Molecular cloning of an orphan G-protein-coupled receptor that constitutively activates adenylate cyclase

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A human gene encoding an orphan G-protein-coupled receptor named ACCA (adenylate cyclase constitutive activator) was isolated from a genomic library using as a probe a DNA fragment obtained by low-stringency PCR. Human ACCA (hACCA) is a protein of 330 amino acids that exhibits all the structural hallmarks of the main family of G-protein-coupled receptors. Expression of hACCA resulted in a dramatic stimulation of adenylate cyclase, similar in amplitude to that obtained with other G_s-coupled receptors fully activated by their respective ligands. This stimulation was obtained in a large variety of stable cell lines derived from various organs, and originating from different mammalian species. hACCA was found to be the human homologue of a recently reported mouse orphan receptor (GPCR21). The mouse ACCA (mACCA) was therefore recloned by PCR, and expression of mACCA in Cos-7 cells demonstrated that the mouse receptor behaved similarly as a constitutive activator of adenylate cyclase. It is not known presently whether the stimulation of adenylate cyclase is the result of a true constitutive activity of the receptor or, alternatively, is the consequence of a permanent stimulation by a ubiquitous ligand. The tissue distribution of mACCA was determined by RNase protection assay. Abundant transcripts were found in the brain, whereas lower amounts were detected in testis, ovary and eye. Various hypotheses concerning the constitutive activity of ACCA and their potential biological significance are discussed.

INTRODUCTION

G-protein-coupled receptors recognize extracellular signals as diverse as single photons, odorants, peptides, amino acids, nucleotides, lipids, proteinases and ions. They form a family of molecules which display a characteristic seven- membrane-spanning-domain topography. The stimulus generated by the ligand is transmitted to transducer coupling proteins, which transfer it to effector molecules able to generate intracellular signals. For receptors to biogenic amines, the ligand-binding site is thought to be in the hydrophobic pocket; the binding site for G proteins involves the intracellular loops with special emphasis on the third intracellular loop and the C-terminal intracellular domain [1]. In the classical model of G-protein-coupled receptor function, coupling between receptor and G protein occurs after stimulation of the receptor by its ligand. Recent evidence suggests, however, that some receptors are endowed with a constitutive activity, stimulating the effector in the absence of a ligand. The first demonstration of this phenomenon came from mutagenesis studies performed on the adrenergic receptors. Specific mutations in the third intracellular loop of these receptors resulted in an enhanced basal activity, presumably resulting from the alteration of their natural conformation [2,3]. For the α1B-adrenoceptor, all possible amino acid substitutions at position 293 led to some level of constitutive activation [4]. This increase in basal activity is accompanied by an increase in the affinity for agonists as compared with the wild-type receptor. These observations led Lefkowitz [5] to postulate that the native receptor is in a constraint inactive form which can be relaxed either by the ligand or by specific mutations of residues implicated in the maintenance of this constraint form. Recent studies have identified naturally occuring mutations of G-protein-coupled receptor genes that are associated with human diseases. As an example, the presence of mutations of the thyrotropin (TSH) receptor gene in human hyperfunctioning thyroid adenomas was correlated with the constitutive activity of the mutant receptors [6]. It was also demonstrated that G-protein-coupled receptors may exhibit some level of constitutive activity even in the absence of activating mutations. δ-opioid receptors exhibit a basal activity, inhibited by inverse agonists, in membranes isolated from NG108-15 cells [7]. Similarly, the $5HT_{2C}$ receptor increases the basal phospholipase C activity when transfected into NIH3T3 fibroblasts [8]. This basal activity can be further demonstrated by the action of $5HT_{2C}$ receptor antagonists that possess an inverse agonist activity. The wild-type TSH receptor stimulates adenylate cyclase to some extent when expressed in COS-7 cells [6,9], as does the dopamine D1B receptor in 293 cells [10].

Alternatively, the apparent constitutive activity of a receptor can be due to the chronic stimulation by its specific ligand. The expression of the dog adenosine A_{2a} receptor in transfected cells, resulted in a sustained stimulation of adenylate cyclase [11]. The role of adenosine released continuously by the transfected cells in

Abbreviations used: TSH, thyroid-stimulating hormone; MSH, melanocyte-stimulating hormone; KRH, Krebs–Ringer/Hepes; PFU, plaque-forming units; CHO, Chinese-hamster ovary.

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The nucleotide sequence data reported here will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X83956.

the apparent constitutivity of the A_{2a} receptor, was demonstrated by the inhibitory effect of adenosine deaminase on the accumulation of intracellular cyclic AMP.

