations used are: LRR, leucine-rich repeat(s); cDNA, complementary DNA; poly(A)$^+$, polyadenylated; kb, kilobase(s); kDa, kilodalton(s); ADNIV, autosomal dominant familial neovascular inflammatory vitreoretinopathy; ADFEVR, autosomal dominant familial exudative vitreoretinopathy; SDS, sodium dodecyl sulfate.

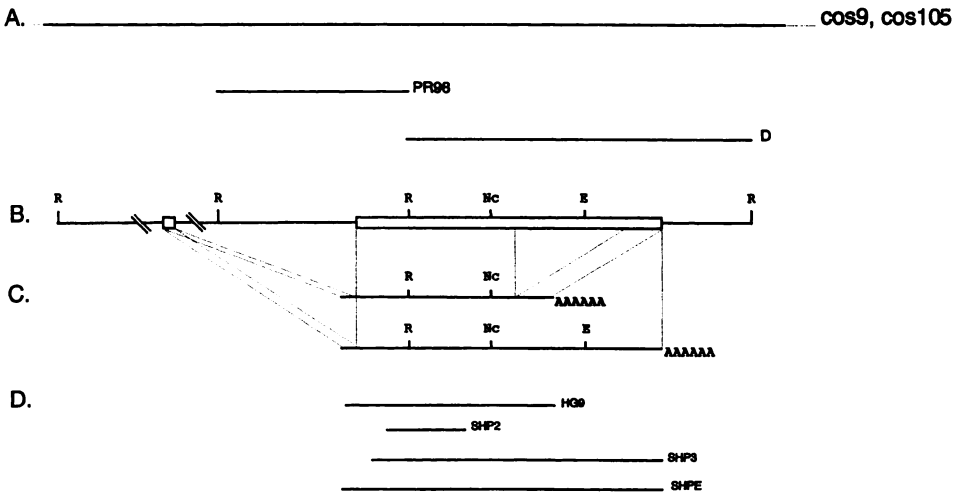

Fig. 1. Structure of the human *GARP* gene. *A*, the inserts of cosmid clones cos9 and cos105 are partially represented. Two genomic fragments derived from cos9 and cos105, PR98 and D, of 2.4 and 4.5 kb, respectively, are shown *below*. Subclones of these fragments (described in Ref. 27) were derived to screen a cDNA library. *B*, tentative physical map of the *GARP* gene. *Boxes*, the two exons. The location of the first exon within an *Eco*RI fragment of 7 kb is not known. Selected restriction enzymes are as follows: *E*, *Eco*RV; *Nc*, *Nco*I; *R*, *Eco*RI. *C*, representation of the two forms of *GARP* complete cDNAs. The positions of some restriction enzymes indicated in *B* are shown. *D*, inserts of overlapping cDNA clones.

terminates the primary translational product of the *GARP* gene. The deduced amino acid sequence of the putative GARP protein, shown in Fig. 2, thus exhibits several specific features of a transmembrane molecule. It has a calculated molecular weight of 72,000. In the long form, a long 3' untranslated tail of 2052 nucleotides is followed by a polyadenylation signal. In the short cDNA form, which was also sequenced, a region of 1545 nucleotides is absent (Fig. 2, *arrowheads*). The nucleotide sequence of the *GARP* gene was also obtained from genomic clones and allowed us to determine the organization of the gene. Most of the extracellular region, the transmembrane domain, and the intracellular region of the putative GARP protein are encoded within one large exon (Fig. 1*B*).

The 20 leucine-rich units are grouped in two blocks, separated by a short proline-rich segment, and constitute about 70% of the molecule. They are aligned for comparison in Fig. 3. They show a typical structure with interspersed hydrophobic (predominantly leucine) and hydrophilic residues.

