Gene and cDNA cloning and characterization of the mouse V3/V1b pituitary vasopressin receptor

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ABSTRACT

The gene of the mouse V3/V1b receptor was identified by homology cloning. One of the genomic clones contained the entire coding sequence. The cDNA presented high identity with rat (92%) and human (84%) sequences. Southern blot analysis indicated the existence of a single gene. Tissue distribution was studied by RT-PCR. The major site of expression was the pituitary. A faint signal was also present in hypothalamus, brain, adrenal, pancreas and colon. The mouse corticotroph cell line, AtT20, did not express the transcript. In order to confirm the identity of the sequence, the V3/V1b receptor cDNA was cloned and stably expressed in CHO-AA8 Tet-Off cells under the control of tetracycline. When transfected cells were treated with arginine vasopressin (AVP), inositol phosphate

production increased in a dose-dependent manner, indicating that the V3/V1b receptor couples to phospholipase C. Moreover, AVP did not stimulate cAMP production. Binding studies with [³H]AVP indicated that the affinity of the mouse V3/V1b receptor ($K_d=0.5$ nM) is similar to that reported for rat and human receptors. The rank order of potency established in competition binding experiments with different analogues was representative of a V3/V1b profile, distinct from V1a and V2. However, significant differences were found between human and mouse receptors tested in parallel. Thus the pharmacology of V3/V1b receptors can not be transposed among different species.

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INTRODUCTION

The activation of the pituitary–adrenal axis is an essential component of the response to stress, both in man and in rodents. Glucocorticoid secretion from the adrenal gland depends on release of adrenocorticotrophic hormone (ACTH) at the pituitary level, which, in turn, is under hypothalamic control. Corticotrophin releasing hormone (CRH) is the positive effector, able to increase both the synthesis and the release of ACTH. In addition, vasopressin (AVP), a nonapeptide coexpressed with CRH in parvocellular neurones of the paraventricular nucleus, potentiates the ACTH-releasing effect of this hormone (Gillies *et al.* 1982). The existence of a pituitary subtype of vasopressin receptors, distinct from the phospholipase C (PLC)-coupled V1a and the adenylyl cyclase-coupled V2 receptor subtypes, had been suspected for a decade on the basis of pharmacological studies

(Jard *et al.* 1986). In 1994, the corresponding human cDNA was cloned simultaneously by RT-PCR of pituitary RNA (Sugimoto *et al.* 1994) and by screening a cDNA library constructed from a corticotroph adenoma (de Keyzer *et al.* 1994). As expected from its coupling to PLC, it belongs to the serpentine family of G-protein-coupled receptors and is expressed mainly in the pituitary, albeit at a low level. We have cloned the murine isoform as a first step towards obtaining animal models including additive or subtractive transgenesis, in order to establish the relevance of the V3/V1b vasopressin receptor in pituitary function.

MATERIALS AND METHODS

Materials

Standard reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA). Cell culture

media and antibiotics were obtained from Boehringer Mannheim (Meylan, France). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Restriction and modification enzymes were obtained from New England Biolabs (Beverly, MA, USA) or Gibco-BRL Life Technologies (Cergy-Pontoise, France). Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium). [³H]Arginine-vasopressin (specific activity 59 Ci/mmol) and labelled nucleotides were purchased from New England Nuclear-DuPont (Le Blanc Mesnil, France). Myo-[2-³H]inositol (10–20 Ci/mmol) was purchased from Amersham (Les Ulis, France). Vasopressin (AVP) and analogues were obtained from Bachem (Torrance, CA, USA).

The mouse corticotroph cell line AtT20 was kindly provided by Dr Drouin (Montreal), and the mouse pancreatic β -cell lines β TC1 and β TC6 by Dr Scharfmann (Paris). African green monkey fibroblasts COS-7 cells were obtained from the American type culture collection (Rockville, MD, USA).

Cloning of a mouse V3/V1b genomic DNA fragment by nested PCR

Standard molecular biology techniques were carried out as described elsewhere (Ausubel *et al.* 1998), unless otherwise stated.

Degenerated primers based on the human V3/V1b sequence previously cloned in the laboratory (de Keyser *et al.* 1994) were used to perform a nested PCR on mouse genomic DNA. The first round of PCR included the 5' primer, 5'-ATGT (A/T)(C/T)GCITCICCTA(C/T)ATG-3' (position 352–372 in transmembrane domain (TMD) III of human V3/V1b), and the 3' primer, 5'-TT (G/A)TCCCA(A/C/G/T)AC(A/C/G/T)(C/G)(A/T)CCACAT-3' (position 904–923 in TMD VI of human V3/V1b). The PCR reaction, including 50 ng mouse genomic DNA, 200 ng each primer and 2.5 mM MgCl₂, was run for 35 cycles (94 °C for 45 s, 42 °C for 120 s and 72 °C for 90 s), followed by 5 min extension at 72 °C.

