Molecular cloning of a novel G protein-coupled receptor that may belong to the neuropeptide receptor family

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A novel cDNA encoding a putative G protein-coupled receptor was isolated from a rat forebrain cDNA library by homology screening. Sequence comparison demonstrates that the encoded polypeptide containing seven transmembrane domains shows highest homology with tachykinin receptors, particularly in the transmembrane domains and the first intracellular loop. The mRNA for this new receptor seems specifically expressed in brain and is prominently localized in specific nuclei of the rat brain, including thalamus, cerebral cortex and hippocampus. We speculate that a neuropeptide may be the natural ligand of this novel G protein-coupled receptor.

G protein receptor; Tachykinin receptor; Neuropeptide receptor

1. INTRODUCTION

A variety of neurotransmitter and neuropeptide receptors elicit their intracellular responses via biochemical pathways that involve transduction elements known as guanine nucleotide regulatory proteins (G protein) [1]. Molecular cloning studies have led to the identification of a growing number of these receptors, including the adrenergic receptors [2,3], several subtypes of muscarinic [4-6] and serotoninergic receptors [7-9] and, among the neuropeptide receptors, the tachykinin receptor [10]. Sequence comparison of the G protein-coupled receptors suggests that all members of this gene family possess similar tertiary structure, characterized by the common structural feature of seven hydrophobic transmembrane domains which are separated by six sequence divergent stretches of hydrophilic amino acids predicted to form intracellular and extracellular loops [11]. For each of these receptors, a particularly well conserved sequence motif occurs within the transmembrane region TM6. We have taken advantage of this motif to isolate cDNAs encoding new receptors. Here we report the cloning, sequencing and brain distribution of a new member of the G protein-coupled receptor family. This putative receptor shows a structural similarity with the tachykinin receptors and hence might represent a new member of the large family of the neuropeptide G-protein coupled receptors.

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2. MATERIALS AND METHODS

A directional lambda ZAP cDNA library derived from rat forebrain was screened under low-stringency conditions (hybridization at 30% formamide, 5 × SSC, 25°C). Filters were washed at 42°C, (1 × SSC) with a 24-mer oligonucleotide (5'-GCAGTTGATGATATGCAGGGGCAGCCAGCAGAGGGCGAAGAG-3') designed to a conserved sequence within the sixth transmembrane region of G protein-coupled receptors [11]. The recombinant plasmid pBluescript was rescued from the bacteriophage lambda ZAP and the cDNA inserts were subcloned into M13 phage vectors for DNA sequence analysis [12].

Total cellular RNA from various rat tissues was isolated according to Chomszynsky and Sacchi [13]. Poly(A)$^+$-selected RNA was prepared by using oligo(dT) cellulose. RNA was resolved on a 0.9% agarose gel containing formaldehyde, blotted onto a nylon membrane (N-bond, Amersham) and baked for 2 h at 80°C. Hybridization was performed in 30% formamide, 2× SSC at 42°C with a 5'-end-labelled oligonucleotide probe to the extracellular loop of FC5 between the TM4 and TM5 domains (5'-ATACTTGTCCTTGAACGCCGCAAGTGATACATTTGGAAG-3'). The blots were washed to a final stringency of 0.5 × SSC, 0.1% SDS at 55°C and then exposed to Kodak XAR-5 film for 3 days.

For in situ hybridization, two 40-mer oligonucleotides directed against the extracellular loop TM4-TM5 (5'-ATACTTGTCCTTGAACGCCGCAAGTGATACATTTTGGAAG-3') and (5'-TCGTTTGGTCTCACTGGACCTGTACTTACTGTCCCTGATTTTGTC-3') were 3'-end-labelled by terminal deoxynucleotidyl transferase [^{35}S]dATP, 1200 Ci/mmol, New England Nuclear) to an average tail length of 30 AMP residues and used as probes in different experiments. Cryostat sections (15 μM) of adult rat brain tissue were fixed in 4% paraformaldehyde and hybridized overnight at 42°C with the ^{35}S-labelled oligoprobes (1 pg/μl) in 50% formamide, 4 × SSC, 10% dextran sulphate. Hybridized sections were washed in 1 × SSC at 60°C before dehydration and then exposed to Kodak XAR-5 film for 4 weeks and/or dipped in Kodak NTB2 emulsion for 8 weeks at 4°C. After development of emulsion autoradiograms, the sections were stained in thionin. Specificity of the signal was checked by displacement with 20 fold excess of unlabelled oligonucleotides.

Cellular expression data were obtained using the host vector system and transformation procedures as described by Pritchett and

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coworkers [9]. Cell membranes for binding assays were prepared 48 h after transfection.