Comparison of the *GARP* Gene Product with Other LRR Proteins. Due to the presence of leucine-rich repeats, the GARP protein (hereafter designated as Garpin) has structural similarities to other proteins. Fig. 4*A* shows comparative alignments of the consensus motifs found in some LRR proteins. Some of the LRR proteins are known to share amino acid similarity extending in either amino-terminal or carboxy-terminal LRR-flanking regions, or both. Comparison of these flanking regions is shown in Fig. 4*B*. Specific residues found in other LRR molecules in the NH_2-terminal LRR-flanking region, as well as in the COOH-terminal LRR-flanking region (especially four cysteines that are highly conserved among these proteins), were also found in Garpin. Therefore, in all portions of the molecule, Garpin resembles the other LRR proteins. This can be seen in the schematic representation of some of these proteins (Fig. 5).

Biochemical Characterization of the *GARP*-encoded Product. The cDNA insert SHPE (see Fig. 1), subcloned into a Bluescript vector to obtain pC-huGARP, was used as a template for *in vitro* translation experiments. pC-huGARP was transcribed *in vitro*, either from the T3 (antisense transcript) or T7 (sense transcript) promoters. RNAs were translated *in vitro* in a rabbit reticulocyte lysate (see "Materials and Methods"). The results are shown in Fig. 6*A*. One band with an apparent molecular mass of 72 kDa was detected in the sense lane. This corresponds to the calculated molecular mass of the unglycosylated *GARP* gene product.

NIH 3T3 cells were transfected with a *GARP*-containing plasmid. Clones expressing the *GARP* mRNA (data not shown) were selected, and one such clone, called NG-14, was radiolabeled with [^{35}S]methionine. Immunoprecipitation of the *GARP* gene product from NG-14, carried out using the rabbit antiserum, is shown in Fig. 6*B*. A band of 80 kDa was observed. As a control, *in vitro* translated products were immunoprecipitated with the rabbit anti-Garpin immune serum. A band with the expected molecular mass of 72 kDa was observed.

The difference between the molecular mass of the *in vitro* translated product and the immunoprecipitated protein could be explained by the presence of *N*-linked glycosylation. This was directly tested by treating NG-14 *GARP*-expressing cells with tunicamycin, an inhibitor of *N*-linked glycosylation. After such a treatment, the apparent molecular weight of Garpin was reduced to 72,000, consistent with the predicted molecular weight of the amino acid backbone.

Expression of the *GARP* Gene in Human Tissues. Expression of *GARP* in human tissues was studied by hybridizing a commercial Northern blot filter containing poly(A)$^+$ RNAs, from eight different tissues, with a human probe (Fig. 7). As previously observed (3), two bands of 4.4 and 2.8 kb, respectively, were detected in placenta. The same level of expression was observed in lung and kidney. Weaker signals were detected in heart, liver, skeletal muscle, and pancreas. No expression was evidenced in brain.

Discussion

We have characterized a new gene which codes for a putative protein with leucine-rich repeats. The deduced pri-