Reaction products (1 μ l) were subjected to a second round of PCR under the same conditions, except that annealing was carried out at 48 °C for 120 s and extension at 72 °C for 60 s, with the 5' primer, 5'-TGGCCATG(A/T)C(A/G)(G/C)(C/T)(G/C)GACCG-3' (position 377–395 in the second extracellular loop of human V3/V1b), and the 3' primer, 5'-(A/G)(A/C)TGAAGAA(G/A/T)GG (C/T)G(C/T)CCAGCA-3' (position 877–898 in the third intracellular loop of human V3/V1b).

PCR products were subcloned into a PCR II vector with the TA cloning kit (Invitrogen, San Diego, CA, USA). Positive clones were identified by low-stringency hybridization of a 1.2-kb fragment of human V3/V1b cDNA labelled by random priming (Megaprime, Amersham).

Screening of a lambda phage mouse genomic library

A lambda FIX II mouse genomic library (Stratagene, La Jolla, CA, USA) was screened both at high stringency with the 534-bp fragment of the mouse V3/V1b receptor obtained by PCR (see above) and at low stringency with a fragment of the human V3/V1b receptor cDNA. These two probes correspond respectively to the 5' and 3' regions of the coding sequence and were intended to hybridize with each of the two coding exons of the mouse gene hypothesized by analogy with the structure of the human gene.

Inserts of the positive lambda clones and their partial BamHI and EcoRI digests were subcloned into Bluescript (Stratagene) for sequencing with the Sequenase II sequencing Kit (US Biochemical Corp, Cleveland, OH, USA).

RT-PCR cloning of the mouse V3/V1b cDNA

Mouse pituitary RNA was purified with RNA Plus reagent according to the instructions of the manufacturer (Bioprobe, Montreuil, France). Mouse pituitary cDNA was synthesized from 2 μ g RNA with Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL Life Technologies). A fragment of the mV3/V1b receptor cDNA including the entire coding sequence was obtained by PCR with the 5' primer, 5'-CATCCACCTTCCCCTCCTTCT-3' (positions –36 to –15 of the mouse cDNA sequence according to the first ATG deduced from the gene sequence), and the 3' primer 5'-CACTAAGCCC GCCTTGACA-3' (position +1415 to +1436 of the mouse gene sequence). The PCR reaction procedure, with one-tenth of the RT reaction as template, was performed as described above, except that the annealing reaction was carried out at 58 °C. PCR products were subcloned into the pT7 vector (Novagene, Abingdon, UK) and sequenced. The pT7 plasmid containing the entire wild-type cDNA sequence of the mouse V3/V1b receptor was called pMV3–9.

Expression of mV3/V1b receptors

An EcoRI–XbaI fragment of the pMV3–9 plasmid corresponding to the insert was subcloned into the

tetracycline-regulated expression vector, pTRE (Clontech, Palo Alto, CA, USA), and the plasmid was called pTREmV3. The transient expression of mV3/V1b receptors was first tested by [³H]AVP binding (see below) to Cos-7 cells cotransfected with pTREmV3 and pTet-Off (Clontech) by lipofection (Dosper liposomal transfection reagent, Boehringer Mannheim). Subsequently, a cell line stably expressing mV3/V1b receptors was generated by cotransfection of CHO-AA8 Tet-Off cells (Clontech) with pTREmV3 and pTK-Hyg (Clontech), using the calcium phosphate precipitation method. Cells were grown in F12 medium supplemented with 10% FBS and G418 (100 µg/ml), selected with Hygromycin (200 µg/ml) and purified by the limiting dilution technique. Clones grown in the presence and absence of 1 µg/ml tetracycline for 48 h were selected by [³H]AVP binding (see below).

Radioligand binding assays

The cells were grown to confluence in 24-well dishes, washed with PBS, 10 mM MgCl₂ and 0.2% BSA, pH 7.4, incubated with [³H]AVP and unlabelled AVP or analogues for 1 h at room temperature in the same buffer, washed three times with cold PBS and recovered with 0.1% SDS, 0.1 N NaOH into liquid scintillation vials for counting. Non-specific binding was measured in the presence of 2 µM AVP. Kinetic constants (K_d and B_{max}) were derived from saturation experiments analysed with the Ebda-Ligand program (Elsevier Bio-Soft, Cambridge, UK). IC₅₀ values were derived from competition experiments by non-linear least squares analysis and K_i values were calculated with the equation of Cheng & Prusoff ($K_i = IC_{50} / (1 + (Lf / K_d))$) using the PRISM program (GraphPad).

