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Characterization of the RDC1 gene which encodes the canine homolog of a proposed human VIP receptor

Expression does not correlate with an increase in VIP binding sites

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We have isolated a portion of the canine gene encoding the orphan receptor RDC1 [1]. The complete coding sequence is contained in a single exon, and an intron divides the 5′ untranslated region of RDC1 mRNA. The RDC1 protein is 94% homologous to the gene product of GPRN1, which has been proposed to serve as a VIP receptor when expressed in CHO-K1 and COS-7 cells (Sreedharan, S.P. et al. (1991) Proc. Natl. Acad. Sci. USA 88, 4986–4990). Northern analysis indicates that CHO-K1 cells endogenously express a 2.1 kb RDC1 mRNA. However, while CHO-K1 cells possess detectable low affinity [^{125}I]VIP binding sites, VIP binding is not altered in membranes of CHO-K1 cells expressing varying amounts of the RDC1 gene construct. Further, endogenous VIP binding is not increased by transient expression of RDC1 in COS-7 cells. Taken together, the data suggest that RDC1 is not a canine homolog of the proposed VIP receptor.

RDC1; VIP receptor; Receptor expression; Receptor cloning; G-protein

1. INTRODUCTION

The putative G-protein-coupled orphan receptor RDC1 was originally cloned from a dog thyroid cDNA library [1], and its amino acid sequence has highest homology to receptors for peptide ligands. While the RDC1 sequence bears little similarity to the secretin receptor sequence [3], it shares a sequence identity with a recently cloned human VIP receptor of 91% and 94% at the nucleic acid and amino acid level, respectively [2]. The high degree of both nucleic acid and protein sequence identity between the canine RDC1 and the human VIP receptor suggested that RDC1 is the dog homolog of the proposed VIP receptor encoded by the GPRN1 cDNA [2].

In searching for the ligand for RDC1 and characterizing this gene, we observed that the transcript was ubiquitously expressed in clonal cell lines from a variety of lineages. In this communication we tested whether the RDC1 construct encodes a canine VIP receptor when expressed in the CHO-K1 and COS-7 cells used to clone the putative human VIP receptor [2].

Abbreviations: VIP, vasoactive intestinal peptide; DMEM, Dulbecco's modified Eagle medium; BSA, bovine serum albumin; fMLP, *N*-formyl-Met-Leu-Phe; IL-8, interleukin-8; 1×SSPE, 0.18 M NaCl, 0.01 M NaH_2PO_4, pH 7.7, 0.1 mM EDTA; WT, wild-type (untransfected) cells; FBS, fetal bovine serum.

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2. MATERIALS AND METHODS

2.1. *Materials*

All enzymes were purchased from either Promega (Madison, WI) or Boehringer Mannheim (Indianapolis, IN). [^{125}I]VIP (2000 Ci/mmol), [^{3}H]NMS (79 Ci/mmol), [γ-^{32}P]ATP, [α-^{32}P]dCTP and [α-^{35}S]ATP were purchased from New England Nuclear (Boston, MA). VIP was purchased from Bachem (Torrence, CA).

2.2. *Cell culture*

CHO-K1 cells (American Type Tissue Culture (Rockville, MD)) were maintained in DMEM containing 5% FBS. COS-7 and HT29 cells were maintained in DMEM containing 10% FBS.

2.3. *Isolation, sequencing, and subcloning of the RDC1 gene fragment*

An α-^{32}P-labeled oligonucleotide (45-mer: GCA GCA CGC TTT TGT TGG GCA TGT TGG GGC ACA GCA CGG TGT CCA) derived from the first putative transmembrane region (bp 176–220) of RDC1 [1] was used to screen a canine spleen genomic library in λ DASH (Stratagene, La Jolla, CA) using standard procedures [4]. A single clone was isolated, containing a 2.7 kb *Pst*I fragment that hybridized to this oligonucleotide and an oligonucleotide derived from the carboxy terminus (bp 1105–1149) of the RDC1 sequence [1] (45-mer; denoted RDC1B: CTC CGT CTC CGA CAC CCT GGA GGC ATC GAT GAG CTT GGT GAG ACC). This *Pst*I fragment was subcloned into pBluescript KS II (Stratagene) and sequenced (Se-