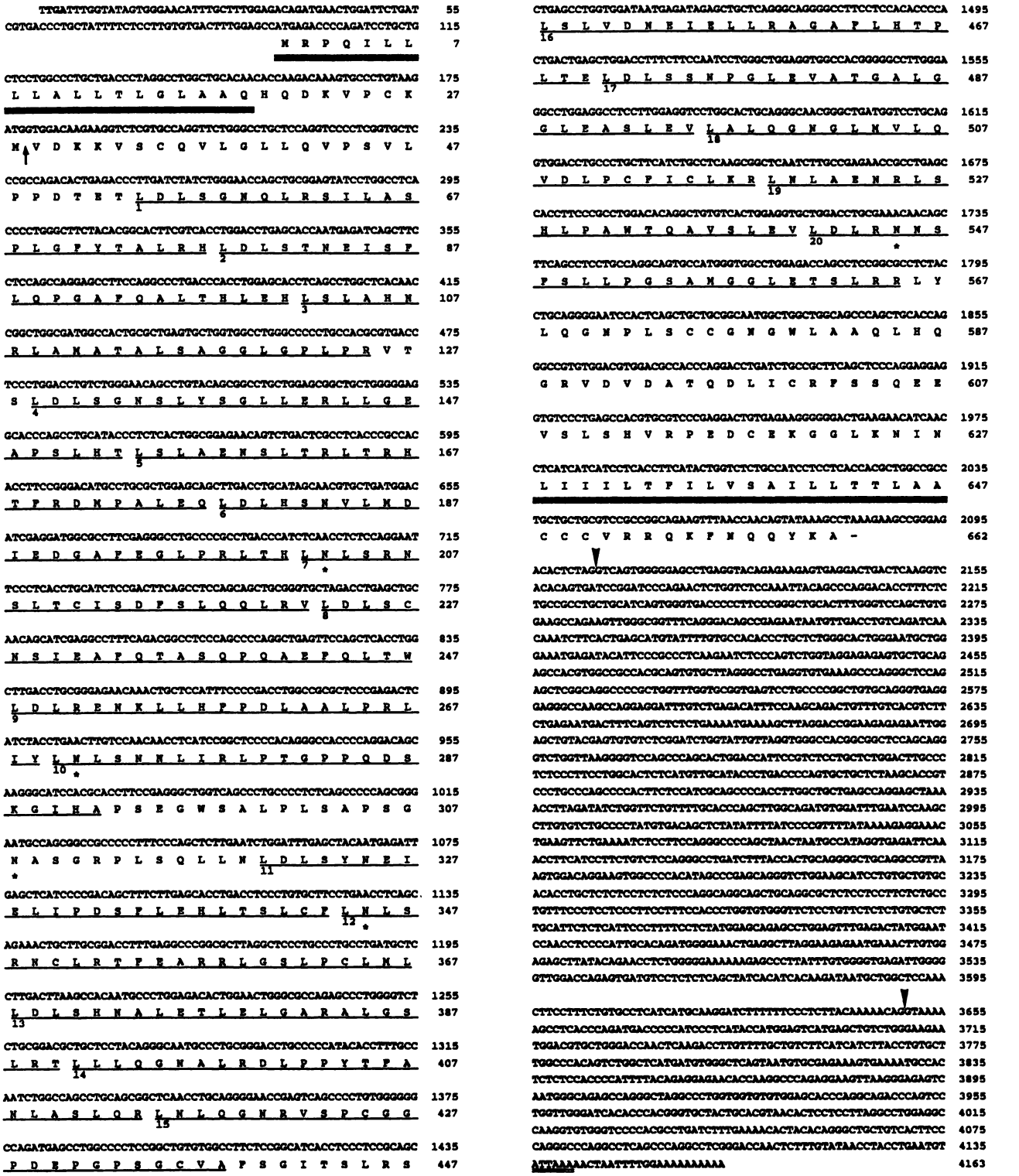

Fig. 2. Nucleotide and deduced amino acid sequence of the human *GARP* cDNA. The *GARP* nucleotide sequence codes for a putative transmembrane protein. The coding sequence is characterized, from 5′ to 3′, by the presence of a putative signal peptide (*underlined* in *bold*), two series of 10 blocks of LRR (*underlined* and *numbered*), separated by a proline-rich sequence, a putative transmembrane domain (*underlined* in *bold*) followed by a short, potentially intracellular, carboxy-terminal tail. *Asterisks*, five potential *N*-linked glycosylation sites. In the 3′ untranslated region, a polyadenylation signal is indicated (*underlined*). The *arrow* at position 178 indicates the 3′ limit of exon 1 and the 5′ limit of exon 2. *Arrowheads* delimitate the 3′ untranslated region spliced out in the short form of the *GARP* transcripts. *Numbers* relative to nucleotide and amino acid positions are shown at *right*.

