Vol. 201, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1994 Pages 458-465

# Characterization of the G-protein Linked Orphan Receptor GPRN1/RDC1

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Received April 8, 1994

**SUMMARY:** Site specific antibodies were raised against the second intracellular loop of the G-protein coupled orphan receptor GPRN1 for analysis of receptor protein expression from a number of sources. Immunoblot analyses showed GPRN1 to be a 60 kDa membrane bound glycoprotein. It was found in AR4-2J, PC12 and SK-N-MC cell lines. High amounts of GPRN1 were found in rat stomach, liver, lung, brain, small intestine and pancreas, with lower amounts in spleen and kidney: none was detectable in rat heart. This distribution differs markedly from that of the closely related orphan receptor RDC1. The receptor shows high evolutionary conservation: immunoreactive 60 kDa GPRN1 was detected in membrane glycoprotein fractions from the livers of rat, toad, chick, stingray and shark. It is therefore predicted that GPRN1 and RDC1 represent two tissue specific subtypes of a 60 kDa receptor for a ligand which has likely been highly conserved throughout evolution and which is active upon many organs of the body. 

RDC1 was originally cloned from a dog thyroid library as a putative G-protein coupled orphan receptor, based on its homology to the superfamily of hormone receptors with seven transmembrane helices (1). It is very similar to a recently identified human receptor GPRN1 (91% identity of nucleotide sequence, 94% identity of protein sequence) (2). RDC1 is therefore the dog homolog of GPRN1 or an extremely closely related receptor subtype. GPRN1 was cloned from a human library and claimed to be a receptor for vasoactive intestinal peptide (VIP) (2). However, several groups have failed to observe $^{125}$I-VIP binding to RDC1 transfected cells (3, 4). In addition, RDC1 and GPRN1 bear little homology to the receptor for secretin, which is related to VIP (5); neither is there significant homology between RDC1 and the recently cloned receptor for VIP (6). Further attempts at elucidating the ligand for RDC1 have proved fruitless; it is not a receptor for bradykinin, parathyroid hormone, parathyroid hormone related peptide, calcitonin, fMet-Leu-Phe, cholecystokinin, thrombin, bombesin, endothelin-1,

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0006-291X/94 $5.00

neuropeptide Y nor $arg^8$-vasopressin (4). As an attempt to provide clues to the possible identity of GPRN1 and RDC1 we have raised polyclonal antibodies to a peptide based on the deduced protein sequence of GPRN1 and used these antibodies to characterize the distribution of this receptor from a number of tissues and species.

## METHODS

*Preparation of Anti-peptide Antibodies*

An undecapeptide corresponding to residues 147-156 of the second cytoplasmic loop of the human GPRN1 sequence (Fig. 1) with an additional $NH_2$-terminal cysteine was synthesized in the protein chemistry facility in the Department of Biochemistry and Molecular Biology, MUSC.

The peptide was coupled to *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester-derivatized keyhole limpet hemocyanin (KLH) essentially as described by Lerner et al., (7). Two rabbits (Nadia and Olga) were immunized with peptide conjugates containing 200 µg peptide per aliquot, first using multiple subcutaneous sites of injection in Freund's complete adjuvant. They were then boosted subcutaneously with immunogen in incomplete Freund's adjuvant, followed by monthly intraperitoneal boosts of immunogen in alum with bleeding at two weeks post each boost. Serum was routinely affinity purified by chromatography on receptor peptide-Sepharose (8). Affinity supports were prepared using CH-Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturers instructions. Routinely, 10 ml of serum was diluted with 20 ml of 0.05 M glycine, pH 7.5 containing 0.5 M NaCl (9) and added to a one ml slurry of receptor-peptide-Sepharose. Following overnight incubation the slurry was packed into a column and the matrix-bound antibody eluted with 0.2 M glycine, pH 2.5, containing 0.5 M NaCl (9). The fractions were neutralized, pooled, dialyzed against water and BSA (0.1% final concentration) was added as carrier. The affinity purified IgG was stored in aliquots at -20°C. Typically, 0.2-0.5 mg (BioRad Protein Assay, IgG as standard) of purified IgG was obtained from 10 ml of serum.

