G PROTEIN-COUPLED RECEPTOR SIGNALLING IN NEUROENDOCRINE SYSTEMS

Transcriptional and post-transcriptional mechanisms regulating the rat pituitary vasopressin V1b receptor gene

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Abstract

The number of V1b vasopressin receptors (V1bR) in the anterior pituitary plays an important role during adaptation of the hypothalamic–pituitary–adrenal axis to stress in rats. Regulation of V1bR expression involves transcriptional and translational mechanisms. One of the elements mediating transcriptional activation of the rat V1bR gene is a long stretch of GAGA repeats (GAGA box) in the promoter located near the transcription start point capable of binding a protein complex of 127 kDa present in pituitary nuclear extracts. There is a lack of correlation between changes in V1bR mRNA and the number of VP binding sites, suggesting that V1bR expression depends on the efficiency of V1b R mRNA translation into protein. Two mechanisms by which the 5′ untranslated region (5′-UTR) of the rat V1bR mRNA can mediate either inhibition or activation of V1bR mRNA translation have been identified. First, upstream open reading frames (ORF) present in the 5′-UTR repress translation of the major ORF encoding the V1b receptor, and secondly, an internal ribosome entry site (IRES) activates V1bR translation. Stimulation of IRES activity through protein kinase C-mediated pathways results in V1bR mRNA translation increasing V1bR protein levels. The existence of multiple loci of regulation for the V1bR at transcriptional and translational levels provides a mechanism to facilitate plasticity of regulation of the number of pituitary vasopressin receptors according to physiological demand.

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Introduction

Adaptation of the organism to a changing environment involves activation of the hypothalamic–pituitary–adrenal (HPA) axis and changes in pituitary responsiveness depending on the type and duration of the stimulus (Dallman *et al.* 1992, Aguilera 1994). This requires rapid regulation of hypothalamic hormones and their plasma membrane receptors controlling adrenocorticotrophin (ACTH) secretion from the pituitary corticotroph. An important factor in this regulation is the hypothalamic peptide, vasopressin (VP), which is

co-secreted with corticotrophin-releasing hormone (CRH) from parvocellular neurons in the paraventricular hypothalamic nucleus (Antoni 1993, Aguilera 1994). VP binds in the pituitary corticotroph to VP V1b receptors (V1bR), a 7 transmembrane domain receptor coupled to protein kinase C-calcium dependent mechanisms (Jard *et al.* 1987, Peter *et al.* 1995). The VP V1bR is the major subtype in the pituitary corticotroph and mediates the stimulation of ACTH secretion by VP.

Production and secretion of ACTH is under the stimulatory effect of CRH and VP and the

inhibitory influence of adrenal glucocorticoids (Antoni 1993, Whitnall 1993, Aguilera 1994). In humans and rodents, vasopressin alone is a weak ACTH secretagogue, but through its interaction with CRH it appears to be critical for the maintenance of corticotroph responsiveness even in the presence of elevated glucocorticoid levels during chronic stress (Gillies *et al.* 1982, Antoni 1993, Aguilera 1994). Physiological states involving activation of the HPA axis, such as stress or adrenalectomy, are associated with increased expression and secretion of vasopressin from parvocellular neurons which also express CRH (Whitnall 1993, Aguilera 1994). Studies in the rat have shown that about 50% of CRH-containing perikarya in the parvocellular hypothalamic paraventricular nucleus co-express vasopressin, and this proportion increases substantially during acute and chronic stress paradigms associated with increased pituitary ACTH responsiveness to a novel stress (DeGoeij *et al.* 1991, Whitnall 1993). This increase in hypothalamic VP is thought to contribute to the sensitisation of the HPA axis during chronic stress (Aguilera 1994).