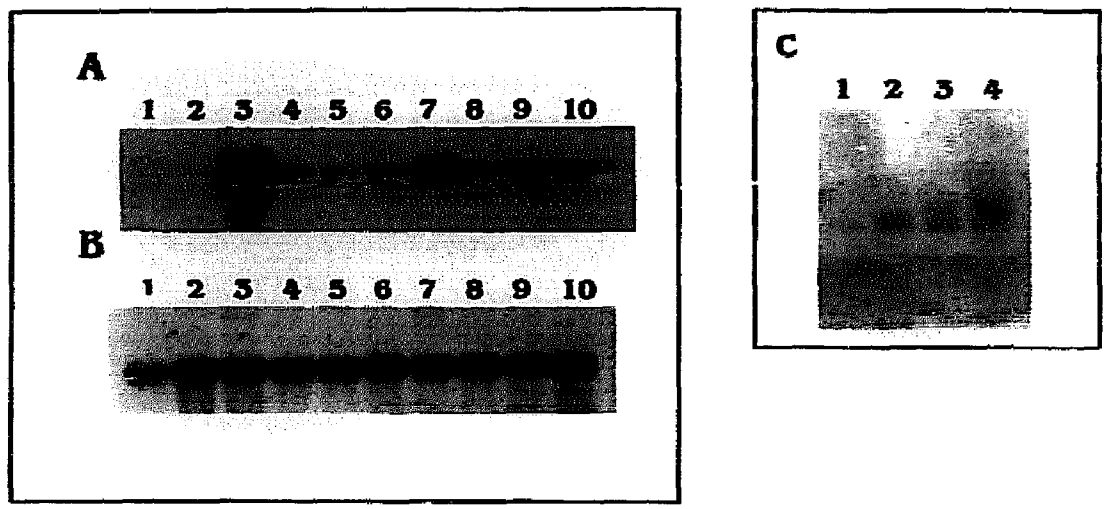

Fig. 1. Northern analysis. Poly A⁺ RNA (2 μg) from a variety of CHO-K1 clones transfected with the RDC1 gene fragment was electrophoresed as described in section 2 and probed with either RDC1B (A) or actin (B). A mouse actin cDNA probe [11] was labeled with [α-^{32}P]dCTP using the multiprime DNA labeling reaction (Amersham, Arlington Heights, IL). Lane 1, the pool of RDC1-transfectants; lanes 2–10 represent individual clones. CHO-RDC1-2 and CHO-RDC1-6 are in lanes 3 and 7, respectively. Exposure = 17 h. (C): Poly A⁺ RNA from either WT (1 or 5 μg, lanes 1 and 2, respectively) or the pool of RDC1-transfectants (1 or 4 μg, lanes 3 and 4, respectively) was used and probed with RDC1B. Exposure = 48 h. The band appearing in the WT cells is 2.13 kb.

quenase, US Biochemicals, Cleveland, OH). The RDC1 coding sequence contained in the *NaeI–SpeI* fragment $RDC1_{614-2190}$ was then subcloned into the mammalian expression vector pCDNA1 (Invitrogen, San Diego, CA) to create the pCDNA-RDC1 plasmid used for the expression experiments. In all cases, orientation and integrity of subclones was verified by sequence determination.

2.4. *Expression of RDC1 into CHO-K1 and COS-7 cells*

CHO-K1 cells were co-transfected with both the pCDNA-RDC1 and pSV2neo (ATCC, Rockville, MD) plasmids by the calcium phosphate method [5]. Stable transfectants were selected in 0.4 mg/ml G418 (Gibco), and cloned by limited dilution. Transient transfection of COS-7 cells was achieved as follows: 1.5×10^6 exponentially growing COS-7 cells were transfected with 8 μg of DNA using electroporation (Bio-Rad) at settings of 300 V, 125 μF, and 1000 Ω. COS-7 cells were also transfected with the human muscarinic cholinergic receptor gene (Hm1) [6] in pCDM8 (Invitrogen) as a positive control. The binding assays were carried out 48 h after transfection.

2.5. *[^{125}I]VIP receptor binding in CHO-K1 and COS-7 cells*

Binding assays in CHO-K1 cell membranes (60–100 μg protein) were conducted in the presence of a protease inhibitor cocktail [7] with 0.02–2 nM [^{125}I]VIP ± 10^{-6} M VIP for 30 min at 25°C in a volume of 0.2 ml.

Binding assays in whole COS-7 or HT29 cells were as previously described [8,9]. Muscarinic receptor expression was measured in transfected COS-7 cell monolayers with 2 nM [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) ± 1 μM atropine [6].

2.6. *RNA isolation and Northern blot hybridization analyses of RDC1 mRNA*

Total RNA was isolated from cells grown in roller bottles using RNAzol (Cinna/Biotec, Friendswood, TX). Poly A⁺ RNA was isolated using oligo dT cellulose chromatography [10], fractionated on an 0.8% agarose gel containing 2.2 M formaldehyde, transferred to Hybond-N membrane (Amersham, Arlington Hgts, IL), and immobilized by UV cross linking (Stratalinker, Stratagene). Membranes were hybridized to the 5′ end-labeled RDC1B oligonucleotide in 5 × SSPE, 2× Denhardts, 0.1 mg/ml salmon testis DNA, and 50% formamide at 37°C for 16 h, followed by a final wash in 0.25 × SSPE/0.2% SDS at 37°C before autoradiography.