In the present work, we describe the cloning and expression of a human orphan receptor, belonging to a group of G-protein-coupled receptors that includes the central (CB1) [12,13] and peripheral (CB2) [14] cannabinoid receptors, as well as the orphan receptors edg1 [15], rCNL3 [16], AGR16 [17] and R334 [18]. When expressed in COS and Chinese-hamster ovary (CHO) cell lines, this receptor stimulated adenylate cyclase to a level similar to that reached with other G_s-coupled receptors when fully activated by their respective ligand. This orphan receptor, which we propose to name ACCA (adenylate cyclase constitutive activator), is the human counterpart of the mouse GPCR21 orphan receptor reported recently [19].

MATERIALS AND METHODS

Cloning and sequencing

A human genomic DNA library constructed in λ charon 4a was screened at high stringency [20] with the HGMP06 probe, a 600 bp PCR fragment amplified from genomic DNA by low-stringency PCR [21,22]. A 1982 bp *Eco*RI fragment containing the whole coding region of hACCA was subcloned in pBluescript SK+, and sequencing was performed on both strands after subcloning in M13mp derivatives, using fluorescent primers and an automated DNA sequencer (Applied Biosystem 370A). Sequence analysis was carried out using DNASIS/PROSIS Software (Hitachi) and the GCG/VMS Software Package (University of Wisconsin Genetics Computer Group, Madison, WI, U.S.A.).

The coding region of mouse ACCA was cloned by PCR from mouse genomic DNA using primers (5′-TCGTAAGCTTACAGGTACCATGATGTGG-3′ and 5′-CTACGGATCCAGGATGAAACTAGACA-3′) synthesized on the basis of the published GPCR21 sequence [19]. The amplified product was cloned in pBluescript SK+ (Stratagene) through the *Hin*dIII and *Bam*HI restriction sites included in the primers. After verification of the sequence on both strands, the mACCA coding region was transferred into the expression vector pSVL (Pharmacia).

RNase protection assays

A 193 bp fragment corresponding to the 3′-end of the open reading frame of GPCR21 was amplified by PCR and cloned through synthetic *Hin*dIII and *Bam*HI restriction sites in pBluescript SK+. The resulting plasmid was used as a template for the synthesis of an antisense ^{32}P-labelled probe ([α-^{32}P]UTP; 800 Ci/mmol; Amersham International). Total RNA was prepared from various mouse tissues by the guanidium thiocyanate/caesium chloride gradient method, and RNase protection assays were performed as described in [20]. RNA samples (20 μg) were denatured (85 °C, 10 min) and hybridized overnight with the probe (180000 c.p.m.) at 45 °C. The next day the samples were digested with RNases A (40 μg/ml) and T1 (2 μg/ml), separated by electrophoresis on a 6%-polyacrylamide denaturing gel, and autoradiographed. All RNA samples were hybridized in parallel experiments with a β-actin antisense probe as control. In order to demonstrate the absence of contamination with genomic DNA, an antisense probe corresponding to another intronless receptor (results not shown) was hybridized with all RNA samples and distinct specific signals were obtained.

Expression in cell lines

The coding region of hACCA (−11 to +1035 relative to the start codon) was reconstructed in pBluescript SK+ by linking together 322 bp of *Pst*I–*Esp*I and 723 bp *of Esp*I–*Eco*RI restriction fragments. An *Xba*I–*Eco*RV fragment was further transferred into the eukaryotic expression vector pSVL. The resulting construct was transfected in COS-7 cells as previously described [23]. The pSVL construct was also co-transfected with pSV2Neo in CHO-K1 cells as described [24] and G418-resistant cell lines expressing hACCA were selected by RNase protection assays. COS-7 and CHO-K1 cells were cultured using Dulbecco's modified Eagle's medium and Ham's F12 medium respectively [25]. COS-7 cells trypsin-treated 2 days after transfection, or CHO cells (10^5 cells in 500 μl of culture medium) were seeded in 24-well culture plates 24 h before proceeding to cyclic AMP accumulation assays.

Cyclic AMP accumulation assays

Cells were washed with 1 ml of Krebs–Ringer/Hepes (KRH) (5 mM KCl, 1.25 mM $MgSO_4$, 124 mM NaCl, 25 mM Hepes, 8 mM Glucose, 1.25 mM KH_2PO4, 1.5 mM $CaCl_2$ and 0.5 mg/ml BSA) and 200 μl of incubation medium (KRH) was added, containing 0.1 mM Ro20-1724 (gift from Hoffmann–LaRoche, Nutley, NJ, U.S.A.) and various agents. The cells were incubated for 1 h at 37 °C and 500 μl of 0.1 M HCl was added to stop the accumulation of cyclic AMP. The medium was transferred to a fresh glass tube, vacuum-dried overnight, and cyclic AMP was measured by radioimmunoassay as described by Brooker et al. [26].