In addition to hormone levels, target tissue sensitivity to a hormonal regulator depends on the number of receptors for the ligand present in the cell, as well as on the efficiency of coupling mechanisms linking receptor activation to cellular effects (Catt *et al.* 1979, Collins *et al.* 1991). An important aspect of the vasopressinergic regulation of ACTH secretion is that, in contrast to CRH, its stimulatory effect is relatively refractory to glucocorticoid feedback inhibition (Aguilera 1994, Scott & Dinan 1998). Studies in the rat have shown that glucocorticoid administration decreases V1b receptor content but facilitates coupling of the receptor to phospholipase C by increasing cell levels of the G protein Gq/11 (Rabadan-Diehl & Aguilera 1998). Increased coupling efficiency of the V1bR by glucocorticoids could contribute to maintaining ACTH responses during stress in spite of elevations in plasma glucocorticoids. Activation of the V1b VP receptor could also affect glucocorticoid feedback in the pituitary through induction of transcription factors capable of modulating genomic transcriptional activation or repression by the glucocorticoid receptor, e.g. relative expression of c-jun and c-fos (Gottlicher *et al.* 1998). In addition, a direct correlation between ACTH responses and VP binding suggests that the regulation of the

number of V1b receptors plays a role in controlling corticotroph responsiveness (Aguilera *et al.* 1994). The number of V1b receptors in the pituitary depends on the rate of receptor synthesis as well as receptor desensitization, internalization and degradation following activation by VP (Fig. 1).

Understanding of the molecular mechanisms of regulation of the receptor has been facilitated by the isolation of the V1b receptor cDNA (De Keyser *et al.* 1994, Sugimoto *et al.* 1994, Lolait *et al.* 1995), and more recently by the identification and characterization of genomic clones and cis acting elements in the 5' flanking region potentially controlling V1b receptor transcription (Ventura *et al.* 1999, Rabadan-Diehl *et al.* 2000, Rene *et al.* 2000). This article will focus on mechanisms regulating the number of V1b receptors in the rat pituitary during adaptation to stress.

Characterization of the vasopressin V1b receptor gene

In the rat, the V1b receptor gene contains three exons and two introns (Fig. 2A). A first short intron (161 bp), similar to that described for the oxytocin receptor, is located in the 5' untranslated region (5'-UTR) from bp +124 to +285 (+1 was assigned to the proximal transcriptional start point (tsp)). The position of the second intron, at the end of the 6th transmembrane domain, is characteristic of a large intron found in other members of this family of receptors (Rozen *et al.* 1995, Thibonnier *et al.* 1996, Ventura *et al.* 1999). 5'-RACE and RNase protection analysis has mapped two major putative transcription initiation points, the proximal assigned +1, and a distal located at -31. The 5'-flanking region shows no indication of a proximal TATA box, but there are several AP1 and AP2 sites, a cluster of Sp1 sites upstream of the AP2 sites, and a glucocorticoid response element (GRE), all of which are potential sites for AP1, cAMP and glucocorticoid regulation (Fig. 2B). The human promoter shows a low degree of homology with the rodent V1bR promoter. As in rodents, it also lacks a TATA box and contains several SP1 sites in proximity to the transcription start point (Rene *et al.* 2000). The human V1bR promoter contains atypical one half palindromic sequences for oestrogen- and cAMP-response elements and a GRE, which is located more than 1000 bp

Figure 1 Sites of regulation of the number of V1b receptors in the pituitary. The number of functional V1b receptors depends on receptor protein synthesis (1 and 2) and receptor desensitization and internalization following interaction with the ligand (3). The role of phosphorylation, G protein receptor kinase (GRK) and scaffolding proteins such as beta-arrestin (Ar) on the V1b receptor fate after VP activation by VP binding has not been studied. An active V1bR gene transcription (1) is required to maintain V1bR mRNA levels but regulation of V1bR translation (2) appears to be the major determinant for receptor synthesis. Based on *in vivo* studies it is likely that V1bR regulation involves signalling by VP itself, corticotrophin releasing hormone and glucocorticoids (Aguilera & Rabadan-Diehl 2000). VP, vasopressin; CRH-R1, corticotrophin releasing hormone type 1 receptor; Ar, β -arrestin; GRK, G protein-coupled receptor kinase; GR, glucocorticoid receptor; Gq and Gs are the guanyl nucleotide binding proteins coupling the VP receptor to phospholipase C and the CRH receptor to adenyl cyclase, respectively.

upstream from the transcription start point. The promoter region of the rat and mouse but not human V1b receptor includes structural features unusual in G protein-coupled receptors such as CA repeats (CACAA box) and CT repeats (inverted GAGA box) (Ventura *et al.* 1999, Rabadan-Diehl *et al.* 2000).

