Folding and cell surface expression of the vasopressin V2 receptor: requirement of the intracellular C-terminus

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Abstract We characterized truncations of the human vasopressin V2 receptor to determine the role of the intracellular C-terminus (comprising about 44 amino acids) in receptor function and cell surface expression. In contrast to the wild-type receptor, the naturally occurring mutant R337X failed to confer specific [^{3}H]AVP binding to transfected cells. In addition, no vasopressin-sensitive adenylyl cyclase was detectable in membrane preparations of these cells. Laser scanning microscopy revealed that c-*myc* epitope- or green fluorescent protein-tagged R337X mutant receptors were retained within the endoplasmic reticulum. Increasing the number of C-terminal residues (truncations after codons 348, 354 and 356) restored G protein coupling, but revealed a length-dependent reduction of cell surface expression. Replacement of positively charged residues within the C-terminus by glutamine residues also decreased cell surface expression. A chimeric V2 receptor with the C-terminus replaced by that of the β_2-adrenergic receptor did not bind [^{3}H]AVP and was retained within the cell. These data suggest that residues in the N-terminal part of the C-terminus are necessary for correct folding and that C-terminal residues are important for efficient cell surface expression.

Key words: Surface expression; Transport; G protein-coupled receptor; Diabetes insipidus

1. Introduction

The vasopressin V2 receptor (V2 receptor) belongs to the large family of G protein-coupled receptors (GPCRs), structurally defined by seven transmembrane domains, an extracellular N- and an intracellular C-terminus. The lengths of the intracellular C-termini are highly variable throughout the protein family. In a comparative analysis of 204 different GPCRs it was found that the C-termini carry minimally 12 and maximally 162 amino acids [1]. Only the GnRH receptor, not considered in this study, completely lacks an intracellular C-terminus.

It was shown that the C-terminus of bovine rhodopsin is important for correct folding: a mutant with six residues after the seventh transmembrane domain was functional, whereas a mutant with only one residue was unable to bind retinal. The length of the C-terminus appeared to be more critical than the presence of specific residues [2]. In the V2 receptor a naturally occurring mutant with only nine amino acids after the seventh transmembrane domain (R337X), found in patients with X-linked nephrogenic diabetes insipidus [3], was demonstrated to be only core-glycosylated and thus likely to be retained within the endoplasmic reticulum (ER) [4].

We expressed the mutant R337X transiently or stably and investigated functional properties (ligand binding, activation of adenylyl cyclase) of intact cells and membrane preparations and analyzed the cellular localization of the mutant in fixed and living cells. In addition, the role of the length of the intracellular C-terminus and of basic residues for efficient cell surface expression was examined.

2. Materials and methods

2.1. Radiochemicals

[^{3}H]AVP (64.8 Ci/mmol), and [α-^{32}P]ATP ($\sim$30 Ci/mmol) were from NEN (Boston, MA, USA).

2.2. Cell culture

Madin-Darby canine kidney (MDCK), COS.M6 and human embryonic kidney (HEK) cells were cultured in DMEM and Chinese hamster ovary (CHO) cells in F12 medium containing 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml), and in case of stably transfected cell clones G418 (400 μg/ml).

2.3. Transient and stable expression of V2 receptors

For transient transfection, COS.M6 or HEK cells were plated at a density of 500 000 cells/60 mm dish, washed once with serum-free DMEM and incubated with a mixture of 30 μl SuperFect (Qiagen, Hilden, Germany) and 5 μg of plasmid DNA in 1.1 ml serum-free DMEM. After 2 h the mixture was replaced by complete medium. For binding experiments with intact cells, cells were passaged the following day onto 24 well plates ($4–5\times10^5$ cells/well) and investigated 2 or 3 days after transfection. For experiments with membranes, cells were kept on the dishes and grown for 2 or 3 days after transfection. Stable transfection of MDCK or CHO cells was performed as described [5].

2.4. Receptor constructs

Site-directed mutagenesis of the V2 receptor cDNA was performed as described recently [5]. Oligonucleotide sequences are available upon request. For the construction of the V2 receptor/β_2-adrenergic receptor chimera the region encoding amino acids 327–413 of the β_2-adrenergic receptor was amplified by PCR, using plasmid pBC-β2 as template (kindly provided by Martin Lohse, Würzburg, Germany). Oligonucleotides used for the amplification introduced restriction sites for *Nsi*I at the 5′ end and for *Xba*I at the 3′ end after the termination codon. The plasmid pRCDN2 [5] carrying the V2 receptor cDNA and the PCR product were cut with *Nsi*I and *Xba*I, and the cut PCR product was cloned in frame into the *Nsi*I/*Xba*I cut plasmid. For the construction of fusions consisting of the wild-type V2 receptor and the autofluorescent red-shifted variant of the green fluorescent protein (EGFP), the V2 receptor cDNA was cut from the plasmid pEU367.PhoA [6] with *Sac*I and *Bam*HI and cloned into the *Sac*I/