Infection of cell lines with a recombinant adenovirus

In order to generate replication-defective adenoviruses expressing hACCA, a *Bam*HI–*Hin*dIII fragment of the pBluescript SK+ construction containing the coding region of hACCA (see above) was inserted in the pACCMV adenoviral Ad5 vector [27] under the control of the CMV (cytomegalovirus)-1 promoter. Adenovirus expressing the human TSH receptor constructed as described [28] was used as control.

Expression of hACCA was verified by transient transfection of the plasmids into CHO-K1 cells. A recombinant virus was constructed by homologuous recombination with the plasmid pJM17 after co-transfection into 293-cells, as described previously [29,30]. After plaque purification, the viruses were propagated in subconfluent 293-cells grown in 75 cm^2 flasks to give a viral stock of approx. 10^8 plaque-forming units (PFU)/ml. Infection with recombinant viruses was performed in an identical manner for all cell types cultured in their respective incubation media. Cells (5×10^4) were seeded in 48-well microtitre plates for a minimum of 18 h before infection. Cells were rinsed once with 1 ml of serum-free media, then approx. 2.5×10^6 PFU of viral particles in 0.1 ml of the same media were added for 1 h at 37 °C. Cells were washed with 1 ml of serum-containing media and the incubation was continued for a minimum of 24 h before proceeding to cyclic AMP accumulation assays, as described above.

For experiments with conditioned media, 25×10^3 B16F cells were seeded in 48-well microtitre plates and infected 24 h later with 5×10^6 PFU of viral particles in 0.1 ml of serum-free media for 1 h at 37 °C. Cells were then washed twice with 1 ml of serum-containing media and then incubated with 5×10^4 trypsin-treated COS cells or fibroblasts. The mixture was incubated for 18 h before proceeding to cyclic AMP accumulation assays.

RESULTS

Low-stringency PCR using degenerate primers corresponding to the conserved regions of the second, third, sixth and seventh transmembrane segments of G-protein-coupled receptors was