Proximal promoter sequences of the rat V1bR (bp -2161 to +377, corresponding to 2.1 kb of putative promoter, exon 1, intron 1 and 92 bp of exon 2) induced transcriptional activation of luciferase gene when transiently transfected into cells, irrespective of their ability to express endogenous V1b receptors. This DNA fragment

shows promoter activity in the hypothalamic cell line, H32 (Volpi *et al.* 2002) and in MCF-7 cells which express endogenous V1b receptor, as well as in cells not expressing the receptor such as in COS-7, CHO and PC12 cell lines (Rabadan-Diehl *et al.* 2000). Promoter activity in the pituitary tumor cell line AtT20, however, remains controversial (Rabadan-Diehl *et al.* 2000, Nomura *et al.* 2001). A construct containing a -2161 to +72 fragment, without the intron and distal sequence, has no promoter activity in any cell line tested, suggesting that intron 1 is important for receptor gene activation (Rabadan-Diehl *et al.* 2000, Volpi *et al.* 2002). Deletion of sequences upstream of -294

Figure 2 (A) Structure of the rat V1b vasopressin receptor gene extrapolated from the genomic clone (Rabadan-Diehl *et al.* 2000) and reported cDNA sequences. The 3 exons (EX) are represented by the box and the shaded area shows the coding regions. Two major transcription start points identified by 5'-RACE and RNA protection assay are indicated by arrows (tsp1 and tsp2). IN, intron. (B) Putative regulatory elements in the V1b receptor promoter determined by computer analysis. Exon 1 and intron 1 are required for basal promoter activity in transfected cells. The GAGA box is also required for basal promoter activity and mediates transcriptional activation of the V1bR promoter. The role of other regulatory elements on the transcriptional activation of the V1bR promoter has not been studied.

increases promoter activity without changes in transfection efficiency, strongly suggesting that the region between bp -294 and -2161 of the V1bR promoter contains repressor elements (Nomura *et al.* 2001, Volpi *et al.* 2002). The genomic sequences responsible for tissue-specific expression of the V1b receptors have yet to be elucidated.

Transcriptional regulation of the vasopressin V1b receptor

No information is available on the transcriptional regulation of the V1bR *in vivo*, but the rapid increases in V1bR mRNA following experimental stress strongly suggest that stress stimulates V1bR

gene transcription. The characterization of the V1bR gene including the 5'-flanking region has made possible *in vitro* studies to determine those elements responsible for the transcriptional regulation of the gene. Of the cis-elements identified by computer analysis, the GAGA box is important for activation of V1bR gene transcription, as indicated by the proximity of the GAGA box to the transcriptional start point, and the dramatic reduction of promoter activity following deletion of 213 bp containing the GAGA box (Volpi *et al.* 2002). This suggests that in rats and probably in mice, the GAGA box is required for initiation of V1bR transcription, as has been postulated for the AT1 receptor and serine protease inhibitor genes

Figure 3 Evidence for GAGA box-mediated transcriptional activation of V1bR transcription. (A) Effect of *Drosophila* GAGA binding protein on V1bR promoter activity. H32 cells were co-transfected with either the luciferase reporter plasmid driven by the V1bR promoter, V1 bRp 2.5, or the V1bR promoter with deletion of the GAGA box, V1 bRp(-)GAGA, and the expression vector containing *Drosophila* GAGA factor (pCR3.1-GAGA) or the empty expression vector (pCR3.1). Promoter activity is expressed as a percentage of pGL3-basic luciferase activity. (B) Effect of GAGA binding protein on endogenous V1bR mRNA expression in H32 cells. Cells were transfected with the empty expression vector (pCR3.1) or the expression vector containing *Drosophila* GAGA factor. V1bR and cyclophilin (Cyph) mRNAs were measured by quantitative RT-PCR. RNA samples without the reverse transcription step were used as controls to exclude genomic contamination. Quantitative analysis of the bands was performed using a KODAK ID Image Analysis system. The quantitative analysis of the mean of 3 experiments is depicted by the bars below. (C) Effect of restraint stress on GAGA binding activity of pituitary nuclear proteins evaluated by EMSA. Rats were killed 15, 30 and 90 min after initiation of the stress. Bars represent the mean and s.e. of the values obtained by Phosphor Imager analysis of the shifted bands in 3 experiments using pools of 2 pituitaries per experimental group. * $P < 0.01$ compared with control (basal).