Total inositol phosphate determination

Confluent control and transfected CHO-AA8 cells pretreated or not with 1 µg/ml tetracycline for 1 week were labelled with [³H] myo-inositol (2 µCi/well, 12-well dishes) for 24 h and washed with Krebs–Ringer–phosphate buffer, 6 mg/ml glucose, 0.04% BSA and 30 µg/ml bacitracin (complete KRP). After a 10-min preincubation at 37 °C with complete KRP containing 10 mM LiCl, the cells were incubated with various concentrations of AVP for 30 min in the same buffer. Total inositol phosphates were separated on Dowex 1X8 (200–400 mesh) columns as described elsewhere (de Keyser *et al.* 1994).

cAMP production

The cells were incubated with complete buffer plus 1 µM AVP or 10 µM forskolin for 4 min at 37 °C in presence of 5 µM indomethacin and 1 mM 3-isobutyl 1-methyl xanthine (IBMX) as described elsewhere (Klinger *et al.* 1998). The cAMP content of each sample was measured by radioimmunoassay (cAMP kit, Immunotech, Marseille, France) according to the instructions of the manufacturer.

RT-PCR analysis of mouse tissues and cell lines

cDNAs from different tissues and cell lines obtained by reverse transcription, in addition to control RNAs, were subjected to PCR analysis. Mouse V3/V1b receptor cDNA was amplified with a 5' primer, 5'-CTGGCCTACATTGCTTGCTG-3', and a 3' primer, 5'-GGTTGAGTTTCCTGATTTCC-3', in positions 853–873 and 1449–1469 of the coding mV3/V1b sequence. These positions were chosen respectively in the first and second exon, to distinguish cDNA from genomic DNA amplification. The Southern blot was hybridized with the internal oligonucleotide, 5'-CTCAGCCTCCATGCAAAGCC-3' in position 1172–1192 of the coding mV3/V1b sequence.

Control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) PCR was performed with the 5' primer, 5'-TGAACGATTTGGCCGTATTGG-3', in position 62–83 and the 3' primer, 5'-GAAGGCCATGCCAGTGAGCTT-3', in position 718–739. The Southern blot was hybridized with an internal oligonucleotide in position 199–221: 5'-CGGCAAATTCAACGGCACAGTC-3'.

Statistical analysis

The ANOVA of cAMP data was carried out with the Statview program (Abacus, CA, USA). Sequence comparisons were performed in the Infobiogen facility (INSERM, Villejuif, France) with the Bisanse program. Data are presented as mean ± S.E.M. (*n*).

RESULTS AND DISCUSSION

Cloning of mouse V3/V1b receptor gene

The initial 534-bp fragment of the mouse V3/V1b receptor gene was obtained by nested PCR of mouse genomic DNA using degenerated oligonucleotide primers issued from the first coding exon of the human V3/V1b sequence. Seven of the ten positive

clones hybridizing with a fragment of the human V3/V1b cDNA at low stringency contained sequences identical to mouse V1a receptor (Y Onodera, K Maekawa and Y Arai, Database Accession Number D49729, 1995, unpublished), whereas the three others contained a 534-bp insert showing 86% and 94% homology to the corresponding fragment of the human (de Keyzer *et al.* 1994, Sugimoto *et al.* 1994) and rat (Saito *et al.* 1995, Lolait *et al.* 1995) V3/V1b cDNA.

The 534-bp fragment of the mouse V3/V1b receptor was used to screen a mouse genomic library at high stringency. One of five different positive clones also hybridized at low stringency with a human probe corresponding to the 3' part of the coding sequence. This clone was subcloned and sequenced to establish the structure of the gene. In the rat V3/V1b gene, the coding sequence is interrupted by a 9-kb long intron between TMDs VI and VII (Saito *et al.* 1995). Similarly, in the mouse gene, two exons were identified, corresponding to 931 and 332 bp of the coding sequence respectively and separated by an approximately 9-kb long intron. The genes of other members of the AVP/oxytocin family also contain an intron between TMDs VI and VII (Barberis & Tribollet 1996).

Upstream of the first codon (ATG), an approximately 5-kb genomic fragment was present in the lambda clone and therefore was sequenced (not shown). This sequence contained several repeated stretches, such as a CT repeat followed by a CA repeat at positions -1023 to -847. In addition, a 19-bp duplication was observed at positions -560 to -521, immediately upstream of the 5' end of the longest rat cDNA sequence published to date (Lolait *et al.* 1995).

The characterization of the 5' flanking region of the gene requires the identification of the transcription start site. Rapid amplification of cDNA ends PCR experiments were unsuccessful, probably because of the scarcity of the mouse V3/V1b mRNA (see below). However, several arguments suggest that the 5' untranslated region extends at least 600 bp upstream of the first coding ATG. When compared with that of the mouse, the 521-bp long 5' untranslated region of the rat sequence was found to be 83% identical. Moreover, the positive RT-PCR of pituitary RNA carried out with a 5' primer at position -633 to -612 and a 3' primer at position +124 to +145 clearly indicates that this region belongs to the first coding exon. However, no experimental evidence indicates that the first coding exon is the first exon of the gene. A putative splice acceptor site, the sequence CCCTCCTCTG CAG/G, is present at position -641. Moreover, the analysis of the sequence upstream of the putative

splice position does not show any characteristic promoter structure. These data suggest that other non-coding exon/s exist upstream of the first coding exon in the mouse V3/V1b gene, as is the case for the rat and human oxytocin receptors (Rozen *et al.* 1995, Inoue *et al.* 1994). However, for both the rat V1a (Murasawa *et al.* 1995) and V2 (Mandon *et al.* 1995) receptor genes, the transcription start site has been localized 300–500 bp upstream of the first ATG codon in the first coding exon. This possibility can not be excluded for V3/V1b receptor genes. Additional work is therefore required to identify formally the eventual non-coding exons, the transcription start site and the 5' flanking regulatory regions of these genes.