3. RESULTS AND DISCUSSION

Of seven cDNA clones analyzed, five were found to contain sequences encoding G protein-coupled receptors. DNA sequence and restriction analysis indicated that three lambda cDNA clones were siblings of approx. 2.5 kb. This clone, named FC5, specified an open reading frame of 349 amino acids (Fig. 1). The predicted polypeptide contains seven segments of 20–25 hydrophobic residues, separated by stretches of hydrophilic residues, typical of G protein-coupled receptors [11]. Comparison of the complete sequence for the novel putative receptor with that of other G protein-coupled receptors shows that the largest concentration of identical residues was found in putative transmembrane regions and identified the presence of amino acid residues that are conserved among different G protein-coupled receptors (Fig. 2). The amino- and carboxyl-terminal regions of FC5, although differing in length and amino acid sequence, also show structural characteristics of G protein-coupled receptors, including potential *N*-glycosylation sites (Asn-11, Asn-17 and Asn-21) in the amino-terminal region and the presence of serine and threonine residues as possible phosphorylation sites in the carboxyl-terminal region.

The highest FC5 overall similarity is to the tachykinin receptor family, possessing within the putative transmembrane domains (sequence corresponding to amino acids 38–324 of the FC5) 33% and 31% amino acid homology with neuromedin K and substance K receptors, respectively. Fig. 2 shows that this high degree of similarity is particularly remarkable in not only the putative transmembrane domains and their flanking sequences, but also the first cytoplasmic loop, which is extremely conserved among the three tachykinin receptors. One other feature common to the FC5 and tachykinin receptors is the presence of a single glutamate residue in TM2, while other G protein-coupled receptors generally contain several acidic residues within the seven hydrophobic transmembrane regions [10].

A further sequence divergence notable between FC5 and other G protein-coupled receptors is the short consensus sequence flanking TM3, usually Asp-Arg-Tyr, that is replaced with the homologous Glu-Arg-His sequence in FC5. The presence of conserved amino acid residues at analogous positions among all members of

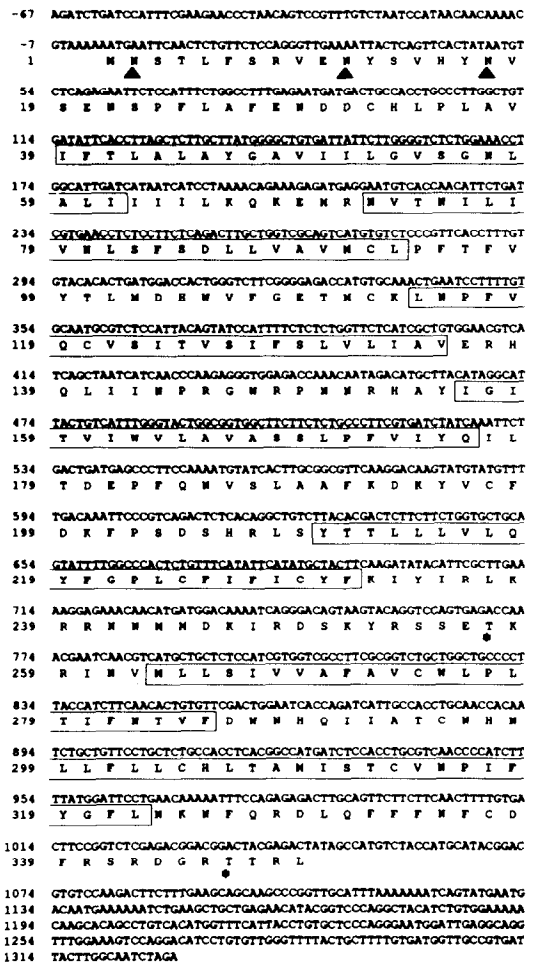


Fig. 1. cDNA and predicted amino acid sequence of the putative FC5 receptor. The termini of each of the putative transmembrane segments I–VII are tentatively assigned on the basis of a hydropathicity profile. Triangles indicate potential N-linked glycosylation sites in the amino-terminal region and asterisks indicate threonine residues in protein kinase C consensus phosphorylation sites.

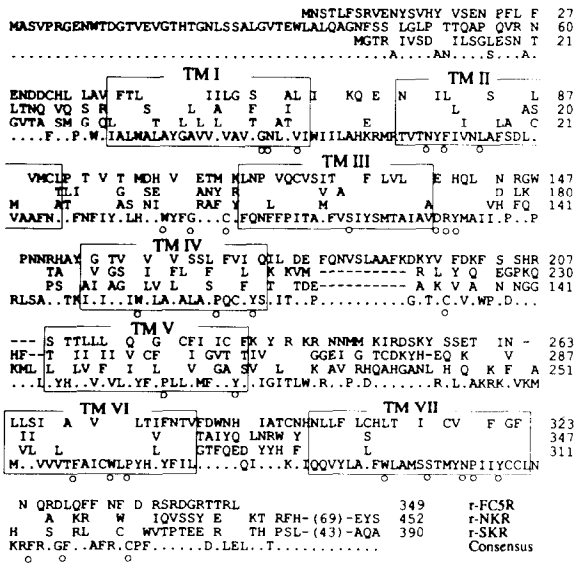


Fig. 2. Alignment of the putative FC5 receptor and the rat neuromedin K (NK) and substance K (SK) receptor polypeptide sequence. Gaps (–) were introduced for optimal alignment. A consensus sequence (bottom line) indicates two out of three amino acids at homologous positions. Dots highlight conserved amino acids in tachykinin, adrenergic and muscarinic receptors.