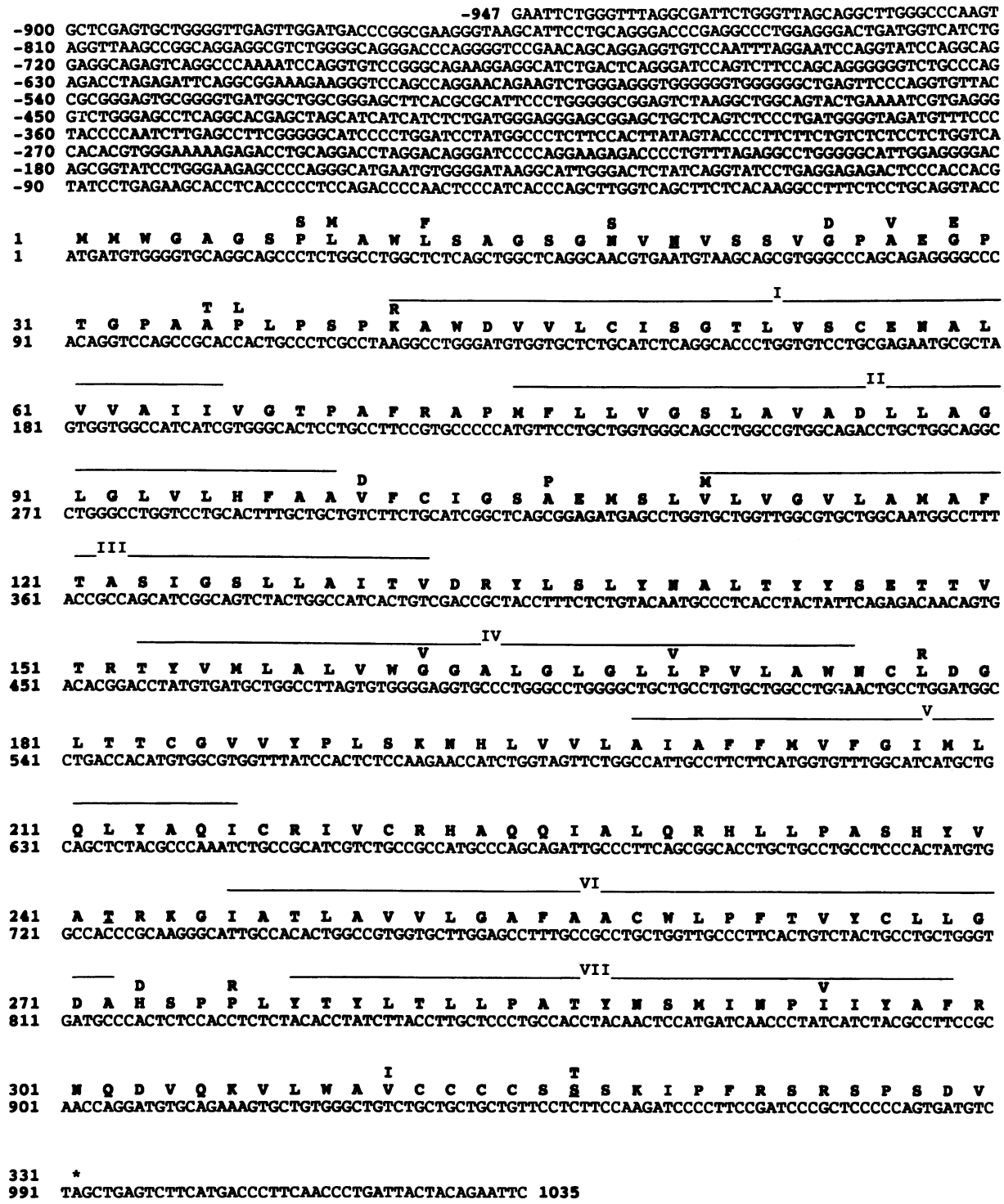

Figure 1 Nucleotide sequence of the human ACCA clone and deduced amino acid sequence of the coding region

Divergent amino acids in the corresponding mouse sequence [19] are indicated above the human sequence. Putative transmembrane segments are overlined and numbered I–VII. Asparagine residue constituting potential acceptor site of N-linked glycosylation and serines residues that may be phosphorylated by the protein kinase C are underlined.

performed on human genomic DNA in order to isolate new members of this large gene family [21,22]. HGMP06, one of the gene fragments encoding unknown receptors, was used as a probe to screen a human genomic library. Five clones corresponding to a single locus were isolated, and a 1982 bp *Eco*RI restriction fragment common to all five clones, and hybridizing with the probe, was subcloned in pBluescript SK + and sequenced entirely. This fragment was shown to contain an open reading frame of 990 nucleotides, in addition to 947 bp of 5′ and 45 bp of 3′ non-coding sequence. The encoded 330-amino-acid protein

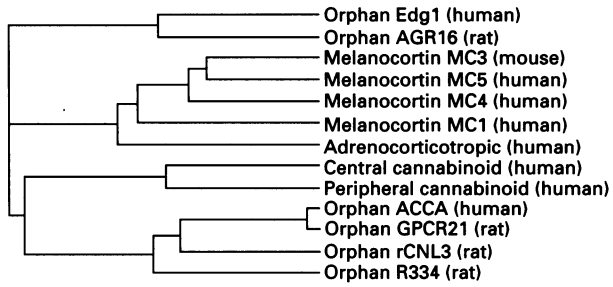

Figure 2 Dendrogram representing sequence similarities between human, mouse ACCA and related receptors

(referred to as hACCA; see below) has a predicted molecular mass of 35 kDa. hACCA displays all the features common to G-protein-coupled receptors, with seven 20–25-amino-acid hydrophobic segments [31]. hACCA contains one potential N-linked glycosylation site in the extracellular N-terminal domain. Glycosylation at this site could therefore increase the actual molecular mass of the mature protein. It also contains two potential sites of phosphorylation by protein kinase C, one in the third intracellular loop and one in the C-terminal intracellular domain. Several serine and threonine residues located in the same regions could also constitute targets for the family of G-protein-coupled receptor kinases and be involved in the desensitization of the receptor [32]. Sites potentially phosphorylated by protein kinase A are not present in the hACCA sequence (Figure 1).

After publication of the sequence of the mouse orphan receptor GPCR21 [19], it appeared that hACCA was the human counterpart of GPCR21 with an amino acid identity score of 91% (Figure 1). Comparison of hACCA sequence with that of other G-protein-coupled receptors demonstrated that it belongs to a cluster of receptors including the orphan receptors edg1 [15], R334 [18], rCNL3 [16], AGR16 [17] and the human central and peripheral cannabinoid receptors [12,14] (Figure 2).

In order to test for potential ligands, the coding sequence of this human orphan receptor gene was inserted in the expression vector pSVL and stably transfected CHO cell lines were established. One cell line was selected, based on the highest amount of messenger RNA coding for hACCA, as detected by RNase protection assay. A number of potential ligands (including the cannabinoid agonists CP55940 and anandamide) were tested for their ability to stimulate or inhibit intracellular cyclic AMP accumulation, or stimulate $InsP_3$ accumulation. None of the tested ligands was able to modify significantly the basal levels of either cyclic AMP or $InsP_3$in this stably transfected cell line. However, it was reproducibly observed that the basal intracellular cyclic AMP levels of the hACCA expressing CHO-K1 cell line was greatly increased in comparison with the wild-type CHO-K1 cells or stable CHO-K1 cell lines transfected with the pSVL vector alone, or with other G protein-coupled receptors. Basal cyclic AMP levels in wild-type CHO-K1 cells were approx. 0.2 pmol of cyclic AMP per tube, whereas in hACCA transfected cells it ranged between 40 and 55 pmol of cyclic AMP under the same experimental conditions. Forskolin (10 μM) was able to stimulate cAMP accumulation in both cases, with values ranging from 20–40 pmol for wild-type CHO-K1 and 80–120 for hACCA expressing cells. In order to investigate the possibility that cloning the transfected cells could have incidentally resulted in the selection of a CHO-K1 cell with an impaired cyclic AMP cascade, we measured the basal cyclic AMP level in other stably transfected CHO-K1 cells expressing lower levels of hACCA. The basal cyclic AMP level was also significantly increased in these cell lines as well, and correlated grossly with the level of mRNA encoding the hACCA, as determined by RNase protection assay (results not shown).