(Simar-Blanchet *et al.* 1998, Wyse *et al.* 2000). Computer analysis of the human V1bR promoter reveals short atypical GAGA binding elements but their relevance remains to be elucidated.

GAGA repeats were first described in *Drosophila* heat shock protein and histone gene promoters, where they were found to bind a protein called GAGA factor (Biggin & Tjian 1988, Gilmour *et al.* 1989, Soeller *et al.* 1993, Farkas *et al.* 1994). Recent studies show that *Drosophila* GAGA factor stimulates

V1bR promoter activity when co-transfected with a V1bR promoter-luciferase construct in H32 or MCF-7 cells (Volpi *et al.* 2002). This increase in V1bR promoter activity by *Drosophila* GAGA factor is mediated by the GAGA box in the promoter, since the effect was abolished by deletion of the main stretch of GAGA repeats (Fig. 3A). In addition, transfection of the GAGA factor into cell lines expressing the V1b receptor increased the expression of endogenous V1bR, providing strong evidence

that a GAGA binding protein is involved in the regulation of the V1bR (Volpi *et al.* 2002) (Fig. 3B).

A GAGA binding protein has also been implicated in the transcriptional regulation of other vertebrate genes, including the human type 1 angiotensin II receptor (AT1R), the rat serine protease inhibitor 1 and the *Xenopus* stromelysin-3 gene (ST-3) (Li *et al.* 1998, Simar-Blanchet *et al.* 1998, Wyse *et al.* 2000). In the case of the serine protease inhibitor 1 and the AT1R, GAGA binding activity is stimulated by growth factors, and the GAGA box appears to mediate growth factor-mediated gene transcription (Simar-Blanchet *et al.* 1998, Wyse *et al.* 2000). For the serine protease 2 gene, GAGA repeats appear to act as enhancers or as a target for Janus kinase activation (Simar-Blanchet *et al.* 1998). GAGA box-mediated transcriptional activation of the V1bR promoter could also involve growth factors since recent experiments in our laboratory have shown increases in GAGA binding activity after incubation of H32 cells with epidermal growth factor (S Volpi and G Aguilera, unpublished observations).

There is evidence that endogenous pituitary nuclear proteins bind to the GAGA box of the V1bR with high association and dissociation rates. In addition to the pituitary, nuclear extracts of several other tissues show GAGA binding activity, which is consistent with the view that GAGA repeats may be involved in the transcriptional regulation of a variety of mammalian genes (Benyajati *et al.* 1997). Preliminary characterization of the GAGA binding protein interacting with the V1bR promoter indicates that DNA binding activity requires the formation of a protein complex, which can be dissociated by deoxycholate. The native nuclear protein complex shows an apparent molecular mass of $\sim 127 \pm 12$ kDa after size exclusion chromatography, while deoxycholate treatment abolished binding in the gel shift assay. Reconstitution of the complex after fractionation of deoxycholate-dissociated extract with NP-40 shows that the binding activity is recovered in the ~ 70 kDa range, suggesting that the active complex corresponds to a homo- or heterodimer of proteins of about ~ 70 kDa (Volpi *et al.* 2002).