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Abbreviations: AVP, arginine-vasopressin; EGFP, red-shifted variant of the green fluorescent protein; ER, endoplasmic reticulum; GPCR, G protein-coupled receptor; V2 receptor, vasopressin V2 receptor

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*Bam*HI cut vector pEGFP-N1 (Clontech, Heidelberg, Germany). The resulting fusion protein consisted of the V2 receptor, lacking the C-terminal four amino acids, and the EGFP (pV2R.EGFP). For construction of the fusion consisting of the R337X mutant and the EGFP we used PCR. The V2 receptor cDNA fragment was amplified with a 5′ oligonucleotide (corresponding to nucleotides 412–429 of the V2 receptor cDNA) and a 3′ oligonucleotide introducing a restriction site for *Bam*HI after codon 336 of the V2 receptor. The PCR product was then cut with *Pml*I/*Bam*HI and cloned in frame into the *Pml*I/*Bam*HI cut pV2R.EGFP plasmid, resulting in plasmid pL336.EGFP. All clones were verified by sequencing. The construction of N-terminally c-*myc* epitope-tagged receptors (insertion of the decapeptide EQKLISEEDL after methionine 3) and their functional characterization is described elsewhere (Andersen-Beckh, B., Dehe, M., Müller, H., Liebenhoff, U., Schülein, R., Oksche, A. and Rosenthal, W., in preparation). The c-*myc* epitope did not alter the functional properties of the V2 receptor (affinity for [^{3}H]AVP, EC_{50} of AVP for stimulation of adenylyl cyclase).

2.5. [^{3}H]AVP binding

Binding of [^{3}H]AVP to intact cells was performed as described recently [5] but using 24 well plates. The procedure for transfection and plating of the cells is described above. For membrane binding, cells were washed in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4, pH 7.4), harvested with a rubber policeman and centrifuged at $400\times g$ for 10 min. The pellet was resuspended in Tris-BAME buffer (50 mM Tris, 0.15 mM bacitracin, 0.0015% aprotinin, 10 mM $MgCl_2$, 2 mM EGTA, pH 7.3), homogenized with a glass/teflon homogenizer (10 strokes), and centrifuged at $26\,000\times g$ for 30 min. The pellet was rehomogenized in Tris-BAME. Membranes (50 μg) were incubated in a final volume of 200 μl with [^{3}H]AVP (5–50 nM) in Tris-BAME without or with unlabelled AVP (5 μM) for 2 h at 25°C at 300 rpm in a rotary water bath. The samples were then transferred onto GF/C filters (Whatman) pretreated with 0.1% polyethyleneimine and washed rapidly twice with PBS using a Brandel cell harvester. Filters were finally transferred into 5 ml vials, and 4 ml of liquid scintillation cocktail was added. Radioactivity was determined in a liquid scintillation counter.

2.6. Adenylyl cyclase assay

The preparation of nuclei-free crude membrane fractions from CHO and HEK cells and the adenylyl cyclase assay were performed as described [5].

2.7. Immunofluorescence microscopy

COS.M6 cells grown on glass coverslips (12 mm) were washed twice with PBS and fixed for 30 min with freshly prepared paraformaldehyde (2.5% (v/v)) in Na-cacodylate buffer (100 mM Na-cacodylate, 100 mM sucrose, pH 7.4). After two rinses with PBS, coverslips were transferred either immediately or after permeabilization (0.1% Triton X-100 in PBS for 3 min) into a humidifying chamber and incubated at 37°C for 45 min with 20 μl of the monoclonal c-*myc* antibody (9E10, Dianova, Hamburg, Germany; 1:100 dilution in PBS). After three rinses with PBS at room temperature cells were incubated at 37°C for 45 min in the humidifying chamber with 20 μl of a polyclonal cy3-conjugated rabbit anti-mouse IgG antibody (Dianova, Hamburg, Germany; 1:400 dilution in PBS). After three final rinses cells were mounted with a mixture of PBS/glycerol (1:1) containing 100 mg/ml DABCO (1,4-diazobicyclo[2,2,2]octane, Sigma, Munich, Germany), to reduce photobleaching. The samples were analyzed at $\lambda_{exc} = 525$ and $\lambda_{em} > 568$ nm with the LSM 410 invert laser scanning microscope (Carl Zeiss, Jena, Germany). For microscopy of living cells, COS.M6 or HEK cells expressing the V2R.EGFP or L336.EGFP fusions were grown on 18×18 mm glass coverslips and incubated for 15 min at 37°C with rhodamine 6G (5 μM) or BODIPY TR ceramide (5 μM) (both from Molecular Probes, Leiden, The Netherlands) for the staining of the ER or of the Golgi apparatus respectively. The samples were analyzed with the LSM 410 at $\lambda_{exc} = 488$ and $\lambda_{em} > 515$ nm (EGFP), $\lambda_{exc} = 543$ and $\lambda_{em} > 570$ nm (rhodamine 6G, BODIPY TR ceramide).