2.7. *Analysis of sequence homology between RDC1 and previously cloned G-protein-coupled receptors*

The alignments were performed using the program FPATTERNALIGN (written by P.E. Correa). The suspected transmembrane helical portions of the RDC1 sequence were used as the pattern. Gaps were adjusted to optimize the alignments of the hydrophobic transmembrane domains.

3. RESULTS AND DISCUSSION

3.1. *RDC1 gene sequence and constructs*

Comparison of the sequence of the published cDNA [1] and the *PstI* genomic fragment revealed that the latter contained the complete coding sequence for the RDC1 protein, and an additional 601 bp preceding a potential intron/exon junction at 28 bp upstream of the initiation codon. This novel sequence is characterized by multiple in-frame termination codons and a C+T-rich region preceding an AG at base pairs 602 + 603 (corresponding to base pairs 63 + 64 of the cDNA sequence), indicating the location of an intron of at least 601 bp in the 5′ untranslated region of the RDC1 gene. The location of this intron is similar to that found in the gene of another orphan G-protein-coupled receptor, RTA [12].

The 3′ untranslated region is characterized by a palindromic (ATAAATAT) sequence which extends for 63 and 55 bp in the genomic and cDNA sequence, respectively. Since A+T repeats in the 3′ untranslated region are thought to influence mRNA stability [13], we chose to delete this putative destabilization sequence for the expression experiments.

3.2. *Northern analysis*

Fig. 1A illustrates the accumulation of the RDC1

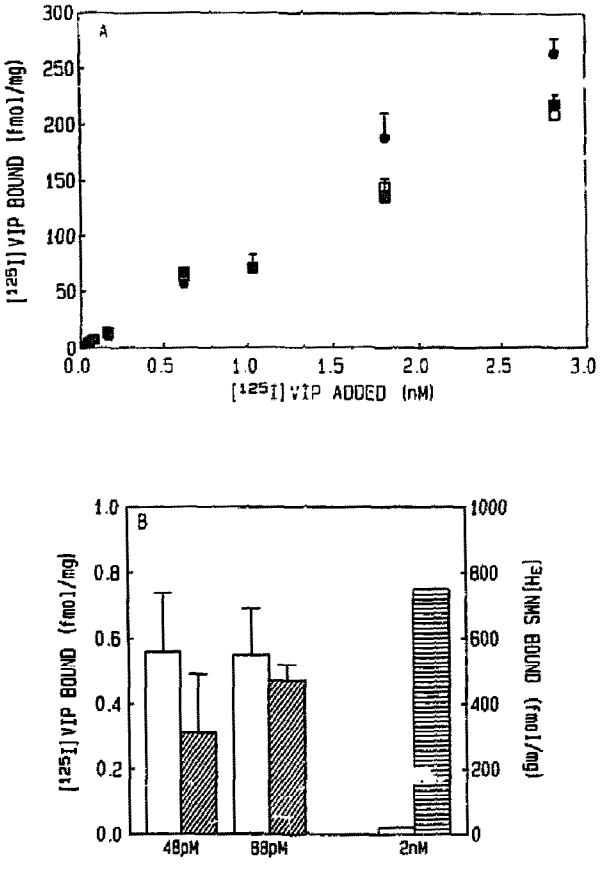

Fig. 2. Lack of alteration in endogenous [^{125}I]VIP specific binding with RDC1 transfection. A. Specific binding in CHO-K1 cells. Binding was performed in membrane homogenates from either WT (□) or RDC1-transfected cells as described in Methods. CHO-RDC1-6 (■) and CHO-RDC1-2 (●) exhibit an enhancement in RDC1 mRNA expression of 3- and 30-fold, respectively, in comparison to that observed in cells not taking up the RDC1 gene fragment (Fig. 1). Data represent the mean ± S.E.M. for triplicate determinations. Similar results were obtained comparing either WT and the two clones, or WT and CHO-RDC1-6, in one and three additional experiments, respectively. B. Specific binding in COS-7 cells. Binding was performed in whole cells transfected with either nothing (□) or RDC1 (▨), at two different [^{125}I]VIP concentrations as described in section 2. [^{3}H]NMS binding (▤) is illustrated as a positive control for transfection (see section 2). Data with [^{125}I]VIP represent the mean ± S.D. for duplicate determinations. The experiment was repeated once with similar results.

