Identification of the glycosylation sites utilized on the V_{1a} vasopressin receptor and assessment of their role in receptor signalling and expression

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Most of the large family of G-protein-coupled receptors (GPCRs) possess putative N-glycosylation sites within their N-termini. However, for the vast majority of GPCRs, it has not been determined which, if any, of the consensus glycosylation sites are actually utilized or what the functional ramifications are of modification by oligosaccharide. The occurrence and function of glycosylation of the V_{1a} vasopressin receptor (V_{1a}R) has been investigated in this study. Using a combination of translation systems that are either glycosylation-competent or do not support glycosylation, we established that of the four putative N-glycosylation sites at Asn¹⁴, Asn²⁷, Asn¹⁹⁸ and Asn³³³ only the first three sites are actually modified by carbohydrate. This was confirmed by disruption of consensus sites by site-directed mutagenesis, individually and in combination. The V_{1a}R is not O-glycosylated. The functionality of a series of glycosylation-defective V_{1a}R constructs was characterized after expression in

HEK 293T cells. It was found that carbohydrate moieties are not required for the receptor to bind any of the four classes of ligand available, or for intracellular signalling. The glycosylation status of the V_{1a}R did, however, regulate the level of total receptor expression and also the abundance of receptor at the cell surface. Furthermore, the nature of this regulation (increased or decreased expression) was dictated by the locus of the oligosaccharide modification. Modification of any one of the consensus sites alone, however, was sufficient for wild-type expression, indicating a redundancy within the glycosylation sites. A role for the carbohydrate in the correct folding or stabilization of the V_{1a}R is indicated. Glycosylation is not required, however, for efficient trafficking of the receptor to the cell surface. This study establishes the functional importance of N-glycosylation of the V_{1a}R.

Key words: GPCR, *in vitro* translation, peptide, *Xenopus*.

INTRODUCTION

The neurohypophysial nonapeptide hormone [arginine⁸]-vasopressin (AVP) has a diverse range of physiological effects, including contraction of vascular smooth muscle, antidiuresis, increased corticotropin release by the adenohypophysis, modulation of insulin secretion and regulation of testicular function. In addition, AVP-containing neurons project to sites other than the neural lobe and in these fibres AVP acts as a neurotransmitter/neuromodulator [1]. These various effects of AVP are mediated by specific vasopressin receptors expressed by target cells. Three different vasopressin receptors (VPRs), classified as V_{1a}, V_{1b} and V₂, have been characterized pharmacologically [2,3]. In addition, AVP is a high-affinity agonist at the oxytocin receptor (OTR). The V_{1a} vasopressin receptor (V_{1a}R), V_{1b} vasopressin receptor (V_{1b}R) and OTR stimulate phospholipase C but differ in the binding characteristics of certain AVP analogues, whereas V₂ vasopressin receptors (V₂Rs) stimulate adenylate cyclase and display a fourth pharmacological profile [3]. The V_{1a}R is widely distributed and mediates nearly all of the physiological actions of AVP, with the notable exceptions of antidiuresis (V₂R) and corticotropin secretion (V_{1b}R). All three VPRs [4–8] and the OTR [9] have been cloned and exhibit the characteristic motifs of G-protein-coupled receptors (GPCRs), including seven transmembrane domains [10]. Furthermore, sequence homology and related pharmacology establish these neurohypophysial hormone receptors as a subfamily of GPCRs.

Sequence analysis reveals that most GPCRs have at least one putative N-linked glycosylation site in their N-terminus based on

the Asn-Xaa-Ser/Thr consensus sequence [11], although exceptions do occur. For example, the A_{2a}-adenosine receptor only contains putative glycosylation sites on the second extracellular loop [12], whereas the α_{2b} -adrenergic receptor does not contain any putative sites at all [13]. For most GPCRs it has not been determined experimentally whether the putative sites are actually utilized. Interestingly, for some receptors where this has been ascertained, it has been shown that not all putative glycosylation sites are actually modified. For example, the gonadotropin-releasing hormone (GnRH) receptor contains putative glycosylation sites on its N-terminus and on its extracellular loops but only sites at the N-terminus are glycosylated [14].

Studies into the function of glycosylation have been undertaken for relatively few GPCRs. Nevertheless, recent reports demonstrate that receptor glycosylation can be important for many aspects of receptor signalling, although the functional ramifications of glycosylation are not predictable from receptor to receptor. Consequently, glycosylation has been shown to be important in maintaining high-affinity binding and/or receptor coupling for rhodopsin [15], β_2 -adrenergic receptor [16], somatostatin receptor [17] and the gastrin-releasing peptide receptor (bombesin BB₂) [18]. In contrast, glycosylation of the receptors for vasoactive intestinal peptide (VIP-1) [19], luteinizing hormone [20], thyroid-stimulating hormone [21], follicle-stimulating hormone [22] and GnRH [14] is important for receptor expression and/or stability.

In this report, we establish that three potential N-linked glycosylation sites in the V_{1a}R are utilized and identify them. Furthermore, we show that the receptor is not O-glycosylated.