Table 1
Binding of [^{3}H]AVP to intact COS.M6 cells expressing wild-type and mutant V2 receptors

	B_{max} (% of wild-type)	K_D values (nM)
Wild-type	(100)	2.8 ± 0.4
R337X	n.d.	–
L339X	n.d.	–
V2β2	n.d.	–
P349X	40 ± 10	2.6 ± 0.6
D355X	45 ± 12	2.1 ± 0.1
S357X	63 ± 9	2.7 ± 0.6
R337Q	52 ± 8	1.5 ± 0.5
K367Q	73 ± 5	2.9 ± 0.9
ΔposV2R	30 ± 7	3.0 ± 1.0

n.d.: not detectable.
B_{max} and K_D values of the wild-type and mutant V2 receptors are shown. Data represent mean values ± S.D. from at least three independent experiments.

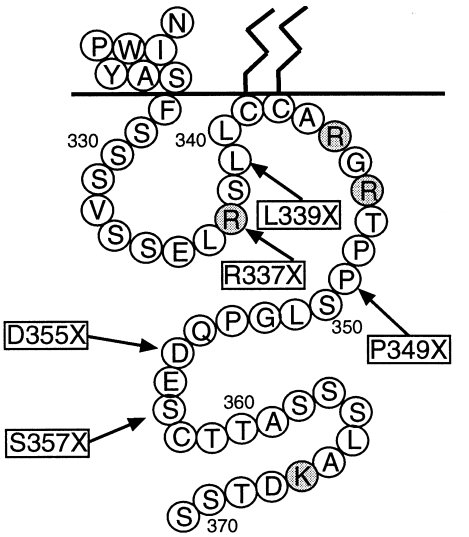

Fig. 1. Primary sequence of the intracellular C-terminus of the V2 receptor. Posttranslational modifications depicted are palmitoylation at cysteine residues 341 and 342. Truncations investigated in this study are marked with arrows. Basic residues of the C-terminus replaced by glutamines are marked as gray circles.

3. Results

To elucidate the role of the intracellular C-terminus of the V2 receptor in mediating cell surface expression and G protein activation we used truncated receptors lacking 15–35 amino acids (Fig. 1). The truncations are designated according to the amino acid replaced by the nonsense codon and its position in the V2 receptor. The shortest truncation mutant, R337X, has been found in patients suffering from nephrogenic diabetes insipidus [3]; it encodes a V2 receptor mutant truncated nine residues after the seventh transmembrane domain. Additional truncations investigated were L339X (includes the first basic residue after the seventh transmembrane domain), P349X (harbors three of four basic residues within the C-terminus), D355X (additionally includes a proline-rich stretch), and S357X (additionally includes two acidic residues). Full length receptors in which basic residues in the C-terminus were replaced by glutamines were also constructed.

Intact COS.M6 cells transiently expressing the naturally occurring mutant R337X lacked any specific binding using 10–50 nM [^{3}H]AVP (see Table 1). Similar results were obtained for the L339X mutant. In contrast, the receptors truncated after residues 348, 354 and 356 displayed specific binding and revealed K_D (see Table 1) and EC_{50} values (Fig. 2A)