similar receptor families (i.e. the Asp-113 in all the G protein-coupled receptors that bind to protonated amines) may be related to similarities in function [14]. Thus the feature of this divergent tripeptide may set the FC5 receptor apart from other G protein-coupled receptors, and may characterize it as a member of a new subfamily.

To examine the expression of FC5 mRNA in rat brain and peripheral tissues, Northern blot analysis was performed. A prominent RNA species, approx. 4 kb in size, was found in poly(A)$^+$ RNA from rat hippocampus and cerebral cortex (Fig. 3). No signal was observed in any of the peripheral tissues we examined, suggesting that FC5 mRNA expression is restricted to brain. The distribution of the FC5 mRNA in brain was analyzed by in situ hybridization. Inspection of film autoradiograms of horizontal and coronal rat brain sections revealed that regions most prominently expressing FC5 mRNA are several thalamic nuclei (including the anteromedial, the anterolateral, the ventral posterolateral, the ventral posteromedial and the laterodorsal nuclei), the pyramidal cell layer of the hippocampus and the dentate granule cells, layer II and layer VI of the cerebral cortex, the anterior olfactory nucleus, various amygdaloid nuclei and a small hypothalamic nucleus, possibly the arcuate nucleus (Fig. 4A and B). No signals were detected in caudate-putamen, cerebellum and olfactory bulb. These results were confirmed at the cellular level by emulsion autoradiograms. Fig. 4C shows that neurons in the anterior olfactory nucleus express FC5 mRNA whereas neighboring olfactory bulb granule cells do not. Although it is difficult to predict the nature of the endogenous ligand for FC5 from the mRNA expression pattern, our data suggest that the new gene is mainly a thalami-cortical transcript, and that the thalamic signals might function as presynaptic regulatory receptors of this pathway.

To determine the functional identity of the putative receptor encoded by the cloned FC5 cDNA, a transient

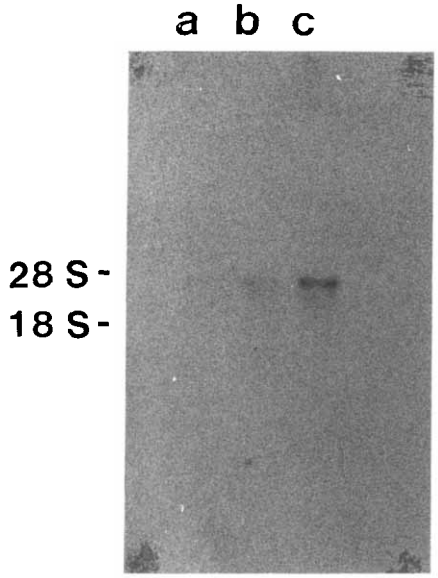


Fig. 3. Blot hybridization analysis of poly(A)$^+$ RNA (10 μg) isolated from rat cerebellum (a), hippocampus (b) and cerebral cortex (c).