amino acid																										
54-77	1	L	D	L	S	G	N	Q	L	R	S	I	L	A	S	P	L	G	F	Y	T	A	L	R	H
78-101	2	L	D	L	S	T	N	E	I	S	F	L	Q	P	G	A	F	Q	A	L	T	H	L	E	H
102-128	3	L	S	L	A	H	N	R	L	A	M	A	T	A	L	S	A	G	G	L	G	P	L	P	R
129-153	4	L	D	L	S	G	N	S	L	Y	S	G	L	L	E	R	L	L	G	E	A	P	S	L	H	T
154-177	5	L	S	L	A	E	N	S	L	T	R	L	T	R	H	T	F	R	D	M	P	A	L	E	Q
178-201	6	L	D	L	H	S	N	V	L	M	D	I	E	D	G	A	F	E	G	L	P	R	L	T	H
202-222	7	L	N	L	S	R	N	S	L	T	C	I	S	D	F	S	L	Q	Q	L	R	V			
223-247	8	L	D	L	S	C	N	S	I	E	A	F	Q	T	A	S	Q	P	Q	A	E	F	Q	L	T	W
248-269	9	L	D	L	R	E	N	K	L	L	H	F	P	D			L	A	A	L	P	R	L	I	Y	
270-292	10	L	N	L	S	N	N	L	I	R		L	P	T	G	P	P	Q	D	S	K	G	I	H	A	
320-343	11	L	D	L	S	Y	N	E	I	E	L	I	P	D	S	F	L	E	H	L	T	S	I	C	F	
344-367	12	L	N	L	S	R	N	C	L	R	T	F	E	A	R	R	L	G	S	L	P	C	L	M	L	
368-390	13	L	D	L	S	H	N	A	L	E	T	L	E	L	G	A	R	A		L	G	S	L	R	T	
391-414	14	L	L	L	Q	G	N	A	L	R	D	L	P	P	Y	T	F	A	N	L	A	S	L	Q	R	
415-438	15	L	N	L	Q	G	N	R	V	S	P	C	G	G	P	D	E	P	G	P	S	G	C	V	A	
448-470	16	L	S	L	V	D	N	E	I	E	L	L	R	A	G	A	F	L	H	T	P		L	T	E	
471-495	17	L	D	L	S	S	N	P	G	L	E		V	A	T	G	A	L	G	G	L	E	A	S	L	E	V
496-518	18	L	A	L	Q	G	N	G	L	M	V	L	Q	V	D		L	P	C	F	I	C	L	K	R	
519-540	19	L	N	L	A	E	N	R	L	S	H	L	P	A	W	T	Q			A	V	S	L	E	V	
541-565	20	L	D	L	R	N	N	S	F	S	L	L	P	G	S	A	M	G	G	L	E	T	S	L	R	R
CONSENSUS:		L	D	L	S	-	N	-	L	-	-	L	-	-	-	-	L	-	-	L	-	-	L	-	-	

Fig. 3. Comparison of the leucine-rich repeats of the *GARP*-encoded protein. The amino acid sequence of the 24 amino acids of LRR comprising the main body of the extracellular region are aligned to allow for comparison. Leucine residues are *boxed*. Residues other than leucine found in 50% or more of the units are also *boxed*. *Space* is a gap introduced to maximize alignment. As in other proteins of the family, the asparagine residue (*N*) is invariant. A derived consensus sequence is shown *below*.

mary structure of the human protein, which we have named Garpin, shows the presence of several repeats made of 24 amino acids with a remarkable periodicity of leucine residues. *In vitro* translation experiments yielded a single product of 72 kDa, in good agreement with the calculated molecular mass of the *GARP* gene product. A protein of similar size was immunoprecipitated when products from the *in vitro* experiments were treated with a rabbit anti-Garpin immune serum made against a TrpE-Garpin fusion protein. A protein of higher molecular weight was observed in cells stably transfected with a *GARP* full-length cDNA. The difference in molecular masses could be attributed to *N*-linked glycosylation.

Leucine-rich motifs are found in a number of proteins involved in important biological processes such as embryonic development, cell morphogenesis, cell and axon migration, and blood coagulation. In these molecules, LRR often make up a large portion of the protein. In several instances, LRR have been shown to be essential for the specific activity of the protein (24–27, 30). Most of the *GARP* sequence is encoded within one exon. The genomic structure of some other human LRR proteins is known. In the case of OMgp and GP Ibα, two LRR proteins characterized in humans, the coding sequence is not interrupted by introns (20, 31). In contrast, LRR of the extracellular region of the thyroid-stimulating hormone receptor are encoded by separate exons (22).