The coding sequence was compared with human and rat V3/V1b receptor sequences (de Keyzer *et al.* 1994, Sugimoto *et al.* 1994, Lolait *et al.* 1995, Saito *et al.* 1995), and the nucleotide identity was 84 and 92% respectively. Besides single nucleotide changes corresponding to species variations, the only differences corresponding to three to four codon insertions or deletions were found in the middle of the sequence coding for the third intracellular loop, one of the less conserved regions.

Finally, the mouse genomic clone contained 279 bp beyond the stop codon. This region does not contain consensus sequences for polyadenylation sites, indicating that the 3' non-coding region extends beyond the end of the clone.

Southern blot analysis of mouse genomic DNA indicated the existence of a single gene (not shown). This is also the case for human (de Keyzer *et al.* 1994) and rat (Saito *et al.* 1995) V3/V1b receptor genes.

Translation of the open reading frame corresponding to the coding exons resulted in a 421-amino acid protein belonging to the G-protein-coupled receptor family with seven putative TMDs. When compared with other V3/V1b receptor sequences available (Fig. 1), the amino acid identity was found to be 94% with rat and 81% with human. Sequences of mouse members of the AVP/oxytocin family were also compared. The identity decreased to 54% and 51% with mouse oxytocin (Y Kubota, Database Accession Number D86599, 1997, unpublished) and V1a vasopressin (Y Onodera, K Maekawa and Y Arai, Database Accession Number D49729, 1995, unpublished) receptors respectively. In the absence of an available mouse V2 receptor sequence, the mouse V3/V1b receptor sequence was compared with the rat V2 receptor sequence (Lolait *et al.* 1992), which was 49% identical. Finally, comparison with the teleost fish, *Catostomus commersoni*, vasotocin receptor sequence (Mahlmann *et al.* 1994) indicated a 53% identity.