Abbreviations used: AVP, [arginine⁸]vasopressin; GPCR, G-protein-coupled receptor; OTR, oxytocin receptor; PhAc, phenylacetyl; VPR, vasopressin receptor; V_{1a}R, V_{1a} vasopressin receptor; rV_{1a}R, rat V_{1a}R; V_{1b}R, V_{1b} vasopressin receptor; V₂R, V₂ vasopressin receptor; ACTH, adrenocorticotrophic hormone; GnRH, gonadotropin-releasing hormone; VIP-1, vasoactive intestinal peptide; DMEM, Dulbecco's modified Eagle's medium; NYT, acetyl-Asn-Tyr-Thr-amide.

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Disrupting the glycosylation sites by mutagenesis, individually and in combination, allowed us to probe the function of glycosylation at each locus and within each extracellular domain. Our results show that glycosylation is required for efficient folding of the receptor but it does not have a role in ligand recognition or G-protein coupling. It is noteworthy that the effect of glycosylation on V_{1a} R expression was dependent on the locus, such that modification by carbohydrate was observed to increase expression, to decrease expression or to be neutral, depending on the asparaginyl residue(s) in question.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), glutamine, fetal bovine serum, sodium pyruvate and trypsin/EDTA (1×) were from Sigma (Poole, Dorset, U.K.); inositol-free DMEM, PBS and penicillin/streptomycin were from Gibco-BRL (Paisley, U.K.); cell-culture plasticware was from Costar (Cambridge, MA, U.S.A.). [$\text{Phe}^3\text{-3,4,5-}^3\text{H}$]AVP (specific radioactivity 64.2 Ci/mmol), [$\text{Phe}^3\text{-3,4,5-}^3\text{H}$]d(CH₂)₅Tyr(Me)²AVP (specific radioactivity 30.8 Ci/mmol) and 2-*myo*-[^3H]inositol (specific radioactivity 22.3 Ci/mmol) were from DuPont NEN (Stevenage, Herts., U.K.). [^{35}S]Methionine (specific radioactivity 1000 Ci/mmol) and [^{32}P]UTP (specific radioactivity 1.0 mCi/ml) were from Amersham (Little Chalfont, Bucks., U.K.). Linear peptide antagonist [PhAc-D-Tyr(Me)²Arg⁶Tyr(NH₂)⁹]AVP (where PhAc is phenylacetyl) was synthesized by Alta Bioscience (University of Birmingham, Birmingham, U.K.); cyclic antagonist d(CH₂)₅Tyr(Me)²AVP was from Bachem (Torrance, CA, U.S.A.) and non-peptide antagonist SR 49059 [23] was a kind gift from Dr C. Serradiel-Le Gal (Sanofi Recherche, Toulouse, France). The restriction enzymes *Pf*23II and *Bbu*I were obtained from MBI Fermentas (Sunderland, U.K.) and Promega (Southampton, U.K.) respectively. All other reagents used were of analytical grade.

Construction of glycosylation-mutant V_{1a} Rs

To disrupt N-glycosylation sites, individually and in combination, the Asn residue in each consensus sequon was mutated to Gln. Mutations were introduced in the rat V_{1a} R (r V_{1a} R) sequence by a PCR approach with specific sense and antisense oligonucleotides. Mutagenic sense oligonucleotides [incorporating the two base changes AAC → CAG (in bold) for the required Asn → Gln substitution (underlined)] were 5'-CC GTG GGC CAG TCC AGC CCG TG-3', 5'-C GAG GGC TCC CAG GGC AGT CAG-3' and 5'-G GTG AAC CAG GGC ACT AAA ACC C-3' for the N14Q, N27Q and N198Q mutations respectively. For the N14Q and N27Q mutations, *Apal*/*Bbu*I-digested fragments were initially subcloned into pBluescript KSII (Stratagene) containing the V_{1a} R sequence. For mammalian expression, the wild type, and N14Q and N27Q mutants were subcloned into the vector pcDNA3 (Invitrogen) using *Bam*HI and *Eco*RI restriction sites. The N198Q mutant was introduced directly into the V_{1a} R sequence in pcDNA3 utilizing unique *Sac*II restriction sites.

For the [N14/27Q] V_{1a} R double mutation, the N14Q mutant in the vector pcDNA3 was used as a template to incorporate the N27Q mutant by PCR. The PCR fragment (containing the N14Q and N27Q mutations) was subcloned into pcDNA3 using unique *Hind*III and *Pf*23II restriction sites. In the case of the [N14/27/198Q] V_{1a} R triple mutant, the *Sac*II fragment (containing the N198Q mutation) was subcloned directly into pcDNA3 containing the [N14/27Q] V_{1a} R double mutant. Veri-

fication of each construct was by automated sequencing (Alta Bioscience).

In vitro transcription and translation of V_{1a} Rs

Wild-type or mutant V_{1a} Rs were subcloned into the unique *Bg*/II site in the transcription vector pSP64T [24], utilizing unique *Bam*HI restriction sites (generates cDNA fragments with compatible ends) flanking the V_{1a} R cDNA sequence. Linearized pSP64T containing wild-type or mutant cDNA was used as a template to synthesize mRNA based on the protocol described previously [24], using SP6 RNA polymerase for 60 min at 37 °C. Translation of mRNA was carried out using both a rabbit reticulocyte lysate system (supplied by Life Technologies) and a cell-free *Xenopus* egg extract (XE) system. Briefly, the reticulocyte lysate translation reactions were performed according to the supplier's instructions and contained 1 mCi of [^{35}S]methionine and 10 µg/ml mRNA. The *Xenopus* egg extract translation reactions were performed as described in [24], containing 1 mCi/ml [^{35}S]methionine and 25 µg/ml mRNA in a final volume of 20 µl. After incubation at 21 °C for 1 h, *Xenopus* egg extract translations were diluted with 9 vol. of buffer [20 mM Tris/HCl, 50 mM KCl, 10 mM magnesium acetate, 100 mM NaCl and 10% (w/v) glucose, pH 7.6] and the membrane and cytosol fractions separated by centrifugation at 12000 *g* for 10 min at 4 °C. Samples were analysed by SDS/PAGE (10% gels, w/v) followed by autoradiography.