The adenylate cyclase-stimulation properties of hACCA were also investigated by transient expression of the pSVL construct in COS-7 cells. High basal cyclic AMP levels were also observed in COS-7 cells transfected with various concentrations of the plasmid pSVL-hACCA (Figure 3). The intracellular cyclic AMP levels in these cells were compared with those measured in cells transfected with the pSVL vector alone or with constructs encoding known receptors positively coupled to the adenylate cyclase (human TSH receptor, mouse MC5 melanocortin receptor, dog A2a adenosine receptor), negatively coupled to the

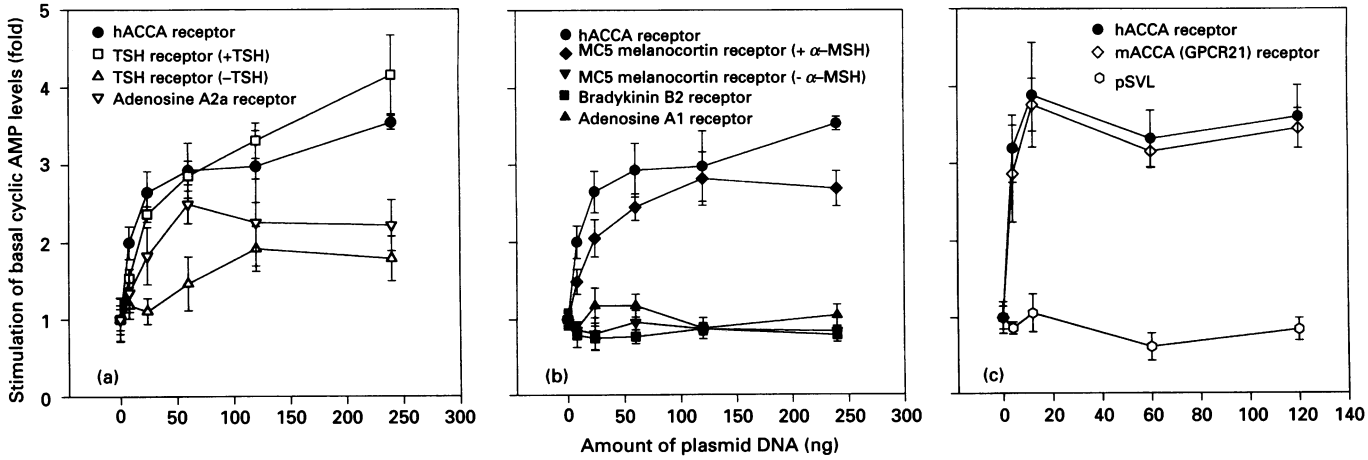

Figure 3 Measurements of intracellular cyclic AMP concentration in COS-7 transfected cells with various amounts of pSVL constructs encoding known G protein-coupled receptors

(**a**) Human ACCA receptor, human TSH receptor stimulated or not with 10 munits/ml TSH or dog adenosine (Ad) A2a receptor; (**b**) human ACCA receptor, mouse MC5 melanocortin receptor stimulated or not with 0.1 μM α-MSH, human bradykinin B2 receptor or dog adenosine A1 receptor; (**c**) human ACCA receptor, mouse ACCA (GPCR21) receptor or pSVL vector alone.

adenylate cyclase (dog A1 adenosine receptor) or coupled to phospholipase C (human B2 bradykinin receptor). A 3 to 4-fold increase in the basal adenylate cyclase activity was observed in COS-7 cells transfected with the hACCA construct, although a fraction only of these cells are transfected. As shown in Figure 3, the maximal cyclic AMP response obtained with the expression of hACCA is in the same range as that observed with characterized G_s-coupled receptors maximally stimulated by their respective ligand: the TSH receptor in the presence of 10 munits/ml TSH (Figure 3a), the MC5 melanocortin receptor in presence of 0.1 μM of α-MSH (Figure 3b). No elevation of the basal cyclic AMP level was observed with the pSVL plasmid alone (Figure 3c), or with the B2 bradykinin receptor, the A1 adenosine receptor or the unstimulated MC5 melanocortin receptor (Figure 3b). In contrast, the TSH receptor, as shown previously, was able to increase less efficiently than hACCA the intracellular cyclic AMP levels in the absence of TSH, as a consequence of an intrinsic G_s stimulation activity [9]. The A2a adenosine receptor was also able to stimulate the accumulation of cyclic AMP in the absence of added adenosine. In this case, the constitutive activity is attributed to the continuous release of adenosine by the cultured cells [11,33].