Owing to the presence of leucine-rich motifs, Garpin has structural similarity with other LRR proteins. It resembles GP Ibα and GP V (32) and the *Drosophila* adhesion molecules Toll, Chaoptin, and Connectin, in which the LRR constitute a large part of the molecule. This suggests that Garpin also could be involved in protein-protein interactions at the cell surface, especially in adhesion processes. The structure and pattern of expression of the *GARP* gene are compatible with a possible role as an adhesion molecule of its encoded product. This hypothesis is currently being investigated.

GARP is located in chromosomal region 11q13.5-q14 (3), which is amplified in about 17% of breast carcinomas (2, 33). The *CCND1/PRAD1* cyclin gene, located in 11q13.3, is thought to be the key gene of the 11q amplification in breast cancer (34). The region containing *GARP* could either define a separate amplicon or merely represent the telomeric extension of a large amplification. For the time being, the role of *GARP* within the amplicon is not known. However, *GARP* does not exhibit structural similarity with the oncogenes, and it is unlikely that it is involved in the cancerous process. It probably merely represents a "passenger" gene in the amplicon. In addition to DNA amplification and rearrangement in malignant diseases, the 11q13-q14 region is involved in several nontumoral pathologies, some of which are hereditary diseases. ADNIV, ADFEVR, and Usher syndrome type I, in particular, appear to map close to or within the 11q14 region (35–37). In that respect, it is interesting to note that Chaoptin and Connectin, to which Garpin is structurally related, are involved in the development of sensory organs and nervous system in *Drosophila* (10). Whether *GARP* is implicated in pathology, and is a possible candidate for ADNIV or ADFEVR, has yet to be determined. Moreover, the human olfactory marker protein OMP is encoded by a gene located at 11q14-q21 which is a candidate for Usher syndrome type I (38). A cluster of genes involved in pathologies in relation with the development of sensory organs might thus exist in the 11q13.5-q21 region. This possibly includes the *GARP* gene.