Cell culture and transfection

HEK 293T cells were cultured routinely in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (100 i.u./ml), streptomycin (100 µg/ml), glutamine (2 mM) and sodium pyruvate (1 mM). Cells were maintained in a humidified 5% (v/v) CO₂ incubator at 37 °C. HEK 293T cells were seeded at a density of approx. 5 × 10⁵ cells/100 mm dish, and transfected after 48 h using the calcium phosphate precipitation method. Briefly, a DNA/calcium phosphate co-precipitate, containing 10 µg of plasmid cDNA for each dish, was prepared 30 min before use. After incubation for 16 h, the media was replaced with growth media and cells incubated for 48 h before harvesting.

Radioligand-binding assays

Total V_{1a} R binding

HEK 293T cell membranes were prepared to determine total receptor expression by radioligand-binding assays as described previously [25]. The protein concentration of membranes was determined using the Pierce BCA protein assay kit (Pierce Chemical Co.) with BSA as standard. Binding assays were essentially as described previously [26,27] and contained membranes (50–300 µg), [$\text{Phe}^3\text{-3,4,5-}^3\text{H}$]AVP (0.3–1.1 nM) as tracer and competing ligand (at the concentration indicated) in a final volume of 0.5 ml. Non-specific binding was defined by unlabelled AVP (10 µM). After incubation for 90 min at 30 °C to establish equilibrium, bound and free ligand were separated by sedimentation at 12000 *g* for 10 min. Membrane pellets were washed and solubilized using Soluene-350 (Packard) prior to liquid scintillation counting using HiSafe3 (Wallac) as cocktail.

Cell-surface V_{1a} R binding

Cell-surface expression was determined by whole-cell binding assays. Briefly, HEK 293T cells were seeded at a density of

1×10^5 cells/well in poly-D-lysine-coated 12-well plates and transfected after 24 h using Transfast[®] (Promega). After 36 h, cells were washed twice with PBS, after which each well received 0.5 ml of binding buffer containing 2% (w/v) BSA and [³H]d(CH₂)₅Tyr(Me)²AVP in the presence (non-specific) and absence (total) of 1 μ M d(CH₂)₅Tyr(Me)²AVP. Plates were incubated for 90 min at 30 °C before removal of the medium by aspiration. After two rinses with ice-cold PBS, 0.5 ml of 0.1 M NaOH was added to each well to extract radioactivity. After 15 min of incubation at 37 °C, the medium from the plates was transferred to scintillation vials containing 10 ml of HiSafe3 scintillation cocktail.

Analysis of binding data

Radioligand-binding results are expressed as a percentage of the control (i.e. specific binding in the absence of the competing ligand). IC₅₀ values were determined by non-linear regression after the fitting of Langmuir binding isotherms to experimentally determined data using the Fig. P program (Biosoft, Milltown, NJ, U.S.A.). Dissociation constants (K_d) were calculated from IC₅₀ values according to the method of Cheng and Prusoff [28], using the experimentally determined value for the affinity of [Phe³-3,4,5-³H]AVP or [Phe³-3,4,5-³H]d(CH₂)₅Tyr(Me)²AVP for each construct.

Measurement of AVP-induced accumulation of InsPs

HEK 293T cells were seeded at a density of 2.5×10^5 cells/well in poly-D-lysine-pre-treated six-well plates. AVP-induced accumulation of InsPs was assayed in transfected cells as described previously [29,30]. Essentially, 24 h post-transfection, medium was replaced with serum-free, inositol-free, DMEM containing 2.5 μ Ci/ml 2-*myo*-[³H]inositol. After 48 h at 37 °C, cells were washed with PBS and incubated with medium containing 10 mM LiCl for 30 min, followed by the addition of AVP at the concentration indicated. Incubations were terminated by adding 0.5 ml of 5% (v/v) perchloric acid containing 1 mM EDTA and 1 mg/ml phytic acid hydrosylate. Cells were pelleted at 12000 *g* for 5 min, supernatants neutralized on ice with 1.2 M KOH, 10 mM EDTA and 50 mM Hepes, and samples loaded on to 0.8 ml Bio-Rad AG1-X8 columns (formate form). After elution of inositol (10 ml water) and glycerophosphoinositol (10 ml of 25 mM ammonium formate), a mixed InsP fraction containing inositol mono-, bis-, tris- and tetrakisphosphates was eluted (10 ml of 1.25 M ammonium formate containing 0.1 M formic acid). Radioactivity was determined using Ultima-Flo AF scintillant (Packard). EC₅₀ values were determined by non-linear regression after fitting of logistic sigmoidal curves to experimental data.