*Membrane Preparation*

Rat lung membranes were prepared according to Provow and Veliçelebi (10). Synaptosomes from rat brains were purified according to Gray and Whittaker (11). Rat pancreata were homogenized in a Dounce homogenizer with ten volumes of 0.3M sucrose containing 0.1% soybean trypsin inhibitor, 1mM PMSF, and 1mM 2-mercaptoethanol. After filtration through cheesecloth, the sucrose concentration of the homogenate was brought to 1.25 M by the addition of 2.0 M sucrose. The homogenate was overlaid with 1.20 M and 0.3 M sucrose and centrifuged for 90 min. at 149,000 x g. Membranes at the 0.3-1.2M interface were collected, washed, and stored in 50 mM Hepes, pH 7.4, 0.15 M NaCl, 5 mM $MgCl_2$ (HMS buffer) containing 0.2% BSA, 1 mM PMSF and 1 mM bacitracin at -80°C. Rat liver plasma membranes were prepared according to Neville to step 11 (12). Membranes from all the other rat organs were prepared by homogenization in 0.25M sucrose, 5mM EDTA, 1mM PMSF, 1mM bacitracin, 1µg/ml aprotinin and 5µM leupeptin with a Tissumizer. The homogenates, excepting the small intestine, were further dispersed in a Dounce homogenizer with 8 strokes of the loose fitting pestle. Debris was pelleted by centrifugation (3,310 x g/15 min/4°C) and the supernatants were centrifuged at 30,000 x g/30 min/4°C. The membrane pellets were suspended in HMS with 1mM PMSF, 1mM bacitracin, 1µg/ml aprotinin and 5 µM leupeptin using a teflon pestle. Liver membranes from 13 day embryonic chicks (*Gallus domesticus*), from marine toad (*Bufo marinus*), from stingray (*Dasyatis americana*) and from dogfish shark (*Squalus acanthias*) were prepared according to Stuart (13).

Cultured cells from subconfluent flasks were washed twice in HMS and harvested by glass beads in an isotonic buffer consisting of HMS containing 0.1% STI, 1 mM PMSF, 1 mM bacitracin, 0.2 μM aprotinin, 5 μM leupeptin and 5 mM EDTA. After centrifugation (8 min/800 x g/4° C) the cells were resuspended in a hypotonic buffer of 50 mM HEPES, pH 7.4, containing the indicated protease inhibitors. The cells were incubated for five minutes at 4° C then passed through a 25 gauge needle ten times to disrupt the cell membranes. The lysate was centrifuged at 800 x g for 8 min at 4° C and the supernatant was recovered and centrifuged (30 min/12,000 x g/4°C) in a Hill microcentrifuge. The membrane pellet was recovered, washed twice in HMS containing protease inhibitors, and stored in aliquots at - 80° C.

*WGA-Agarose Chromatography*

In preparation for chromatography, membranes (1-2 mg) were solubilized in 25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM $MgCl_2$, 0.2% BSA, 0.01% STI, 1 mM bacitracin, 1 mM PMSF, 2% NP-40 by incubation on ice for 10 min and gentle mixing for 5 min. Insoluble material was removed by centrifugation at 15,000 x g for 30 min. The soluble fraction was diluted to 0.2% NP-40 by addition of 50 mM HEPES containing 0.1 M NaCl and added to the WGA-Sepharose beads equilibrated in 50 mM HEPES, pH 7.5 containing 0.1 M NaCl and 0.1% NP-40 and packed in a column. The flow-through was recycled three times and the gel was sequentially washed with 0.1 M NaCl, 0.5 M NaCl, and finally 0.3 M GlcNAc in 0.5 M NaCl all in 50 mM HEPES, pH 7.5 containing 0.1% NP-40. The eluate was concentrated to ~1mg/ml in a Centriprep 30 and frozen as aliquots at -80°C until use.

*SDS Gel Electrophoresis*

Membrane proteins were resolved by SDS-PAGE according to the method of Laemmli (14). Samples were solubilized in 0.125 M Tris, pH 6.95, containing 4% SDS, 10 mM EDTA, 15% sucrose, 0.1 M DTT, and 0.01% bromophenol blue. The stacking gel (3% acrylamide, pH 6.95) and the resolving gel (7.5% acrylamide, pH 8.9) solutions contained 2 mM EDTA. Running buffer was adjusted to pH 8.75 and contained 2 mM EDTA, 25 mM Tris, 0.1% SDS and 0.2 M glycine. Molecular weight markers were myosin ($M_r$ = 205,000), β-galactosidase ($M_r$ = 116,500), phosphorylase b ($M_r$ = 97,400), bovine serum albumin ($M_r$ = 66,000) and ovalbumin ($M_r$ = 45,000).