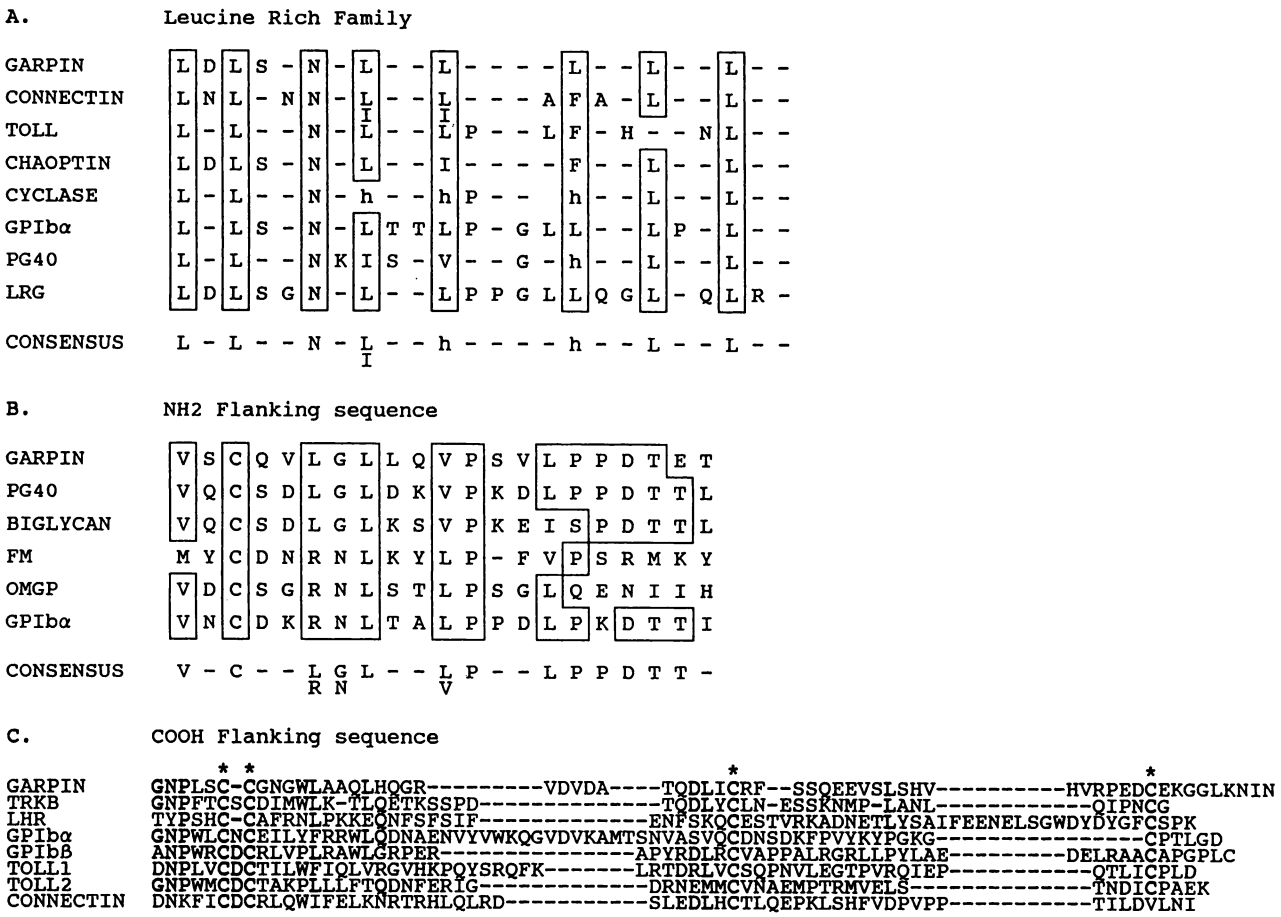

Fig. 4. Comparison of Garpin with other LRR proteins. *A*, alignment of consensus sequences of LRR derived from eight different LRR proteins. *LRG*, leucine-rich α2 glycoprotein of the serum; *h*, hydrophobic residues; *space* is a gap introduced to maximize alignment. *B*, alignment of the amino-terminal LRR flanking region of Garpin with the corresponding regions from other LRR proteins. *FM*, fibromodulin. Conserved residues are *boxed*. *C*, alignment of the carboxy-terminal LRR flanking region of Garpin with the corresponding regions from other LRR proteins. *LHR*, lutropin receptor. The four cysteine residues found in all proteins are in *bold* and indicated by *asterisks*. *Dash*, a gap introduced to maximize alignment.

Materials and Methods

Cells, Tissues, and Embryos. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

cDNA Screening. Two cDNA libraries made from human placenta were screened, and several clones were isolated: HG clone from a Clontech (Palo Alto, CA) library and SHP clones from a Stratagene (La Jolla, CA) library (see Fig. 1). Mouse cDNA clones were obtained by screening a mouse embryo library as described (3). The "BigLow" fragment of 0.5 kb (described in Ref. 3), corresponding to the transmembrane domain, the carboxy-terminal region, and a portion of the 3′ untranslated region, was used as a probe.

Nucleotide Sequencing. Nucleotide sequences were determined with the dideoxynucleotide method, using Bluescript (Stratagene) single-stranded or double-stranded templates and the modified T7 polymerase protocol (Sequenase; U.S. Biochemicals). Both short and long cDNA clones were sequenced, as well as corresponding portions of genomic clones derived from cosmids. Clone SHPE was sequenced on both strands. Sequence analysis was carried out using PC-gene software (Intelligenetics). The accession number for the nucleotide sequence of the *GARP* gene is Z24680.

Antibody Production. A TrpE-*Garp* construct was made by inserting a 1.7-kb *Eco*RI fragment of the murine *Garp* coding region (corresponding, in the human protein, to amino acid residues 467 to 662; see Fig. 2) into the *Eco*RI site of the pATH3 expression vector. A 58 kDa fusion protein was produced in RR1 bacterial cells as described (39). The bacterially expressed protein was injected into rabbits to generate polyclonal antibodies against the product of the *Garp* gene.