An important observation is that GAGA binding activity in nuclear extracts from pituitary is regulated during physiological conditions leading to changes in V1bR expression. Restraint stress, an experimental condition which increases pituitary

V1bR mRNA, also increases GAGA binding activity in pituitary extracts (Volpi *et al.* 2002), supporting a role for the GAGA box in the physiological regulation of V1bR transcription (Fig. 3C). The effect of stress appears to be specific for the pituitary since in the same experiments no differences between control and stressed rats were found in the electrophoresis mobility shift assay (EMSA) analysis of liver nuclear proteins. It is noteworthy that the increase in GAGA binding activity during stress is transient and occurs early (30 min). This is consistent with the possibility of activation of GAGA binding proteins by phosphorylation, as has been reported for growth factor-mediated regulation of the AT1 receptor promoter (Wyse *et al.* 2000). The full characterization of the GAGA binding protein complex responsible for the transcriptional regulation of the V1bR as well as the role of other regulatory elements present in the V1b receptor promoter on transcriptional regulation remain to be elucidated.

Post-transcriptional regulation of V1bR expression

In a number of experimental conditions in the rat, such as chronic osmotic stimulation, adrenalectomy or glucocorticoid administration, VP receptor content in the pituitary shows no correlation with V1bR mRNA levels (Aguilera *et al.* 1994, Rabadan-Diehl *et al.* 1997). This suggests that V1bR content in the pituitary does not primarily depend on the levels of V1bR mRNA but on the efficiency of mRNA translation into protein and post-translational events. Recent studies have focused on the translational regulation of the V1bR and especially on the role of the 5'-UTR (Rabadan-Diehl & Aguilera 2001, 2002, Nomura *et al.* 2001). The rat V1bR 5'-UTR is long, structurally complex, and displays several small open reading frames (ORF) upstream of the initiating methionine (uORF), features likely to reduce the ability of ribosomes to linearly scan the V1b 5'-UTR mRNA according to conventional scanning models (Fig. 4; see also Fig. 6).

The presence of short ORFs in the 5'-UTR of the mRNA can affect translation of the main ORF in a number of transcripts (Kozak 1991, Geballe & Morris 1994, Hinnebush 1997). While this occurs in less than 10% of transcripts in vertebrates, it is

Figure 4 Structure of the V1bR cDNA. The 5'-UTR is unusually long and contains small upstream open reading frames (uORF) encoding potential peptides of 10, 26 and 38 amino acids (aa). (B) Sequence of the 5'-UTR of the V1bR cDNA with uORFs 1, 2 and 3 highlighted in blue, green and pink respectively. (C) Effect of mutation inactivation mutations of uORFs 1 and 2 on *in vitro* translation of the V1bR. The initiating methionine of the V1bR ORF is indicated by the black triangle. Mutation of ATG to ATA in uORF2 and uORF1 increases translation efficiency of the main ORF encoding the V1bR. The image depicts the 46 kDa *in vitro* translated bands corresponding to the V1bR in a representative experiment. The bar graph shows the mean and s.e. of the values in 2 experiments.

more frequent in low abundance proteins such as some members of the G protein-coupled receptor family, including muscarinic, adrenergic, serotonergic, substance P, substance K, angiotensin II and type 1 CRH receptors (Kobilka *et al.* 1987, Kozak 1991, Mori *et al.* 1996, Xu *et al.* 2001). In the CRH type 1 receptor, β_2 -adrenergic and type 1 angiotensin II receptors, AUG triplets which are part of uORFs in the 5'-UTR of the transcript inhibit translation into receptor protein (Parola & Kobilka 1994, Mori *et al.* 1996, Xu *et al.* 2001).

In the V1bR mRNA, sequential mutation of the upstream ORFs increases *in vitro* translation of a 46 kDa band corresponding to the V1bR (Rabadan-Diehl & Aguilera 2002; Fig. 4) or luciferase activity using reporter gene constructs (Nomura *et al.* 2000). The more proximal uORF, encoding a putative peptide of 38 amino acids, can

be translated *in vitro*. Co-translation of the upstream peptide with the wild type V1bR cDNA did not affect translation of the V1bR protein, but attenuated the increased translation of the V1bR with the mutated uORF. This suggests that the upstream peptide mediates inhibition of the V1bR protein translation. Interestingly, *in vitro* translation using canine microsomes and Western blot after transfection of cells with a peptide-V5 tag construct have confirmed computer analysis predictions that the peptide is targeted to the membrane (Rabadan-Diehl & Aguilera 2002). Analysis of the 5'-UTR sequences reveals the presence of upstream ORFs in the mouse but not in human V1bR 5'-UTR.