It was then considered that the cloned hACCA gene could have mutated during the library construction, screening or clone amplification procedures, giving rise to a constitutive activity not shared by the natural receptor. *In vitro*-engineered mutations or naturally occuring mutations leading to constitutive activation of G-protein-coupled receptors have indeed been reported [6,9,34–39]. Moreover, the GPCR21 mouse counterpart of hACCA that had been reported in the meantime, and had been tested in bioassays similar to ours, had not been described as activating adenylate cyclase. The mouse GPCR21 sequence was therefore recloned by PCR and, after verification of the coding sequence, was expressed in COS-7 cells and assayed for cyclic AMP accumulation in parallel with human ACCA. Figure 3(c) shows that both the mouse and human receptors increase the basal levels of cyclic AMP in transfected COS-7 cells to similar levels, demonstrating that this property is not specific for our human clone, nor for the human species.

Experiments were designed to understand better the constitutive activity of hACCA. Two hypotheses were considered: either the receptor had a true constitutive activity, similarly to that observed with the TSH receptor, or the ACCA ligand was present in the assay medium, being produced by the cells or being introduced as a component of the assay itself. In a series of experiments not illustrated here, it was demonstrated: (1) that the effect was not due to the presence of serum in the culture medium, since culturing the cells in 10% or 1% serum, or in serum-free medium, did not significantly change the cyclic AMP levels in hACCA-expressing cells; (2) that the constitutive stimulation of cyclic AMP accumulation could not be decreased significantly by extensive washing of the transfected cells, nor (3) could it be modulated by conditioned medium from a variety of cell types. We then investigated whether the cyclase-stimulation activity was restricted to the two cell lines used (COS-7 and CHO-K1) or could be obtained in cell lines derived from different tissues and species. For that purpose, a recombinant adenovirus, expressing the hACCA under control of the strong CMV promoter, was produced, allowing the infection of a wide variety of cells. A recombinant adenovirus vector expressing the human TSH receptor was used as a control in parallel experiments. The basal cyclic AMP levels were measured after the infection of 12 cell lines of human, mouse, hamster, monkey and dog origins. The level of adenylate cyclase stimulation after infection with the TSH receptor expressing adenovirus and in presence of

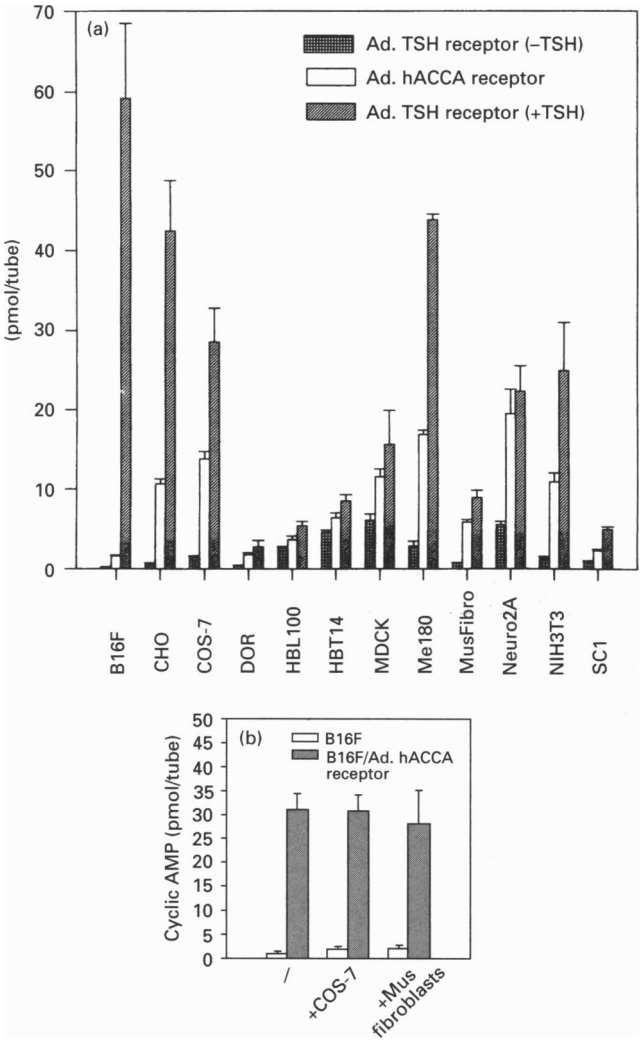

Figure 4 (a) Measurements of intracellular cyclic AMP concentration in various cells infected with recombinant adenovirus expressing human ACCA or human thyrotropin receptor and (b) measurement of intracellular cyclic AMP concentration in B16F cells infected or not with recombinant adenovirus expressing hACCA in the absence of added cells (/) or co-cultured with COS-7 cells or mouse fibroblasts