RESULTS

Establishing the glycoprotein nature of the V_{1a}R

Examination of the sequences of the three VPR subtypes and the OTR, cloned from a range of species, reveals that they all possess putative glycosylation sites at their N-termini (Figure 1). V_{1a}Rs cloned from rat, human and mouse contain four conserved consensus sequences for N-linked glycosylation [4,31,32]. Two sites are present on the N-terminus (Asn¹⁴ and Asn²⁷ for rV_{1a}R), and one site is present on each of the second and third extracellular loops (Asn¹⁹⁸ and Asn³³³ respectively in the rV_{1a}R). For the OTR, three glycosylation sites are present on the N-terminus, whereas V_{1b}R and V₂R contain only one glycosylation site (Figure 1). The V_{1a}R is unique in this family in that it

Figure 1 Location of putative glycosylation sites on the VPR/OTR family of receptors

Putative glycosylation sites are indicated by branched chains.

possesses conserved glycosylation sites within the second and third extracellular loops. The rV₂R has one additional consensus site in the second extracellular loop but this is not conserved in either the human or porcine V₂Rs [5,33].

The existence of a consensus sequence for N-glycosylation (Asn-Xaa-Ser/Thr) in an amino acid sequence does not necessarily result in a glycosylated protein. It was therefore important to establish experimentally which, if any, of the canonical sites of the V_{1a}R are actually utilized by oligosaccharyltransferase. To this end, we employed a *Xenopus* egg extract *in vitro* cell-free translation system, which has been shown to support appropriate N-linked, and O-linked, glycosylation [24,34].

To establish whether the V_{1a}R is indeed glycosylated, V_{1a}R mRNA was translated *in vitro* using both a rabbit reticulocyte lysate translation system, which does not support glycosylation, and the *Xenopus* extract, which does. Inclusion of [³⁵S]methionine generated a labelled translation product that could be analysed by SDS/PAGE. Translation with the reticulocyte lysate system produced a single product with an apparent molecular mass of 35 kDa (Figure 2), corresponding to the non-glycosylated receptor. The 48 kDa band observed using this system with either water or V_{1a}R mRNA is a well-characterized methionine-binding protein present in the lysate [35]. When V_{1a}R mRNA was translated in the *Xenopus* extract, the product migrated with an apparent molecular mass of 46 kDa. The reduced mobility of the translation product generated in this extract compared with the reticulocyte lysate is entirely consistent with glycosylation of the V_{1a}R. This was confirmed using the tripeptide acetyl-Asn-Tyr-Thr-amide (NYT), which corresponds to an N-glycosylation consensus site. Addition of NYT to the *Xenopus* extract completely suppressed V_{1a}R glycosylation, resulting in a translation product that co-migrated with that from the reticulocyte lysate (Figure 2). It was noteworthy that the V_{1a}R was efficiently segregated into membranes as no receptor was detected in the supernatant from translation reactions (Figure 2).

Figure 2 *In vitro* translation and glycosylation of the $V_{1a}R$

Translational products from the reticulocyte lysate after addition of water (RLO) or $V_{1a}R$ mRNA (RLV) were analysed by SDS/PAGE (10% gel, w/v). Translation products from *Xenopus* egg extract were separated by centrifugation to yield membrane and supernatant components. Membranes from translation reactions in the absence (XM-) or presence (XM+) of the tripeptide inhibitor NYT (1 mM) were analysed separately to the supernatant from the reactions without (XS-) or with (XS+) tripeptide. Molecular-mass markers are shown.

Figure 3 Determination of the number of $V_{1a}R$ glycosylation sites utilized

Progressive inhibition of N-linked glycosylation was achieved by titrating increasing concentrations of the tripeptide NYT into the *Xenopus* egg extract system. Translation products of $V_{1a}R$ mRNA were analysed by SDS/PAGE (10% gel, w/v). The number of glycosylation sites utilized in each translation product is indicated. Molecular-mass markers are also shown.

Determination of the number of putative glycosylation sites actually utilized

To assess the number of putative N-linked glycosylation sites actually utilized, the receptor mRNA was translated using the *Xenopus* extract in the presence of increasing concentrations of the tripeptide NYT. Titration with NYT resulted in gradual inhibition of N-glycosylation to reveal four products, each representing a different glycosylation state of the $V_{1a}R$ (Figure 3). Each product exhibited a different apparent mass, reflecting differences in the number of sites modified. Bands were observed at 35 kDa (non-glycosylated), 39 kDa (one glycosylation site utilized), 43 kDa (two sites utilized) and 46 kDa (three sites utilized). In each case, oligosaccharide modification resulted in reduced migration equivalent to an apparent molecular mass of 3–4 kDa. The $V_{1a}R$, therefore, is modified at only three of the four putative N-linked glycosylation sites. As to the identity of

Figure 4 *In vitro* translation of a fully non-glycosylated $V_{1a}R$

Translation of wild-type $V_{1a}R$ (WT) or a non-glycosylated construct mRNA ([N14/27/198Q] $V_{1a}R$) was performed using the *Xenopus* egg extract (XE) and reticulocyte lysate (RL) systems as described in the Materials and methods section. Translation of each receptor mRNA was performed in the absence (–) or presence (+) of the tripeptide NYT inhibitor (1 mM) and analysed by SDS/PAGE (10% gel, w/v). Molecular-mass markers are indicated.

the non-glycosylated site, it was reasoned that Asn³³³ in the third extracellular loop (Figure 1) was the most likely candidate. The glycosylation consensus site (Asn-Xaa-Ser/Thr) at this locus is Asn-Pro-Ser. The prolyl³³⁴ at position Xaa can prevent such a sequence acting as a glycosyl acceptor [36]. Further support is provided by analysing sequence homology for the $V_{1a}R$ cloned from different species. Sheep $V_{1a}R$ has only three putative glycosylation sites, which correspond to Asn¹⁴, Asn²⁷ and Asn¹⁹⁸ of r $V_{1a}R$ [37]. An Asn is conserved within the third extracellular loop of the sheep receptor (corresponding to Asn³³³ in rat); however, an Ala replaces the necessary Ser/Thr of the consensus sequence, rendering this site non-functional.