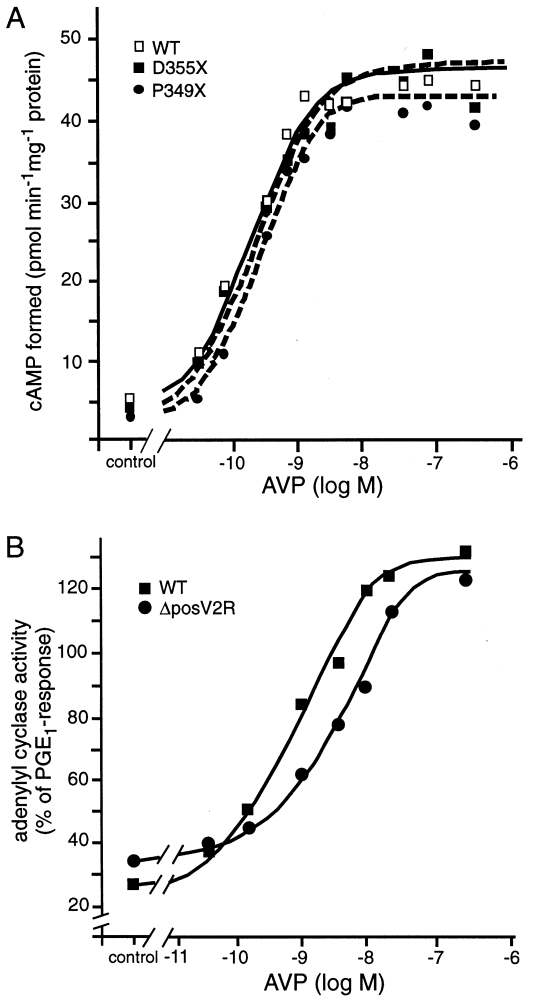

Fig. 2. AVP dose response curves of membrane preparations. A: Adenylyl cyclase activities in membrane preparations derived form CHO cells stably expressing wild-type, P349X or D355X mutant V2 receptors. For each mutant two independently developed clones were investigated. Data represent mean values of duplicates, which differed by less than 5%. EC_{50} values for the wild-type, the P349X and the D355X mutants were 0.1 nM, 0.18 nM and 0.1 nM respectively. B: Adenylyl cyclase activities in membrane preparations derived from HEK cells transiently expressing the wild-type or the ΔposV2R receptors. Data represent mean values of duplicates, which differed by less than 5%. EC_{50} values for the wild-type and ΔposV2R mutants were 0.85 nM and 3 nM respectively. Basal, 250 nM AVP-stimulated, and 100 μM PGE_1-stimulated adenylyl cyclase activities were (in pmol cAMP/mg protein/min): 4.0, 20.3 and 15.1 for the wild-type and 4.0, 14.2 and 11.7 for the ΔposV2R mutant respectively.

similar to those of the wild-type (EC_{50} values are given in the legend to Fig. 2). However, the truncated receptors reveal reduced B_{max} values (see Table 1), which were lowest for P349X and highest for S357X, indicating a relationship between the length of the C-terminus and the extent of cell surface expression.

To detect receptors which are competent to bind [^{3}H]AVP but retained intracellularly, we prepared membranes of COS.M6 and HEK cells transiently or of MDCK and CHO cell clones stably expressing the R337X mutant. In contrast to membranes from cells expressing the wild-type receptor, we were unable to detect specific binding of [^{3}H]AVP (5–50 nM) to membranes from those cells (Fig. 3). Experiments with the L339X mutant yielded similar results (not shown). In agreement with the binding data, adenylyl cyclase in membrane preparations of HEK cells transiently or MDCK cells stably expressing the R337X or the L339X mutant receptors did not respond to AVP up to 1 μM (not shown).

To exclude the presence of normally transported receptors with defective binding sites on the cell surface (R337X, L339X), we performed immunofluorescence on wild-type and truncated V2 receptors carrying a c-*myc* epitope at the extracellular N-terminus. In addition, the truncated receptors with normal function (P349X, D355X) were analyzed. In non-permeabilized COS.M6 cells (Fig. 4A) the wild-type receptor and the mutants P349X and D355X were detected at the cell surface, with the wild-type showing the most prominent signal. In contrast, the R337X mutant was not detected at the cell surface. When cells were permeabilized (Fig. 4B) the mutant R337X was easily detected, like the mutants P349X, D355X or the wild-type V2 receptor. The relative intensities of the fluorescent signals found for permeabilized cells expressing the wild-type or the truncated receptors were similar, indicating that the tagged receptors are synthesized at comparable levels.

The immunoreactive signals of fixed COS.M6 cells expressing the various mutants did not allow the identification of receptor-harboring compartments. We therefore expressed fusion proteins consisting of the wild-type or truncated (after L336) V2 receptor and the autofluorescent green fluorescent protein (EGFP, fused to the C-terminus of the V2 receptor). This approach allowed the analysis of receptors within living cells, and by visualizing organelles with specific fluorescent dyes, we were able to identify the subcellular distribution of the fusion proteins. The wild-type receptor (V2R.EGFP) ex-