mv3	MDSEPSWTATPSPGGTLFVPNTTTPWLGRDEELAKVEIGILATVLV	LATGGNLAVLLILGLQGHKSRMHFLVFLHLAL
rv3	-N-----P--A-----	-----T--RH-----
hv3	---G-L-D-N-T-R---SA--A-----V-----	-----T--QL-R-----
mv1	MSFPRGSHDLPAAGNSPWWPLTTEG-NS-REAGLGGGSP-GDV-N----L-VTV--VIF-	V-VL--SS---A-HRTPR-T-----IR--S-
rv2	MLLVSTVSAVPLF--PSSPSNSQEEELDD--PL-VRA--AL-S-IF-AVA-SN-LV-GA-IRR-RR-R WAP--V-IS--C-	--LS--AC---A-RTTR--H--LFF-MK--SI
moxy	MEGTPAANWSIELDLG-GVPPGAEG-L-AGPPR-N-A--R--VAV-CLI-F	V-VI---S---AMHNTKK-S-----IK--S-
ccvt	MGRIANQTTASND-DPF--N--V--M--TV-SVTFF	
TM1		
mv3	TDLGVALFQVLPQLLDITRYFQGSDDLCAVVKYLQVLSMFASTYMLLAMTLDRLAVCHPLRSLQPSQST	YPLIAAPWLLAAILSLPQVFIF
rv3	-----R-----	-----
hv3	---A-----P-----G---L-----F-----	
mv1	A--A--F-----C-----R-P-W--V--H--FA---S--VV--A--I-----KT---ARRS RLM--S-G-SFV--I--Y---	
rv2	A--A-----A--A-D--H-P-A-----MVG-Y--S--I-----HR-I-R-MLAYRHGGGARWNRPVLA-AFSL--L---	
moxy	A--V--V-----F--Y-P--L-----VG-----L--L-S--C--I-Q-----RRRTD RLAVL-T--GCLVA-V--H--	
ccvt	A-MV--F-----C-E--F--Y-P-F--I--H--G-----MVM-----I-I--KT---T-RA--IM-GST--CSLL--T--Y---	
TM2 TM3 TM4		
mv3	SL REVIQSGSVLDCWADFYFSWGPRAYITWTMTAIFVLPVVLTACYGLICHEIYKNLKVKTQAGREERRGWP	KSSSSA AAAATRGL
rv3	-- --S-----A--S-----RTWD-----V-T-----	
hv3	-- --G-P-----L--L-----TM-----S-----C-----W-VGGG--RTWDRP-P-T L--T---	
mv1	-VIEF--NN-TKAQ---T-IPP--T--V--M-SGV--V--II-GT---F--YH-WR-VRG--AS -QSKG- -G-GE- -GPFHK--LV	
rv2	AQ -D-GN---F---R-AEP--L--V--IALMV--A-ALGIA--QV--FR--HAS- -PGPSE-AG GRRRGRTG-P- EG-H	
moxy	-- --AD--F---V-IQP--K--V--I-L-VYIV--I--A-----SFK-WQ--RL--A-AAAA AEG-D- -GG-G-AA	
ccvt	-- S-IQN--Y-Y--GH-IEP--I-----I-VG--LI--II-MI--F--S-W--I-C--MR-TRNTKDGMI-K	
TM5		
mv3	PSRVSSISTISRAKIRTVKMTFVIVLAYIACWAPFFSVQMWSVDENAPNEDSTNVAFITISMLLGNLSSCCNPWIYMGFNHLLPRSLSHRACCR	
rv3	-----R-----H--T	
hv3	-----N-----K--D-----N-----P-R-L---G	
mv1	TPC--VKS-----S--L--T--I-----T-FVWT--E-PST--TA--AS-N-----F-SG--QDCVQSFP--Q	
rv2	--A AM-- --R--L--IV-VL-----L--L-AA--PE--L RPP-VLL--AS-N--T---AS-S-SVSSE -RSL--A	
moxy	LA--V-KL--K-----I--F-V--T--F-----V--K-A S--I-A--AS-N-----L-TG--FHVLVQRFL--S	
ccvt	V--VTI--L-----L-----V-----I-----FSWD--E-A-V-L-A--AS-N-----L-SG--YDF-RCFP--K	
TM6 TM7		
mv3	GSKPRVHRQLSNSSLASRRITLLTHTCGPSTLRLSLNLSLHAKPKPAGSLKDLEQVDGEATMETSIS	
rv3	-----Q-----T---T-----A--SP-----R--R-----F	
hv3	-PQ--MR-R--DG--S--H-----RSSC-A--S---S-T-SGR-R-EE-PR---LA--G-A--I-F	
mv1	SIAQKFAKDD-D- M---Q-SYNNRS-TNSTGTWKD-PKSSKSIRFIPVST	
rv2	QRHTTHSLGPQDE-C-TASSS-MKD-PS	
moxy	ARYLKGS-PGET-ISKKSNSSTFVLSRCS-SQ-SCSQP-SA	
ccvt	KPRNMLQKED-D-- I--N---KLAAGRMTNDGFGSWRDPNCRKS-QSIGLDCFKSSQCLEHDCSRKSSQCIPLDCSRKSSQCIPLDCSRK	
ccvt	SSQCMSKES	

FIGURE 1. Alignment of the aminoacid sequences of AVP/oxytocin family receptors. Mouse V3/V1b (mv3), rat V3/V1b (rv3), human V3/V1b (hv3), mouse V1a (mv1), rat V2 (rv2), mouse oxytocin (moxy) and fish vasotocin (ccvt) receptor sequences are compared. Amino acids identical to the reference mouse sequence are indicated by hyphens. Underlined regions correspond to putative TMDs. The intron position is indicated by an asterisk.

The most conserved regions were found in TMDs II, III, IV, VI and VII, and in the first and second extracellular loops; in contrast, the third intracellular loop and the N- and C-terminal regions strongly diverged among the different receptor types.

The amino acid sequence shows several common features with other members of the AVP/oxytocin family. Six Gln and Lys residues, present in equivalent positions of TMDs II (Gln⁸⁷, Gln⁹¹), III

(Lys¹¹³, Gln¹¹⁴), IV (Gln¹⁶⁸) and VI (Gln²⁹⁸) of all members of the AVP/oxytocin family, also exist in the mouse V3/V1b receptor sequence. These residues are probably involved in the binding of the cyclic moiety of the hormone, as suggested by molecular modelling and subsequent site-directed mutagenesis studies on the V1a receptor sequence (Mouillac *et al.* 1995). Moreover, Tyr¹¹⁵ in the first extracellular loop, essential for AVP recognition by

V1a receptors (Chini *et al.* 1995), is also present at an equivalent position (Tyr⁹⁸) in the mouse V3/V1b receptor sequence. This residue probably interacts with the AVP side-chain residue, Arg⁸, according to the three-dimensional model cited above.

Nine cysteyle residues out of 11 are conserved in all members of the AVP/oxytocin family at equivalent positions and are involved either in disulphide bridge formation (Pavo & Fahrenholz 1990) or palmitoylation (Sadeghi *et al.* 1997) of this class of receptors. The N-terminal extracellular region contains a unique putative N-glycosylation site at Asn²¹, at variance with other members of the oxytocin/vasopressin family such as the V1a receptor (Burbach *et al.* 1995). As demonstrated for the V2 receptor, this glycosylation is not essential for receptor function (Innamorati *et al.* 1996).