***In Vitro* Translation Experiments.** A 2.2-kb *Bam*HI fragment containing the full-length coding sequence of human *GARP*, derived from the cDNA clone SHPE (see Fig. 1), was subcloned into a Bluescript KS$^+$ vector (Stratagene) to obtain plasmid pC-huGARP and was used for *in vitro* transcription and translation assays. *In vitro* transcription was carried out with the T3 (antisense transcript) and T7 (sense transcript) RNA polymerases from the Stratagene RNA transcription kit, according to the manufacturer's instructions. Aliquots of 0.5 to 1 μg of synthesized RNAs were translated in a rabbit reticulocyte lysate system (Promega), in the presence of [^{35}S]methionine (Amersham) according to the recommended protocol. Products of *in vitro* translation were resolved by electrophoresis in a 10% SDS-polyacrylamide gel.

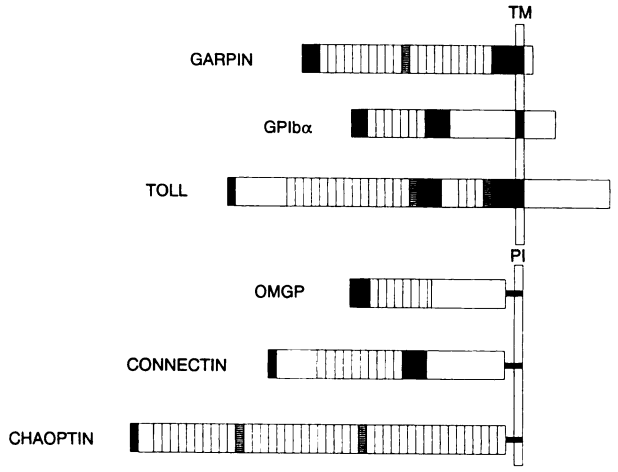

Fig. 5. Schematic representation of Garpin and other LRR proteins. Two types of LRR proteins are represented, *i.e.*, the transmembrane type (*TM*, transmembrane domain) and the PI-anchored type (*PI*, glycosylphosphatidylinositol structure). Three mammalian proteins (Garpin, GP Ibα, and OMgp) and three *Drosophila* adhesive molecules (Toll, Connectin, and Chaoptin) are shown. Putative signal sequences and transmembrane domains are shown in *black*. Blocks of LRR can be separated by a short segment (*horizontal stripes*). Each unit is shown in *open boxes*. LRR flanking regions are *shaded*.

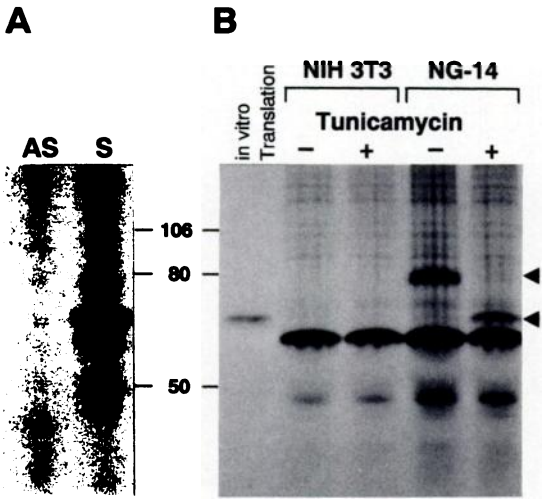

Fig. 6. Biochemical characterization of Garpin. *A*, RNA transcribed *in vitro* from the human *GARP* cDNA clone pC-huGARP were translated in a rabbit reticulocyte lysate system and analyzed by 10% SDS-polyacrylamide gel electrophoresis. Translation was from either antisense (*AS lane*) or sense (*S lane*) transcripts. *B*, immunoprecipitation of Garpin (*arrowheads*) from *GARP*-transfected NIH 3T3 cells (*NG-14*). Control NIH 3T3 cells and *in vitro* translated products are shown. Cells were treated (+) or not (–) with tunicamycin. Migration of molecular mass markers (molecular mass standards from Bio-Rad) is indicated between the two *panels*.