(**a**) Cell lines tested were B16F (mouse melanoma), CHO-K1 (Chinese-hamster ovary), COS-7 (monkey kidney fibroblasts), DOR (human melanoma), HBL100 (human mammary epithelial cells), HBT14 (human glioblastoma), MDCK (dog kidney epithelial cells), Me180 (human cervix carcinoma), Mus (murine fibroblasts), Neuro2A (mouse neuroblastoma), NIH3T3 (mouse embryonic fibroblasts), SC1 (mouse embryonic fibroblasts). Cell lines were obtained from the A.T.C.C. or were provided by Dr. T. Velu. Bars correspond to the cyclic AMP accumulation in cell lines infected with adenovirus expressing the human TSH receptor in absence of TSH, the human ACCA receptor, or the human TSH receptor in presence of TSH (10 munits/ml). (**b**) The virus/cell ratio was four times higher than in the experiment shown in (**a**).

10 munits/ml TSH varied among cell types, but demonstrated effective coupling to the cyclic AMP cascade (Figure 4a). In all these cell lines the hACCA-expressing virus gave rise to a constitutive activation of the adenylate cyclase. However, the intracellular level of cyclic AMP in hACCA expressing cells was generally lower than that in stimulated TSH-receptor-expressing cells, and the ratio of stimulation (stimulated TSH receptor versus hACCA receptor) varied according to the cell line. This result may reflect differences among cell lines to produce and

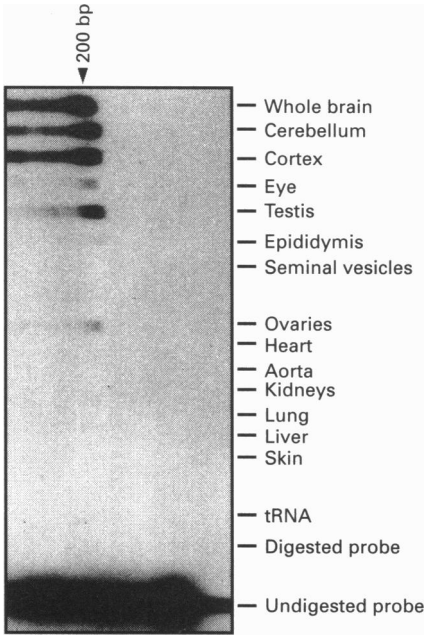

Figure 5 Tissue distribution of mouse ACCA gene expression investigated by RNase protection assays

Protected fragments were separated on polyacrylamide gel. As a positive control, all RNA samples were hybridized with a β-actin probe. Negative controls included yeast tRNA and omission of RNA.

transport the tested receptors to their membranes. Alternatively, the ligand for hACCA receptor may be released by the cells themselves, but with different efficiencies. In order to investigate whether a potential ligand is produced more abundantly by the cell lines in which hACCA appears as strongly constitutive, cell lines displaying low cyclic AMP levels when expressing hACCA (B16F cells) were incubated with Cos-7 and mouse fibroblasts conditioned media. These conditioned media did not modify the cyclic AMP cascade stimulation in hACCA-expressing B16F cells (results not shown). The intracellular cyclic AMP level was also measured in B16F cells infected with the recombinant adenovirus, when co-cultured with COS cells or fibroblasts (Figure 4b). In order to increase the sensitivity of the assay, B16F cells were infected with a higher virus titre than that used in the previous experiments. No significant elevation of the cyclic AMP concentration was obtained in the co-culture conditions. Furthermore, adenylate cyclase assays performed on CHO-cell membranes demonstrated that the enzyme basal activity is greater for hACCA expressing cells than for non-transfected cells (results not shown).

The distribution of ACCA was investigated by RNase protection assay in mouse tissues, using a fragment of the mouse GPCR21 coding sequence. Northern blotting performed on a panel of ten dog tissues had shown that the expression level of ACCA was low to undetectable (results not shown). Using the more sensitive RNA-protection-assay technique applied to a large panel of mouse tissues, a strong signal was found with RNA extracted from total adult or newborn brain, from cerebellum and cortex (Figure 5). Moderate signals were also obtained with RNAs from eye, testis and ovary. Organs where no expression of mACCA could be found included stomach, duodenum, jejunum, caecum, large bowel, liver, kidney, urinary bladder, epididymis, seminal vesicle, heart, aorta, adrenal gland, thyroid gland, spleen, thymus, lymph nodes, bone marrow, fat tissue, lung and skin. These results are consistent with those obtained by Saeki et al. [19].