The third intracellular loop and the C-terminal tail contain putative phosphorylation sites for protein kinase A (Thr³⁷⁵) and protein kinase C (Thr²⁷⁶, Ser³⁴⁸, Ser³⁷¹, Thr³⁸⁵ and Ser⁴⁰³). AVP induces the phosphorylation of Ser-Thr clusters present in the C-terminus of the V2 receptor sequence via G protein receptor-specific kinases (GRK) (Innamorati *et al.* 1997). Potential sites for GRK phosphorylation are also present in the C-terminus of the V3/V1b receptor.

The second (IL2) and third (IL3) intracellular loops are crucial for specific coupling of AVP/oxytocin receptors to G proteins: IL2 of the V1a receptor bears the determinants for Gq coupling, whereas IL3 of the V2 receptor is involved in Gs coupling (Liu & Wess 1996). The IL2 of the mouse V3/V1b receptor showed greater amino acid identity with Gq/11-coupled receptors (V1a, VT and oxytocin) than with the V2 receptor. We identified a six-amino acid motif, Leu-Arg/Lys-Ser/Thr-Lys-Gln/Arg-Gln/Arg, common to Gq/11-coupled receptors and absent from V2 receptors. The comparison of IL3 sequences was hindered by their different length and amino acid heterogeneity.

Pharmacological characterization of mouse V3/V1b receptor

Cloning and sequencing of mouse V3/V1b cDNA by RT-PCR of mouse pituitary RNA confirmed the sequence and the exon-intron structure of the gene. This cDNA sequence was subcloned into the inducible expression vector, pTRE (Resnitzky *et al.* 1994), and a cell line stably expressing the mouse V3/V1b receptor as a function of tetracycline concentration was obtained by transfecting pTREmV3 into CHO-AA8 Tet-Off cells which express the transcriptional activator, tTA. The pharmacological characterization of mouse V3/V1b

FIGURE 2. Binding of [³H]AVP to CHO-AA8 cells expressing the mouse V3/V1b receptor. CHO-AA8 cells transfected with pTREmV3 were grown to confluence. Saturation binding experiments were performed with increasing concentrations of [³H]AVP in the presence or absence of 2 μ M cold AVP. Specific binding is shown. Non specific binding did not exceed 6% of total binding. Inset: Scatchard plot. The data are means $\pm$ S.E.M. of one representative experiment repeated three times, with triplicate determinations.

receptor was carried out by [³H]AVP binding to this cell line. CHO-AA8 Tet-Off non-transfected cells did not bind [³H]AVP (data not shown). Kinetic constants were derived from saturation experiments (Fig. 2). The Scatchard plot was linear, indicating a single class of binding sites. The receptor bound [³H]AVP with the same affinity ($K_d=0.58 \pm 0.10$ ($n=3$) and 0.47 ± 0.03 ($n=3$) nM respectively), in tetracycline-treated and -untreated cells. These values are in good agreement with those reported for human (de Keyser *et al.* 1994) and rat (Lolait *et al.* 1995) cloned V3/V1b receptors – 0.55 and 0.63 nM respectively. In the presence of tetracycline, the number of binding sites decreased from 48669 ± 2990 ($n=3$) to 6089 ± 662 ($n=3$) in 48 h and remained stable for at least 1 week. This indicates either a slow turnover of the receptors or, more probably, some degree of promoter leakage.

Competition binding experiments were carried out in parallel in human and mouse V3/V1b receptor-expressing CHO cells (Fig. 3). Among natural hormones, vasotocin displaced [³H]AVP three times more efficiently than did AVP itself, whereas oxytocin was 70 times less efficient in mouse V3/V1b receptor transfected cells (Table 1). The rank order of potency is AVP>VT>oxytocin for AVP receptors, and AVP=oxytocine>vasotocin for oxytocine receptors (Burbach *et al.* 1995). Thus the pharmacological profile of the expressed receptor corresponds to a vasopressin receptor.

FIGURE 3. Binding of [^3H]AVP to CHO-AA8 cells expressing the mouse V3/V1b receptor. CHO-AA8 cells transfected with pTREmV3 were grown to confluence. Competition binding experiments were performed with 4 nM [^3H]AVP and increasing concentrations of AVP ($\square$) or analogues. Non-specific binding in the presence of 2 μM cold AVP did not exceed 3% of total binding. (A) Competition curves of agonists: vasotocin ($\bullet$), (deamino-Cys 1 , β (3-pyridyl)-D-Ala 2 ,Arg 8)-VP ($\diamond$), oxytocin ($\blacklozenge$) and (deamino-Cys 1 ,D-Arg 8)-VP ($\circ$). (B) Competition curves of antagonists: (phenylac 1 ,D-Tyr(Et) 2 ,Lys 6 ,Arg 8 ,des-Gly 9)-VP ($\blacktriangledown$), (d(CH $_2$) $_5^1$,Tyr(Me) 2 ,Arg 8)-VP ($\triangle$) and (d(CH $_2$) $_5^1$,D-Ile 2 ,Ile 4 ,Arg 8 ,Ala-NH $_2^9$)-VP ($\blacktriangle$). All competitors were tested simultaneously in each experiment, repeated three times. The data (means $\pm$ S.E.M.) are expressed as % of maximal binding in the absence of competitor. One representative experiment with triplicate determinations is shown.