In vitro translation of a non-glycosylated $V_{1a}R$

To verify that the three residues Asn¹⁴, Asn²⁷ and Asn¹⁹⁸ account for all the glycosylation sites of the $V_{1a}R$, the construct [N14/27/198Q] $V_{1a}R$ was engineered, which would ablate any glycosylation at these sites. This non-glycosylated mutant construct was translated in both the *Xenopus* extract and reticulocyte lysate systems and the products compared with wild-type receptor. The [N14/27/198Q] $V_{1a}R$ translation product generated by the *Xenopus* extract was identical with that from the reticulocyte lysate preparation when analysed by SDS/PAGE, thereby establishing that the [N14/27/198Q] $V_{1a}R$ receptor protein was not glycosylated (Figure 4, compare lanes 4 and 6). This conclusion was confirmed by the observation that the presence of the NYT peptide in the *Xenopus* extract had no effect on the apparent size of the [N14/27/198Q] $V_{1a}R$ receptor protein (Figure 4, compare lanes 4 and 5). In marked contrast, when the wild-type receptor was translated in parallel, the *Xenopus* extract product migrated with a higher apparent mass than that of the reticulocyte lysate product. Furthermore, inclusion of the NYT peptide in the extract abolished this difference (Figure 4, lanes 1–3). This is exactly what would be expected for an N-glycosylated protein. The observation that the wild-type $V_{1a}R$ and the [N14/27/198Q] $V_{1a}R$ mutant generated products of the same apparent

Table 1 Pharmacological profile of receptors with ablation of a single glycosylation site

Wild-type and mutant V_{1a}Rs possessing disruption of the single glycosylation site indicated were expressed in HEK 293T cells and assayed for ligand binding as described in the Materials and methods section. Dissociation constants (K_d) were calculated from IC₅₀ values and corrected for radioligand occupancy as described in the Materials and methods. Data shown are the means $\pm$ S.E.M. from three independent experiments each performed in triplicate.

Ligand	Binding affinity (K_d , nM)			
	V _{1a} R	[N14Q]V _{1a} R	[N27Q]V _{1a} R	[N198Q]V _{1a} R
AVP	1.0 $\pm$ 0.1	1.4 $\pm$ 0.2	2.1 $\pm$ 0.3	0.9 $\pm$ 0.1
[PhAc-D-Tyr(Me) ² Arg ⁶ Tyr(NH ₂) ⁹]AVP	0.5 $\pm$ 0.1	1.2 $\pm$ 0.2	1.1 $\pm$ 0.1	0.5 $\pm$ 0.1
d(CH ₂) ₅ Tyr(Me) ² AVP	0.7 $\pm$ 0.2	1.0 $\pm$ 0.1	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1
SR 49059	1.9 $\pm$ 0.2	2.2 $\pm$ 0.3	1.2 $\pm$ 0.1	1.6 $\pm$ 0.2

mass when translated in the *Xenopus* extract plus NYT, and also in the reticulocyte lysate, established that the residues Asn¹⁴, Asn²⁷ and Asn¹⁹⁸ accounted for all the glycosylation sites of the V_{1a}R. In addition, this indicated that the V_{1a}R is not O-glycosylated.

Pharmacological characterization of V_{1a}Rs with glycosylation ablated at a single site

To investigate whether glycosylation of individual asparaginyl residues had a role in V_{1a}R function, a series of three mutant receptors was engineered in which each of the individual glycosylation sites was sequentially disrupted. The constructs [N14Q]-V_{1a}R, [N27Q]V_{1a}R and [N198Q]V_{1a}R were expressed in HEK 293T cells and individually characterized with respect to ligand binding and second-messenger generation. The V_{1a}R is unusual amongst GPCRs binding peptide ligands in that it recognizes a wide range of ligand classes. Four different classes of ligand were used to probe the pharmacology of the glycosylation-defective mutant receptors: (i) cyclic agonist, AVP; (ii) cyclic antagonist, d(CH₂)₅Tyr(Me)²AVP; (iii) linear antagonist, [PhAc-D-Tyr(Me)²Arg⁶Tyr(NH₂)⁹]AVP and (iv) non-peptide antagonist, SR 49059 [23]. The affinity of these ligands binding to [N14Q]V_{1a}R, [N27Q]V_{1a}R and [N198Q]V_{1a}R was determined by competition radioligand-binding studies and the data are summarized in Table 1. In all cases, the binding affinities (K_d) exhibited were very similar to that of wild-type V_{1a}R. The effect of disrupting glycosylation at a single site on intracellular signalling was also investigated. AVP-induced accumulation of InsPs was assayed and the dose-response curves are presented in Figure 5. Disruption of any one of the three glycosylation sites did not perturb second-messenger generation.