*Immunoblot Analysis*

Proteins resolved on SDS gels were transferred onto nitrocellulose using a one-buffer system containing 48 mM Tris, 39 mM glycine, 0.0375% SDS and 20% MeOH. After transfer, the nitrocellulose was quenched for at least 60 min in 10 mM Tris, pH 8.0, 0.15 M NaCl and 0.05% Tween (TBST) containing 5% nonfat dry milk (14). Filters were then incubated with affinity purified antibody (0.5 μg/ml) in the same buffer for 18 h at 4°C. The filters were then incubated with peroxidase-conjugated goat anti-rabbit antibody secondary for 1 h at room temperature and reacted with the ECL Western blotting detection system for 1 min followed by exposure of the blots to Kodak XRP x-ray film for 30 sec to 10 min.

## RESULTS AND DISCUSSION

*Characterization of Antipeptide Serum*

Sera from both rabbits immunized with a peptide from the proposed second intracellular loop of GPRN1 (Fig. 1) yielded specific IgG when purified by antigen-Sepharose affinity chromatography. Antibody from the first bleed showed positive

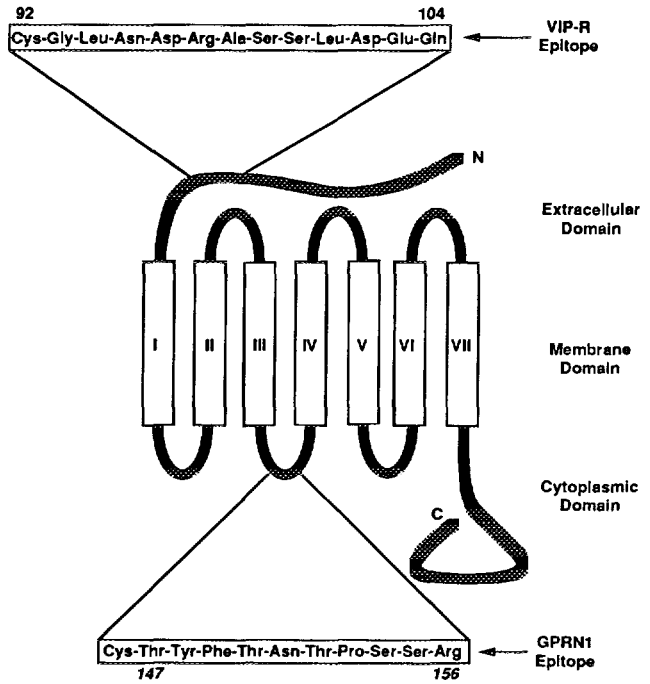


Figure 1. Schematic Diagram of GPRN1 depicting peptide sequence to which the site-specific antibodies were made.

reactivity toward peptide in dot-blot analyses (data not shown). The yields from subsequent bleeds were higher (up to 0.5 mg from 10 ml serum) and all experiments reported in this paper used affinity purified antibody from bleeds 2 and 3.

To test for reactivity against the receptor protein, wheat germ agglutinin agarose enriched membranes from a number of cell types were resolved by reducing SDS-PAGE and immunoblot analysis. As shown in Figs. 2A and 2B, the antibody recognizes a 60 kDa protein band in membranes from rat pancreas, from the rat pancreatic acinar cell line AR4-2J, from the pheochromocytoma line PC12 and from the neuroblastoma line SK-N-MC. The fact that PC12 cells have high amounts of GPRN1 further underscores the unlikelihood of GPRN1 being a receptor for vasoactive intestinal peptide; PC12 cells do not display high affinity binding sites for VIP (16). In addition, whereas SK-N-MC cells were found to express low levels of GPRN1 they exhibited considerable $^{125}$I-VIP binding sites (Law and Rosenzweig, unpublished observations). The presence of GPRN1 in wheat germ agglutinin-agarose enriched membranes indicates that it is a membrane bound glycoprotein as predicted. The nucleotide sequence predicts a 43 kDa protein with 4 potential sites of *N*-glycosylation (2); therefore there is approximately 17 kDa of oligosaccharide associated with the protein.