Interestingly, the affinity of vasotocin and oxytocin was one order of magnitude higher in mouse than in human V3/V1b receptors.

The development of hundreds of peptidic, linear or cyclic, in addition to non-peptidic AVP analogues has allowed classification of the different

physiological responses into three categories, corresponding to three types of receptors: a vascular type, V1a, also present in liver membranes, a renal type, V2, and a pituitary type, V3/V1b (Burbach *et al.* 1995, Barberis & Tribollet 1996). The majority of potent V1a receptor antagonists exhibit lower affinity for the V3/V1b receptor, whereas V1a agonists are equipotent on V3/V1b receptors (Burbach *et al.* 1995). This was the basis for the identification of the V3/V1b receptor in pituitary membranes (Jard *et al.* 1986). In our hands, the affinity of V1a-specific antagonists, (d(CH $_2$) $_5^1$,Tyr(Me) 2 ,Arg 8)-VP and (phenylac 1 ,D-Tyr(Et) 2 ,Lys 6 ,Arg 8 ,des-Gly 9)-VP, was more than two orders of magnitude lower than that of AVP on mouse V3/V1b receptors. Among V2 selective agonists, (deamino-Cys 1 ,D-Arg 8)-VP (dDAVP) has a reasonably good affinity for human V3/V1b: 20 nM (Table 1). The affinity for the mouse receptor was fivefold lower. As expected, the affinity of the V2 antagonist, (d(CH $_2$) $_5^1$,D-Ile 2 ,Ile 4 ,Arg 8 ,Ala-NH $_2^9$)-VP, was more than three orders of magnitude lower than that of AVP itself. Highly selective ligands for the V3/V1b receptor are not currently available, except for the human V3/V1b agonist, (deamino-Cys 1 , β (3-pyridyl)-D-Ala 2 ,Arg 8)-VP, the affinity of which was 14 nM in this study. This compound was 6.5 times less effective on mouse V3/V1b receptors. In conclusion, the pharmacology of the cloned receptor corresponds to a V3/V1b profile and is in good agreement with the data reported in the literature, including relevant species differences (de Keyzer *et al.* 1994, Sugimoto *et al.* 1994, Lolait *et al.* 1995, Saito *et al.* 1995, Thibonnier *et al.* 1997, Burbach *et al.* 1995, Barberis & Tribollet 1996).

Physiological characterization of mouse V3/V1b receptor

Functional coupling of mouse V3/V1b receptors was studied in stably transfected CHO-AA8 cells. Vasopressin activated production of total inositol phosphates in a dose-response manner (Fig. 4), thus demonstrating that the cloned receptor is functionally coupled to the PLC pathway, as in human and rat (de Keyzer *et al.* 1994, Lolait *et al.* 1995). Maximal effects were obtained beyond 100 nM AVP, in either tetracycline-treated (threefold) or -untreated (fivefold) cells. The EC $_{50}$ was similar in both cases: 6.7 ± 1.2 nM and 4.6 ± 0.7 nM respectively. Non-transfected cells did not respond to AVP. Vasopressin (1 μM) did not modify basal cAMP concentrations, but slightly decreased (by 14%, $P < 0.05$) the response to 10 μM forskolin of

TABLE 1. Comparison of the affinity (K_i) of AVP and structural analogues for the mouse (m) and human (h) V3/V1b receptors

Compound	K_i (nM)		
	mV3	hV3	mV3/hV3
Natural hormones			
AVP	1.5 ± 0.20	1.0 ± 0.13	1.50
Vasotocin	0.5 ± 0.09	5.9 ± 0.09	0.09
Oxytocin	103 ± 27.0	910 ± 390	0.11
V1 antagonists			
(d(CH ₂) ₅ ¹ ,Tyr(Me) ² ,Arg ⁸)-VP	340 ± 52.0	540 ± 106	0.63
(phenylac ¹ ,D-Tyr(Et) ² ,Lys ⁶ ,Arg ⁸ ,des-Gly ⁹)-VP	140 ± 23.0	29 ± 5.2	4.83
V2 analogues			
(deamino-Cys ¹ ,D-Arg ⁸)-VP	130 ± 6.0	20 ± 0.40	6.50
(d(CH ₂) ₅ ¹ ,D-Ile ² ,Ile ⁴ ,Arg ⁸ ,Ala-NH ₂ ⁹)-VP	>2000	>2000	
V3 agonists			
(deamino-Cys ¹ ,β(3-pyridyl)-D-Ala ² ,Arg ⁸)-VP	91 ± 7.3	14 ± 5.8	6.50

Mean ± s.e.m. ($n=3$) of the affinity constants derived from paired competition experiments performed in CHO-AA8 cells stably expressing the mouse (mV3) cDNA and CHO cells stably expressing the human (hV3) clone with 4 nM [³H]AVP and 2 μM cold AVP. K_i values were calculated according to the Cheng & Prusoff equation.