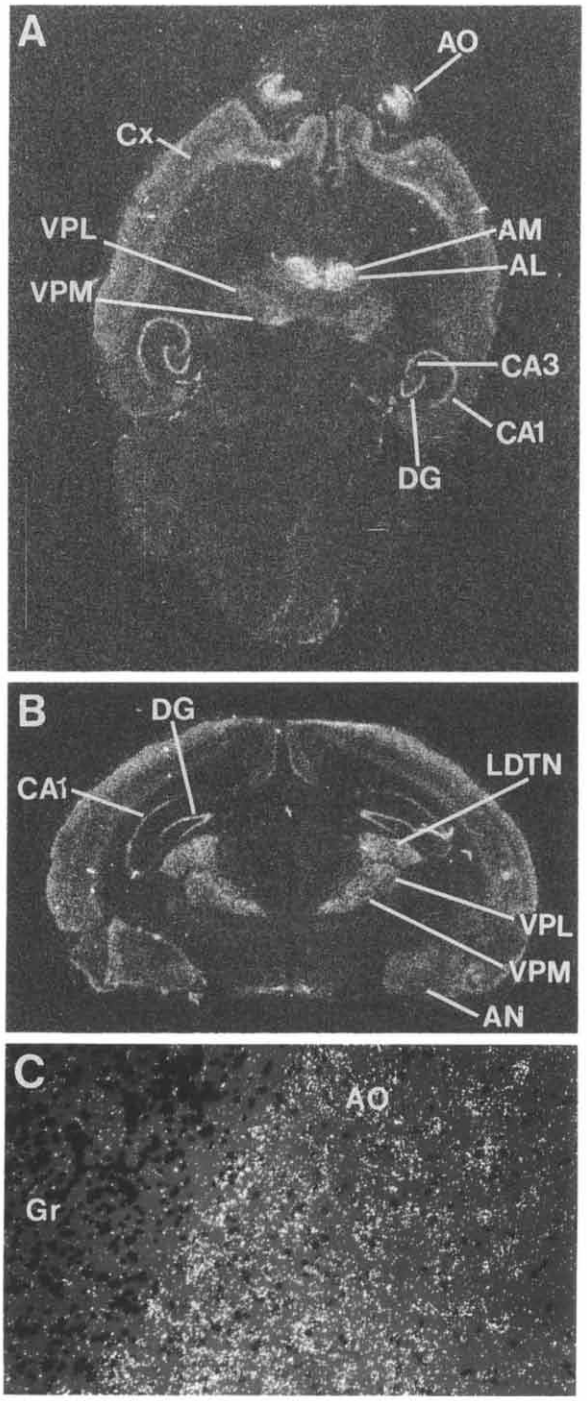


Fig. 4. Autoradiograms from in situ hybridization with FC5 of horizontal (A) and coronal (B) sections of adult rat brain. (C) Dark-field autoradiogram of a horizontal brain section shows neurons in the anterior olfactory nucleus expressing the transcript. AL, AM, LDTN, VPL, VPM: anterolateral, anteromedial, laterodorsal, ventroposterolateral and ventroposteromedial thalamic nuclei; AN: amygdaloid nuclei; AO: anterior olfactory nucleus; Cx, cortex; DG: dentate gyrus; Gr: granule cells of olfactory bulb.

mammalian expression system [9] was utilized to screen for binding to most of the commercially available radiolabelled ligands. None of the drugs we have tested, however, showed any detectable difference in binding to membrane prepared from transfected or untransfected cell (data not shown).

Although we failed to functionally characterize the FC5 receptor, our data suggest that it constitutes a novel neuropeptide receptor subtype. Expression studies showed that the FC5-transfected cells do not bind most of the 'classical' neurotransmitters and autacoids so far identified. Additionally, comparison of the amino-acid sequence of FC5 with that of the other G protein coupled-receptors indicates that the greater overall homology and common structural features occur with the tachykinin receptors. Thus, although further functional and binding studies are required to demonstrate a biological function for this orphan receptor, we speculate that it may represent a member of the large family of the neuropeptide receptors.

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REFERENCES

[1] Gilman, A.G. (1987) Annu. Rev. Biochem. 56, 615–629.
[2] Frielle, T., Collins, S., Daniel, K.W., Caron, M.G., Lefkowitz, R.J. and Kobilka, B.K. (1987) Proc. Natl. Acad. Sci. USA 84, 7920–7924.
[3] Kobilka, B.K., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T.S., Francke, U., Lefkowitz, R.J. and Caron, M.G. (1987) Nature 329, 75–79.
[4] Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H.H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Koijma, M., Matsuo, H., Hirose, T. and Numa, S. (1986) Nature 323, 411–416.
[5] Peralta, E.G., Winslow, J.W., Peterson, G.L., Smith, D.H., Ashkenazi, A., Ramachandran, J., Schimerlik, M.I. and Capon, D.J. (1987) Science 236, 600–605.
[6] Bonner, T.I., Buckley, N.J., Young, A.C. and Brann, M.R. (1987) Science 237, 527–532.
[7] Fargin, A., Raymond, J.R., Lohse, M.J., Kobilka, B.K., Caron, M.G. and Lefkowitz, R.J. (1988) Nature 335, 358–360.
[8] Julius, D., MacDermott, A.B., Axel, R. and Jessel, T.M. (1988) Science 241, 558–564.
[9] Pritchett, D.B., Bach, A.W.J., Wozny, M., Taleb, O., Dal Toso, R., Shih, J.C. and Seeburg, P.H. (1988) EMBO J. 7 (13), 4135–4140.
[10] Shigemoto, R., Yokota, Y., Tsuchida, K. and Nakanishi, S. (1990) J. Biol. Chem. 265 (2), 623–628.
[11] O'Dowd, B.F., Lefkowitz, R.J. and Caron, M.G. (1989) Annu. Rev. Neurosci. 12, 67–83.
[12] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[13] Chomszynsky, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[14] Strader, C.D., Sigal, I.S. and Dixon, R.A.F. (1989) Trends Pharmacol. Sci., December Suppl., 26–30.