Pharmacological characterization of V_{1a}Rs with glycosylation ablated in different domains

The use of peptide mimetics [38], photoaffinity labelling [39] and mutagenesis [40,41] have shown that AVP binding involves extracellular receptor epitopes. In addition, it was demonstrated earlier in this study that glycosylation of V_{1a}R occurs on two discrete extracellular domains, the N-terminal domain and the second extracellular loop. Consequently, it was possible that oligosaccharide modification of these domains may influence ligand recognition by the receptor. The role of glycosylation of each of these extracellular segments was investigated. Three constructs were studied, one non-glycosylated at the N-terminus but glycosylated on the second extracellular loop ([N14/27Q]-V_{1a}R), another non-glycosylated on the second extracellular loop but glycosylated at the N-terminus ([N198Q]V_{1a}R) and a third

Receptor	EC ₅₀ (nM)
Wild-type V _{1a} R	0.4 $\pm$ 0.1
N14Q	0.4 $\pm$ 0.1
N27Q	0.2 $\pm$ 0.1
N198Q	0.1 $\pm$ 0.1

Figure 5 Intracellular signalling by V_{1a}Rs with ablation of a single glycosylation site

(A) AVP-induced accumulation of InsP₁–InsP₄ in HEK 293T cells transfected with wild-type (●), N14Q (□), N27Q (△) or N198Q (▽) V_{1a}Rs. Data shown are means $\pm$ S.E.M. from a representative of three separate experiments each performed in triplicate. Values are expressed as percentage maximum stimulation induced by AVP. The mean 0–100% values for each receptor were 16 600–41 700 d.p.m., 21 000–79 000 d.p.m., 23 600–87 400 d.p.m. and 25 300–51 200 d.p.m. for wild-type, N14Q, N27Q and N198Q V_{1a}Rs respectively. (B) EC₅₀ values of individual glycosylation-defective mutant receptors, means $\pm$ S.E.M. ($n = 3$).

Table 2 Pharmacological profile of receptors lacking glycosylation within the N-terminus ([N14/27Q]V_{1a}R), second extracellular loop ([N198Q]V_{1a}R) or both domains ([N14/27/198Q]V_{1a}R)

Wild-type and mutant V_{1a}Rs with disruption of glycosylation sites were expressed in HEK 293T cells and assayed for their ability to bind ligands. Dissociation constants (*K_d*) were calculated from IC₅₀ values and corrected for radioligand occupancy as described in the Materials and methods. Data shown are the means ± S.E.M. from three independent experiments each performed in triplicate.

Ligand	Binding affinity (<i>K_d</i> , nM)			
	V _{1a} R	[N14/27Q]V _{1a} R	[N198Q]V _{1a} R	[N14/27/198Q]V _{1a} R
AVP	1.0 ± 0.1	1.5 ± 0.3	0.9 ± 0.1	1.0 ± 0.2
[PhAc-D-Tyr(Me) ² Arg ⁶ Tyr(NH ₂) ⁹]AVP	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.1
d(CH ₂) ₅ Tyr(Me) ² AVP	0.7 ± 0.2	1.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
SR 49059	1.9 ± 0.2	0.6 ± 0.1	1.6 ± 0.2	1.2 ± 0.2

non-glycosylated on both domains ([N14/27/198Q]V_{1a}R). The results of radioligand-binding studies using the four different classes of ligand available are presented in Table 2. Non-glycosylation of either the N-terminus or second extracellular loop did not markedly affect the pharmacological profile of the V_{1a}R compared with the wild type. Furthermore, the fully non-glycosylated receptor ([N14/27/198Q]V_{1a}R) exhibited wild-type pharmacology. The intracellular signalling of all of these glycosylation mutants was also unimpaired (Figure 6).

The glycosylation status of the V_{1a}R regulates its expression

The expression of the receptors with differing glycosylation status was determined by radioligand-binding studies (Figure 7). For each construct, the total receptor expression was calculated from experiments using a broken cell (membrane) preparation. Non-glycosylation of Asn¹⁹⁸ ([N198Q]V_{1a}R) had no effect on total receptor expression (76 ± 17 %, *n* = 3, compared with the wild type). In contrast, disruption of either one of the two glycosylation sites located in the N-terminus resulted in an approx. 3-fold increase in total receptor expression, with [N14Q]V_{1a}R and [N27Q]V_{1a}R being expressed at 277 ± 42 % (*n* = 3) and 266 ± 89 % (*n* = 3) respectively compared with the wild type (Figure 7A, white bars). Deletion of both of these sites simultaneously in the [N14/27Q]V_{1a}R construct, however, produced wild-type expression levels (116 ± 9 %, *n* = 3; Figure 7A, white bars). In contrast, the fully non-glycosylated receptor [N14/27/198Q]V_{1a}R had severely impaired expression, being only 27 ± 9 % of the wild-type total expression (Figure 7A, white bars). The role of glycosylation in receptor expression was investigated further by using whole-cell radioligand-binding studies to quantify only those receptors that were efficiently trafficked to the cell surface (Figure 7A, black bars). It was apparent from these studies that the cell-surface expression of the constructs did not parallel the total receptor expression. Consequently, disruption of a single glycosylation site located in either the N-terminus ([N14Q]V_{1a}R or [N27Q]V_{1a}R) or in the extracellular loop ([N198Q]V_{1a}R) had no significant effect on the number of receptors expressed on the surface of the cell compared with the wild type (Figure 7A, black bars). A lack of N-terminal glycosylation in the [N14/27Q]V_{1a}R mutant resulted in increased expression at the cell surface (177 ± 10 %, *n* = 3, compared with the wild type), whereas the totally non-glycosylated receptor ([N14/27/198Q]V_{1a}R) was expressed poorly on the plasma membrane (49 ± 4 % of wild type, *n* = 3; Figure 7A, black bars).