CHO-AA8 cells transfected with pTREmV1b (Fig. 5). This decrease in cAMP might result from the inhibition of adenylate cyclase activity due to

some degree of coupling of the V3/V1b receptor to Gi, as shown for the V1a receptor (Strakova *et al.* 1997).

FIGURE 4. Production of total inositol phosphates (IP) by CHO-AA8 cells expressing the mouse V3/V1b receptor. CHO-AA8 cells, naive (□) or transfected (○, ●) with pTREmV3 were grown to confluence in the presence (■) or absence (□, ○) of 1 μg/ml tetracycline for 1 week. The cells were treated for 30 min with increasing concentrations of AVP in the presence of 10 mM LiCl and total inositol production was determined in triplicate. The data are expressed as % of basal values (means ± s.e.m., $n=3$ experiments).

FIGURE 5. Production of cAMP by CHO-AA8 cells expressing the mouse V3/V1b receptor. CHO-AA8 cells transfected with pTREmV3 were grown to confluence. Production of cAMP was determined in triplicate in cells treated with vehicle, 0.1% dimethyl sulphonate (C), 1 μM AVP and vehicle (A), 10 μM forskolin (F) or both (A+F) for 4 min in the presence of 1 mM IBMX. Means ± s.e.m. ($n=3$ experiments) are shown. In the two-way ANOVA, both the forskolin factor ($P<0.001$) and the AVP factor ($P<0.027$) were significant.

Tissue distribution

The expression of the V3/V1b receptor is probably scarce, as we were unable to detect it by Northern blot. For this reason, tissue distribution was studied by RT-PCR. The presence of V3/V1b transcripts was detected essentially in the pituitary. This is not surprising, as the V3/V1b receptor was first defined pharmacologically as a pituitary-specific subtype (Jard *et al.* 1986). Faint signals were also detected in brain, hypothalamus, adrenal, pancreas and colon, but not in liver or in kidney (Fig. 6). This distribution is an additional argument in favour of the V3/V1b specificity of the cloned cDNA.

Vasopressin binding sites have been described in several structures of the brain, either by direct autoradiography with tritiated or iodinated AVP or analogues, or by *in situ* hybridization with V1a or V2 probes (Burbach *et al.* 1995). The results obtained with the two techniques do not always overlap, suggesting that binding sites different from V1a and V2 could exist. In fact, V3/V1b receptors have been detected by RT-PCR in several rat brain regions, including hypothalamus (Lolait *et al.* 1995). The presence of V3/V1b sites has been reported in rat (Grazzini *et al.* 1996), but not in human (de Keyser *et al.* 1994) adrenal medulla, whereas V1a sites are present in the adrenal cortex of both species. The V3/V1b receptor transcripts found in mouse pancreas are probably involved in the potentiating effect of vasopressin on insulin release by mouse islets (Gao *et al.* 1994). In conclusion, the sequence reported here corresponds to the mouse V3/V1b receptor on the following basis: first, its high degree of homology with the receptor sequences reported for rat and human; secondly, the preferential expression of the transcripts in the pituitary; thirdly, the rank order of potency of analogues obtained in [³H]AVP binding studies; fourthly, the preferential coupling to PLC.

The cloning and characterization of mouse V3/V1b receptor gene and cDNA are the first steps towards obtaining *in vivo* and *in vitro* animal models, including additive or subtractive transgenesis, in order to understand the physiological role of vasopressin in pituitary function.

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FIGURE 6. Distribution of mouse V3/V1b receptor mRNA in adult mouse tissues, by RT-PCR. Two-microgram samples of RNA were incubated with MMLV reverse transcriptase. One-tenth of each reaction was subjected to PCR with primers flanking the intron of mouse V3/V1b receptor (shown here as mV3) or with primers chosen in the mouse G3PDH sequence for comparison. The expected sizes were 616 bp for mV3/mV1b and 677 for G3PDH. Ten microlitres of each PCR reaction were electrophoresed in two separate gels, Southern blotted, hybridized with internal oligonucleotides to either mouse V3/V1b receptor or G3PDH and autoradiographed. Overnight exposures are shown. Ethidium bromide staining gave similar results. Samples not subjected to reverse transcription were negative (not shown). gDNA, genomic DNA; βTC6, βTC1, mouse pancreatic β-cell lines.

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