While upstream ORFs may account for poor translational activity and the low levels of V1b receptors in the pituitary in basal conditions, recent studies in the rat V1bR indicate that the 5'-UTR of

Figure 5 The rat V1bR 5'-UTR contains an IRES element. (A) The 5'-UTR of the V1bR mRNA was cloned in the intercistronic region of the bicistronic vector, SL2 (kindly provided by Dr Rudolf Werner, Miami, FL, USA). Translation of the first cistron, renilla luciferase (rLUC), is cap dependent. A stem loop in the intercistronic region inhibits cap-mediated translation of the second cistron, firefly luciferase (fLUC). (B) fLUC/rLUC ratios after transfection of MCF-7 cells with the vector alone (SL2) or the V1bR 5'-UTR construct (SL2-V1b5'UTR). Insertion of the V1bR 5'-UTR markedly increased IRES activity (B) but had no effect on fLUC mRNA levels (C). AU, arbitrary units. * $P < 0.001$ vs. empty SL2 vector.

the V1bR can mediate positive translational regulation (Fig. 5). In a number of mRNAs with high complexity of the 5'-UTR, cap-dependent translation and mRNA scanning are inefficient, and translation utilizes a cap-independent mechanism mediated by ribosome binding to an internal ribosome entry site (IRES) (Kozak 1991, Sonnenberg 1996, Sachs *et al.* 1997). The complex structure of the 5'-UTR suggested the involvement of an IRES in translational activation of the receptor (Fig. 2). Recent experiments using bicistronic vector technology have shown that insertion of 500 bp of the V1bR 5'-UTR between the coding regions of two cistrons, renilla and firefly luciferase, increased luciferase activity of the second cistron when transfected into MCF-7 cells (Fig. 5), or in an *in vitro* translation system. This stimulation of translation was preserved after inhibition of cap-dependent translation, suggesting that the V1bR 5'-UTR contains an IRES

element capable of initiating translation independently of cap. The exact sequence responsible for IRES activity has not been identified but it appears to require the full 5'-UTR, since deletions of V1bR 5'-UTR fragments reduced the potentiation of firefly luciferase activity. Activation of protein kinase C (PKC) by the phorbol ester, PMA (phorbol myristylacetate), increased IRES-mediated translation, suggesting that this mechanism contributes to V1bR mRNA upregulation induced by PKC-coupled regulators, such as VP itself (Rabadan-Diehl & Aguilera 2001). The 5'-UTR of the mouse and human V1bR is also long, 500 to 600 bp, and computer analysis predicts complex conformation, but no information is available on the presence of IRES activity in these species. The mechanisms regulating the balance between translational inhibition and stimulation by the 5'-UTR of the V1bR mRNA will be the subject of future studies.

Figure 6 Diagram depicting negative and positive regulation of V1bR translation by the 5'-UTR of the mRNA. The complexity of the 5'-UTR and the presence of upstream ORFs (uORF), slow down cap-mediated translation and scanning of the mRNA, and could account for low levels of translation of the V1bR in basal conditions. The presence of an internal ribosome entry site (IRES) in the 5'-UTR and regulation of IRES activity by protein kinase C (PKC) may mediate positive regulation of V1bR translation.

These are likely to involve RNA binding proteins (McCarthy & Kollmus 1995) and interactions of distal elements of the mRNA with the 5'-UTR (Gallie *et al.* 1995, Bergamini *et al.* 2000).

In summary, mRNA translation is an important site for regulation of V1b receptor expression. Two potential mechanisms by which the 5'-UTR of the rat V1bR could mediate negative and positive regulation of V1bR translation have been identified (Fig. 6). While basal translational activity is under inhibition by mechanisms involving upstream ORFs, the presence of an internal ribosome entry site (IRES) could mediate rapid activation of translation to meet physiological demands during stress.

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