*Organ Distribution of GPRN1*

Wheat germ agglutinin-agarose enriched membranes from the major organs of the rat were resolved by SDS-PAGE and immunoblotted with α-GPRN1 antibody. As can

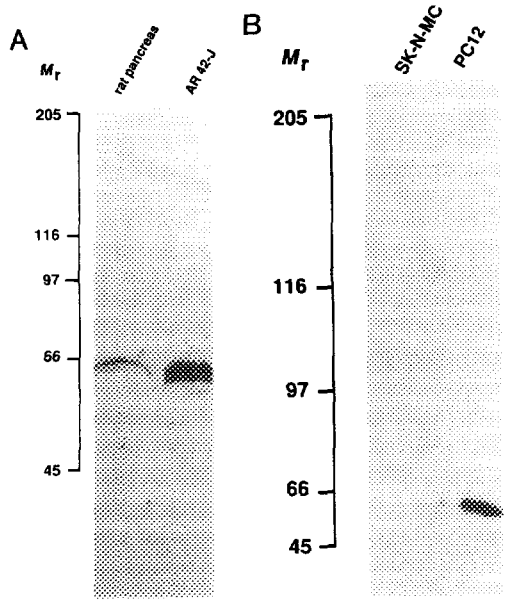


**Figure 2.** Immunoblot analysis of GPRN1 from epithelial and neuronal cells.
**A.** 80 µg of wheat germ agglutinin (WGA)-agarose enriched proteins from rat pancreatic plasma membranes (RPPM) and rat pancreatic acinar cell line, AR4-2J cell membranes were resolved on 7.5% SDS-PAGE and probed with anti-GPRN1 antibody. Shown is a 30s exposure of the immunoblot.
**B.** 20 µg of WGA-agarose enriched membrane proteins from the pheochromocytoma cell line, PC12h and the neuroblastoma cell line SK-N-MC were immunoblotted with anti-GPRN1 antibody. Shown is a 5 min exposure of the immunoblot.

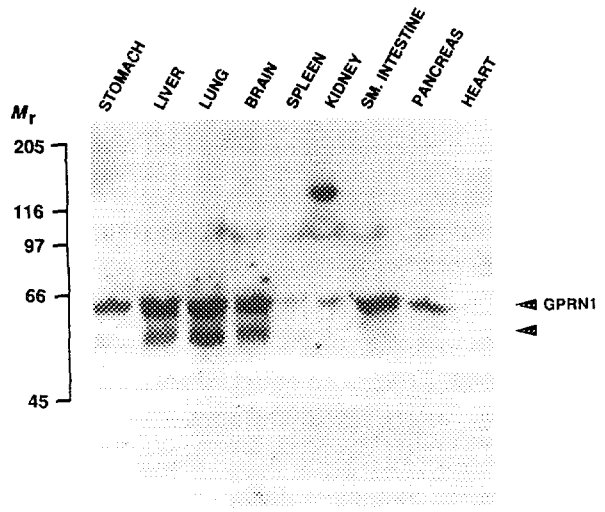


**Figure 3.** Expression of GPRN1 in various rat tissues.
30 µg of WGA-agarose enriched membrane proteins from the indicated rat organs were immunoblotted with anti-GPRN1 antibody. Shown is a 1 min exposure of the immunoblot.

be seen in Fig 3., a 60 kDa species was detected in all organs tested with the exception of the heart. Low, albeit detectable, levels were present in the rat spleen and kidney. A cross-reacting 125 kDa species of unknown identity was also found in kidney. A 55 kDa band was detected in some samples shown in Fig 3. most notably, liver, lung and brain. The amount of this band showing up is very variable and seems to depend upon the age of the membrane preparation; it is therefore likely to represent a proteolytic breakdown product of GPRN1.