In Figure 7(B), the distribution of the receptor between the cell surface and the interior of the cell is presented for each of the receptor constructs. This distribution pattern varies from 90 % of the receptors being located in intracellular compartments for

Receptor	EC ₅₀ (nM)
Wild-type V _{1a} R	0.4 ± 0.1
[N14/27Q]V _{1a} R	0.4 ± 0.1
[N198Q]V _{1a} R	0.1 ± 0.1
[N14/27/198Q]V _{1a} R	0.2 ± 0.1

Figure 6 Intracellular signalling by receptors lacking glycosylation within the N-terminus ([N14/27Q]V_{1a}R), the second extracellular loop ([N198Q]V_{1a}R) or both ([N14/27/198Q]V_{1a}R)

(A) AVP-induced accumulation of InsP₃–InsP₄ in HEK 293T cells transfected with wild-type V_{1a}R (●), [N14/27Q]V_{1a}R (○), [N198Q]V_{1a}R (□) or [N14/27/198Q]V_{1a}R (■). Data shown are means ± S.E.M. from a representative of three separate experiments each performed in triplicate. Values are expressed as percentage maximum stimulation induced by AVP. The mean 0–100% values for each receptor were 16 600–41 700 d.p.m., 26 600–78 300 d.p.m., 25 300–51 200 d.p.m. and 18 800–32 400 d.p.m. for wild-type, [N14/27Q]V_{1a}R, [N198Q]V_{1a}R and [N14/27/198Q]V_{1a}R respectively. (B) EC₅₀ values of glycosylation-defective mutant receptors, means ± S.E.M. (*n* = 3).

B

Construct	Distribution (%)	
	Cell Surface	Internal
Wild-type	43 ± 4	57 ± 4
N14Q	21 ± 6	79 ± 6
N27Q	13 ± 7	87 ± 7
N198Q	42 ± 14	58 ± 14
N14/27Q	66 ± 10	34 ± 10
N14/27/198Q	80 ± 23	20 ± 23

Figure 7 Expression of glycosylation-defective V_{1a}Rs

All receptor constructs were transfected simultaneously, processed and assayed in parallel. (A) Total receptor expression (white bars) and cell-surface expression (black bars) were determined by radioligand-binding experiments, as described in the Materials and methods section, for the constructs indicated. Values are expressed as the percentage of wild-type V_{1a}R (WT) expression. Data shown are the means ± S.E.M. from three separate transfections. **P* < 0.05 and ***P* < 0.01 compared with wild-type V_{1a}R. (B) For each of the constructs indicated, the distribution of the receptor between the cell surface and intracellular compartments is given.

[N27Q]V_{1a}R, to 80% of the receptors being present in the plasma membrane for [N14/27/198Q]V_{1a}R.

DISCUSSION

Analysis of the sequences of the GPCR family reveals that, with very few exceptions, they possess putative glycosylation sites at their N-termini, although the number and location of these sites varies. In addition, some receptors also have putative glycosylation sites within the extracellular-loop regions. This conservation of post-translational modification throughout the GPCR family is suggestive of a functional role. Unfortunately, for the

vast majority of GPCRs, characterization of receptor glycosylation has not proceeded beyond identifying putative glycosylation sites in the amino acid sequence. This information is only of limited use, however, as not all consensus sites are actually utilized by the oligosaccharyltransferase in the endoplasmic reticulum.

In the present study we identified the glycosylation sites utilized in the V_{1a}R using a combination of a rabbit reticulocyte lysate translation system (which does not support glycosylation) and a *Xenopus* egg extract cell-free translation system (which is glycosylation-competent [24,34]). Our data show that there are three residues modified by carbohydrate, Asn¹⁴ and Asn²⁷ in the N-terminus and Asn¹⁹⁸ in the second extracellular loop. Asn³³³ located in the third extracellular loop constitutes part of an Asn-Xaa-Ser/Thr consensus site but is not modified, most probably because of the disruptive effect of the juxtaposed Pro³³⁴ [36]. A recent survey of multi-span membrane glycoproteins revealed that glycosylation of more than one extracellular segment is very unusual [42]. Indeed to our knowledge, within the GPCR family, only the V_{1a}R (this study), angiotensin AT₁ receptor [43] and the histamine H₂ receptor [44] have been shown to possess N-linked oligosaccharide on both the N-terminus and on an extracellular loop. Many GPCRs have consensus sites in these two segments but in most cases the sites have not been shown to be actually modified by carbohydrate. Of the few GPCRs for which glycosylation has been studied experimentally, rhodopsin, the VIP-1 receptor and the GnRH receptor all have consensus glycosylation sites on both the N-terminus and on an extracellular loop. However, rhodopsin has carbohydrate linked only at the N-terminus (Asn² and Asn¹⁵) but not in the second extracellular loop (Asn²⁰⁰) [15]. Likewise, the VIP-1 receptor is glycosylated at the N-terminus (Asn⁵⁸, Asn⁶⁹ and Asn¹⁰⁰) but not at the glycosylation consensus site in the third extracellular loop (Asn²⁹³) [19]. The GnRH receptor is glycosylated at the N-terminus (Asn⁴ and Asn¹⁸) but not at Asn¹⁰², which is within a consensus site in the first extracellular loop [14]. However, the Asn¹⁰² itself is a critical determinant for agonist potency [45]. The glycosylation site in the second extracellular loop of the V_{1a}R (Asn¹⁹⁸ in the rat) is conserved in all species cloned to date. In addition, it is also conserved in the vasotocin receptor, which shares 61% identity with the V_{1a}R and at which AVP is an agonist [46]. We would therefore predict that Asn¹⁶⁹ of the vasotocin receptor (corresponding to Asn¹⁹⁸ of the rV_{1a}R) is also glycosylated.