DISCUSSION

We report here the cloning of a human orphan receptor that belongs to a subgroup of G-protein-coupled receptors, including the central and peripheral cannabinoid receptors, and a number of other orphan receptors. As described by Song et al. [16], besides an overal similarity, these receptors share a number of specific structural features, including the absence of a conserved cysteine residue in the second extracellular loop (which is believed to form a disulphide bridge in most G-protein-coupled receptors), and distinct motifs that diverge significantly from other receptor classes (Glu-Asn rather than Gly-Asn in the first transmembrane segment, and no proline residue in the fifth transmembrane segment). ACCA is expressed mainly in the brain, and to a lesser extent in testis, ovary and eye. The other members of the subgroup are also principally expressed in brain, with the exception of the peripheral cannabinoid receptor, which is expressed in lymphoid tissues [14]. Some of these related receptors are also expressed in other tissues: the central cannabinoid receptor [13] and the R334 orphan receptor [18] are expressed in testis, and the AGR16 orphan receptor is expressed in lung, heart, stomach and intestine [17].

When expressed in cell lines, ACCA was able to activate the adenylate cyclase to a level similar to that obtained for other G_s-coupled receptors fully stimulated by their respective ligands. This property was shared by both the human and the mouse receptors, and was obtained with a large number of cell lines originating from different mammalian species and derived from a variety of cell types (Figure 5). We can therefore exclude the possibility that the constitutive activity is the result of mutations affecting receptor properties. It rather appears as an intrinsic property of ACCA, which is independent from both the species of origin and the cell type in which it is expressed. Two mechanisms could account for the observed basal adenylate cyclase stimulatory activity of ACCA: stimulation by an ubiquitous ligand or basal coupling to G_s in the absence of ligand.

Concerning the first mechanism (ubiquitous ligand), we excluded the likelihood that this putative ligand could be introduced in the assay through the serum used for cell culture. The ligand of ACCA could however be produced to a variable level (Figure 4a) by most, if not all, cell types. Using conditioned media or co-culture experiments, we were unable to demonstrate that cell lines exhibiting a strong adenylate cyclase stimulation upon hACCA expression release potential ligand(s) in the culture medium. The constitutive activity of the receptor could also be demonstrated on isolated membranes. The activation of the receptor by a membrane-bound ligand cannot, however, be ruled out completely. Given the sequence similarity to the cannabinoid receptors and the chemical nature of the natural ligand of cannabinoid receptors (anandamide), it is quite possible that the ligand of ACCA is a membrane-derived or membrane-bound lipid, widely distributed across species and cell types. This situation would be reminiscent of that of the adenosine A2a receptor first isolated in our laboratory as an orphan receptor called RDC8 [21]. Expression of RDC8 in cell lines resulted in the activation of adenylate cyclase, and only after the identification of the ligand was it possible partially to inhibit this putative constitutive activity. Indeed incubation of the cells in presence of adenosine deaminase reduced the activation of adenylate cyclase [11].

The alternative mechanism by which ACCA would stimulate adenylate cyclase in the absence of ligand–receptor interaction is equally possible. It has been shown that some G-protein-coupled receptors may exhibit a basal G-protein-coupling activity. The TSH receptor, for example, activates adenylate cyclase without addition of TSH both in COS-7 and CHO-transfected cells [9]. Barker et al. [8] demonstrated, similarly, that the 5-HT2C receptor transfected into NIH3T3 cells constitutively activates phospholipase C, leading to basal PtdIns formation. Other examples of basal coupling activities involve the β_2-adrenergic receptor [40] and the dopamine D1B receptor [10]. This basal activity can be demonstrated by the inhibitory effect of some antagonists that are referred to as 'inverse agonists' or 'negative antagonists' [35,41]. The hypothesis is that basal effector activity reflects precoupling of the receptor to G-proteins and that inverse agonists reduce this basal activity by uncoupling the receptor–G-protein complex [7]. If the stimulatory activity of ACCA is a true constitutive activity, this activity would differ from that reported for other receptors by the amplitude of the stimulation. The basal activity of ACCA is comparable with that of other fully stimulated G_s-coupled receptors. It is clear, however, that treatment of ACCA-transfected cells with forskolin can increase cyclic AMP accumulation, demonstrating that the cascade can be further stimulated. According to this hypothesis, a constitutively active ACCA receptor could mediate a sustained stimulation of the cyclic AMP cascade in the cells that express it. This elevated cyclic AMP level could be further modulated upwards or downwards by specific ligands acting as agonists or inverse agonists respectively. Future studies will determine which of these two hypotheses holds true.

During the course of this work, a partial sequence of human ACCA was published under the name GPR3 and located to the 1p35-p36.1 region of the human genome [42].

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