The gel was then fixed, treated for fluorography with Amplify (Amersham), dried, and exposed to Fuji or Kodak films.

Cell Transfections. The full-length cDNA clone derived from SHPE (see Fig. 1) and encoding the human *GARP* gene was inserted in both sense and antisense orientations in the mammalian expression vector pMexNeo (40) to obtain pMexNeo-GARP and pMexNeo-PRAG, respectively. NIH

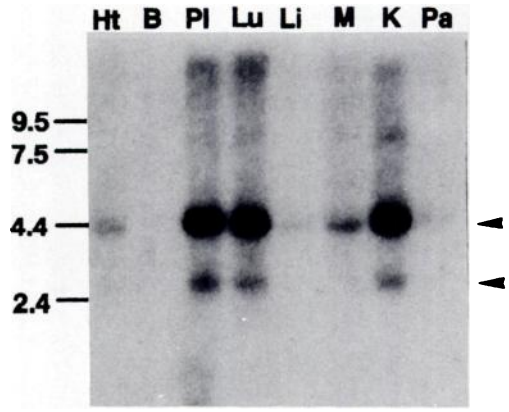

Fig. 7. Expression of the *GARP* gene in human tissues. A commercial Northern blot filter (Clontech), containing 2 μg/lane of poly(A)$^+$ RNA extracted from various human tissues (as indicated *above* each lane), was hybridized with a human *GARP* probe (see "Materials and Methods"). Two major transcripts, of 4.4 and 2.8 kb, respectively, were detected (*arrowheads*). *Ht*, heart; *B*, brain; *Pl*, placenta; *Lu*, lung; *Li*, liver; *M*, skeletal muscle; *K*, kidney; *Pa*, pancreas.

3T3 cells were seeded at 3×10^5 cells/100-mm dish 18 h prior to transfection. Cells were transfected with 10 μg of either pMexNeo-GARP or pMexNeo-PRAG, using Lipofectin reagent (GIBCO-BRL) according to the manufacturer's indications.

Immunoprecipitation Experiments. Approximately 3×10^5 NIH 3T3 cells were labeled for 3 h with 10 μCi [^{35}S]methionine/ml in Dulbecco's modified Eagle's medium minus methionine supplemented with 10% dialyzed fetal calf serum. The labeled cells were lysed in 200 μl of denaturing buffer (50 mM Tris-Cl, pH 7.5-0.5% SDS-70 mM β-mercaptoethanol), boiled for 5 min, and diluted in 800 μl of radioimmunoprecipitation assay buffer (10 mM Tris-Cl, pH 7.5-1% sodium deoxycholate-1% Nonidet P-40-130 mM NaCl-0.25 mM phenylmethylsulfonyl fluoride). All samples were initially preadsorbed with 5 μl of preimmune serum at 4°C for 1 h and then incubated with 40 μl of Protein A-Sepharose CL-4B (Pharmacia) for another 1 h at 4°C. The supernatants of these incubations were then used for immunoprecipitation with anti-Garpin rabbit serum. Samples were incubated with 5 μl of antiserum for 1 h on ice, followed by incubation with 40 μl of Protein A-Sepharose for 1 h at 4°C, on a rocking system. Immune complexes were washed once with buffer A (10 mM Tris-Cl, pH 7.5-150 mM NaCl-2 mM EDTA-0.2% Nonidet P-40), once with buffer B (same as buffer A but with 500 mM NaCl), and once with buffer C (10 mM Tris-Cl, pH 7.5). After boiling in 2× Laemmli buffer, the samples were run in a 7.5% polyacrylamide gel. In some experiments, cells were treated with 10 μg/ml tunicamycin throughout the labeling period.

Northern Blot Hybridizations. A blot filter containing 2 μg/lane of poly(A)$^+$ RNAs extracted from eight human tissues (Clontech) was hybridized with the 2.2-kb insert fragment from pC-huGARP, using the conditions recommended by the manufacturer.

Acknowledgments

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