The distribution of GPRN1 contrasts sharply with the organ distribution found for the closely related receptor RDC1. RDC1 was thought to be the canine homolog of human GPRN1, having 91% identity at the nucleotide level, and 94% identity at the protein sequence level. However, Northern blotting analysis of RNA from the various tissues of the dog with RDC1 probe indicates it is expressed at high levels in heart and kidney, with much lower levels in the other organs (1). This distribution is exactly opposite to that found for GPRN1 so it raises the possibility that GPRN1 and RDC1 are actually tissue-specific subtypes of the same receptor. The peptide sequence to which α-GPRN1 antibodies were raised is considerably different than the sequence in the corresponding region of RDC1, therefore cross-reactivity may not be expected.

*Evolution of GPRN1*

To investigate the evolutionary conservation of this receptor, liver membranes from species from a number of different phyla were immunoblotted with α-GPRN1 (Fig. 4A and 4B). There was an immunoreactive 60 kDa protein observed in liver membranes prepared from rat, marine toad (*Bufo marinus*), day 13 embryonic chick (*Gallus domesticus*), stingray (*Dasyatis americana*) and dogfish shark (*Squalus acanthias*), indicating that this receptor is highly conserved. For comparison, membranes from toad retina were immunoblotted in parallel: toad retina is avascular, therefore all the retinal membranes are neural or glial in origin (17). It is clear that the toad neural retina does contain a small complement of the 60 kDa GPRN1 protein (Fig. 3). In some cases, the 60 kDa protein band can be resolved into two extremely closely migrating bands; whether this heterogeneity is due to differential glycosylation, proteolysis, or some other post-translational modification, remains to be established.

RDC1 was first identified as a putative G-protein linked receptor by a cloning procedure dependent upon homology to other members of the seven transmembrane helix family of receptors. Along with RDC1, several other clones were identified: the $\alpha_1$-adrenergic receptor, the $\beta_2$-adrenergic receptor, the $5HT_{1A}$ receptor and 3 receptors of unknown identity termed RDC4, RDC7 and RDC8. Since this work was published, these last three have been identified as $5\text{-}HT_{1D}$ (18), adenosine $A_1$ (19) and adenosine $A_2$ (20) receptors respectively, leaving RDC1 as the only receptor whose ligand is as yet unidentified. From the abundance and evolutionary conservation of GPRN1 detailed in this paper, we predict that the ligand for GPRN1 and RDC1 is physiologically relevant in a wide range of tissues throughout the animal kingdom.

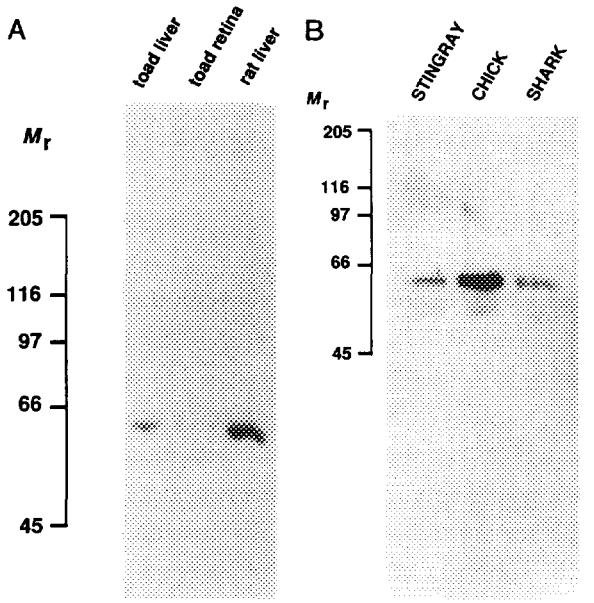


Figure 4. Expression of GPRN1 in evolutionary distant tissues.
A. 50 µg of WGA-agarose enriched membrane proteins from the indicated sources were immunoblotted with anti-GPRN1 antibody. Shown is a 1 min exposure of the immunoblot.
B. 50 µg of WGA-agarose enriched membrane proteins from the livers of chick, stingray and dogfish shark were immunoblotted with anti-GPRN1 antibody. Shown is a 10 min exposure of the immunoblot.

## ACKNOWLEDGMENTS

We thank Pauline Brown and Brad Miller for excellent technical assistance, and the MUSC Audiovisual Service for photographic work; Dr. Don Miller, Department of Cell and Molecular Pharmacology and Experimental Therapeutics, MUSC, for providing the organs from sharks and stingrays. This work was supported, in part, by NIH grant EY-06581 awarded to SAR.

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