Disruption of the three N-glycosylation sites of the V_{1a}R by site-directed mutagenesis, or suppression of N-glycosylation by the NYT peptide, generated a translation product that was indistinguishable from the non-glycosylated receptor produced by the reticulocyte lysate system (Figure 4). These data establish that the V_{1a}R is not O-glycosylated. To date the only GPCR reported to possess O-glycosylation is the V₂R [47].

It has been well established by peptide mimetics [38], mutagenesis [40,41] and photoaffinity radiosequencing [39] that extracellular loops of VPRs have a role in ligand recognition. Furthermore, a subdomain of the N-terminus of the V_{1a}R and OTR is critical for high-affinity agonist binding and signalling [48,49]. Consequently, oligosaccharide side chains modifying the N-terminus and/or the second extracellular segment of the V_{1a}R might influence the pharmacology of the receptor. Radioligand-binding assays and second-messenger assays of a series of glycosylation-defective V_{1a}R constructs indicated that N-linked carbohydrate does not have a role in the receptor binding any of the four classes of ligand available (Tables 1 and 2) or in intracellular signalling (Figures 5 and 6).

Our data establish that the glycosylation status of the V_{1a}R regulates the level of receptor expression. Furthermore, the

nature of this regulation is dictated by the locus of the oligosaccharide modification within the receptor. Glycosylation of the receptor's N-terminus is not required for normal expression as the total expression of a non-glycosylated N-terminus construct ([N14/27Q]V_{1a}R) was not significantly different from wild-type levels (Figure 7A, white bars). This established that glycosylation of a single site in the second extracellular loop (Asn¹⁹⁸) was sufficient for normal V_{1a}R expression. However, glycosylation of Asn¹⁹⁸ was not a prerequisite for wild-type expression as this site could be ablated without detriment (Figure 7A). It seems, therefore, that there is a high degree of redundancy in utilizing three glycosylation sites distributed between two extracellular segments. An unexpected observation was that if the N-terminus of the receptor was glycosylated at only one site rather than two, total expression was increased 3-fold (Figure 7A). This contrasts with the mouse GnRH receptor, where deletion of either one of the two N-terminal glycosylation sites decreased expression [14], and the human GnRH receptor, where expression was increased by engineering in a second glycosylation site [50]. At least one glycosylation site must be modified by oligosaccharide, however, for the receptor to exhibit wild-type expression, as the totally non-glycosylated V_{1a}R ([N14/27/198Q]V_{1a}R) was expressed at very low levels (Figure 7A). This contrasts markedly with the V₃R, which is expressed normally when its single N-glycosylation site is disrupted [51]. However, it is perhaps relevant to note in this regard that, as cited earlier, the V₂R is also O-glycosylated, unlike the V_{1a}R [46]. The related OTR has three glycosylation sites in the N-terminus (Figure 1), which appear similar to the V_{1a}R in that individually they do not seem to have a role in ligand binding or signalling [52]. It is not known whether glycosylation has a role in expression of the OTR, as the fully non-glycosylated OTR has not been studied.

In addition to assaying total receptor expression, we also assayed cell-surface expression of the various constructs using whole-cell binding. These studies revealed that there was not a direct correlation between the total level of expression and the abundance of receptor at the cell surface. Consequently, although total expression of [N14Q]V_{1a}R and [N27Q]V_{1a}R was approx. 3-fold higher than that of wild-type V_{1a}R (Figure 7A, white bars), there was no significant difference in the cell-surface expression of all these receptors (Figure 7A, black bars). Obviously, this means that a smaller percentage of the [N14Q]V_{1a}R and [N27Q]V_{1a}R receptors synthesized by the cell were trafficked to the plasma membrane (Figure 7B). In contrast, total expression of the fully non-glycosylated receptor [N14/27/198Q]V_{1a}R was markedly lower (27%) than wild-type V_{1a}R. However, the vast majority of these receptors were at the cell surface (80%, Figure 7B). This suggests that in the absence of modification by oligosaccharide, there is decreased production of functional receptor by the cell. Nevertheless, once assembled successfully, the non-glycosylated receptor is transported efficiently to the plasma membrane.

In conclusion, the V_{1a}R is glycosylated at three positions, two in the N-terminus (Asn¹⁴ and Asn²⁷) and one in the second extracellular loop (Asn¹⁹⁸). Oligosaccharide must be linked to at least one of these loci for normal receptor expression but this modification by carbohydrate does not influence either ligand recognition or intracellular signalling. Glycosylation is required for the V_{1a}R to be folded efficiently into the functional conformation, or stabilizes such a conformation. However, it is not a prerequisite for trafficking of the receptor to the cell surface.

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