mRNA in 9 neomycin resistant CHO cell clones transfected with the $RDC1_{614-2190}$ construct, and that of the original pool of CHO cell transfectants. Two of these clones (CHO-RDC1-2 and CHO-RDC1-6; Fig. 1A, lanes 3 and 7) showed an enhanced expression of 30- and 3-fold, respectively, when normalized to actin mRNA (Fig. 1A and B). Two different transcripts are noticeable in the RDC1 transfectants with sizes of 2.5 and 2.1 kb resulting from the use of alternate SV-40 splice sites in the pCDNA1 vector. Fig. 1C compares the expression of RDC1 mRNA in both the WT (untransfected) CHO cells and the pool of CHO-RDC1 cell transfectants. A 2.1 kb RDC1 mRNA is observed in WT cells (Fig. 1C), which corresponds to the mRNA observed in the neomycin resistant CHO cells that did not appear to express RDC1 to a large extent (Fig. 1A). A minor 2.3 kb RDC1 mRNA was also observed in WT COS-7 cells (data not shown). These data indicate that the RDC1 gene is constitutively expressed in the two mammalian cell types most commonly used for expression and analysis of G-protein-coupled receptors.

3.3. *[^{125}P]VIP Receptor binding in RDC1-transfected CHO-K1 and COS-7 cells*

We investigated whether CHO-K1 and COS-7 cells contained a low level of endogenous VIP receptors. Fig. 2A illustrates the [^{125}I]VIP binding observed in membrane homogenates derived from WT and RDC1-transfected CHO-K1 cells. Specific binding is linear and does not saturate over the 2 nM concentration range. This low affinity binding is in contrast to the subnanomolar affinity in nerve cell lines [14] and lymphocytes [15]. The low affinity VIP binding sites may represent binding to other unrelated peptide receptors to which VIP has access [16].

A similar low level of [^{125}I]VIP binding was observed in whole COS-7 cells (Fig. 2B). In contrast, [^{125}I]VIP binding in HT29 cells was 20-fold higher (10–12 fmol [^{125}I]VIP bound/mg protein), as expected from previous results [8,9].

Endogenous binding was not increased in either cell line transfected with the RDC1 gene (Fig. 2A and 2B). Furthermore, VIP binding did not correlate with mRNA expression and was identical in each of the CHO-K1 clones examined (Figs. 1A and 2A). The pCDNA1 vector was highly effective in COS-7 cells as illustrated by the extensive expression with the transfected Hml muscarinic receptor (Fig. 2B and [6]). However, a lack of RDC1 receptor antibody precludes our ability to reach a definitive conclusion regarding expression of the RDC1 protein in either CHO-K1 or COS-7 cells. Nevertheless, when taken together, the data indicate that RDC1 does not appear to encode a VIP receptor.

Similar circumstances, i.e. low-affinity binding and lack of correlation between binding and mRNA expression, have resulted in the reassignment of the identity of the F3R gene product. The F3R cDNA, originally identified as a rabbit fMLP receptor [17], was subsequently identified as an IL-8 receptor [18]; a result that was supported by its sequence homology to members of the IL-8 receptor family [19].

3.4. *Homology of RDC1 to cloned G-protein coupled receptors*

The sequence identity between the secretin receptor [3] and the proposed VIP receptor GPRN1 [2] is rather low in the highly conserved transmembrane regions (47.6%), whereas RDC1 is clearly the canine homolog of GPRN1 with a sequence identity of 97.5% in these

regions. If VIP were indeed the ligand for RDC1, one would have expected greater identity to the related secretin receptor in the ligand binding pocket. In contrast, RDC1 exhibits a stronger homology (73.3%) to the type I angiotensin II receptor [20] in the transmembrane regions. However, inositol mono- and polyphosphate responses to angiotensin II were not observed in the RDC1-transfected CHO-K1 cell clones, nor was an enhancement of endogenous angiotensin II-stimulated calcium-dependent chloride current seen in *Xenopus* oocytes injected with RDC1 mRNA (data not shown). These data suggest that RDC1 does not encode a novel subtype of angiotensin receptor. Similarly, responses obtained in the inositol monophosphate production assay (CHO-K1 cells) and/or the *Xenopus* oocyte system were negative for bradykinin, parathyroid hormone, parathyroid hormone-related peptide, calcitonin, fMLP, cholecystokinin, VIP, thrombin, bombesin, endothelin-1, neuropeptide Y and arg^8-vasopressin (data not shown).

It is expected that the canine and human VIP receptors will have a high degree of sequence identity. Even though RDC1 is nearly identical to the proposed human VIP receptor GPRN1 [2], our data indicate that it does not encode a canine VIP receptor and that it should still be considered an 'orphan' G-protein-coupled receptor.

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