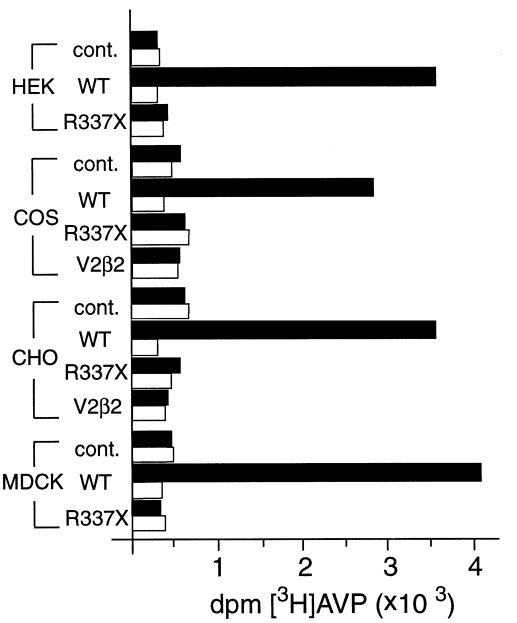

Fig. 3. [^{3}H]AVP binding to membrane preparations of untransfected cells (contr.) or of cells expressing the wild-type (WT), the R337X mutant or the V2β2 chimeric receptors. Binding was performed with 5 nM [^{3}H]AVP in the presence (white bar) or absence (black bar) of 1 μM AVP. In case of CHO and MDCK cells stably transfected clones were analyzed. The data represent mean values of duplicates, which differed by less than 5%. Similar results were obtained in at least three independent experiments and in the case of stably transfected cells from two independently derived cell clones.

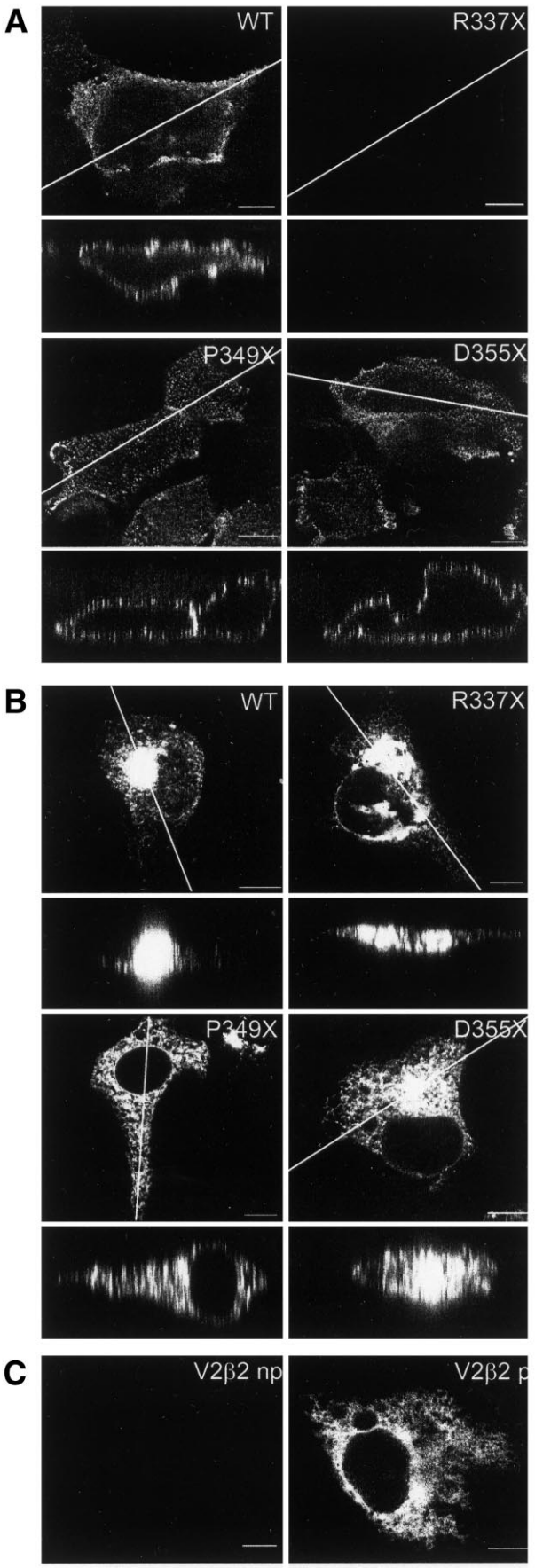

Fig. 4. Immunolocalization of epitope-tagged wild-type or mutant V2 receptors in COS cells. The wild-type or mutant receptors, with an N-terminal c-*myc* epitope, were detected with a primary monoclonal anti-c-*myc* antibody and a secondary polyclonal cy3-conjugated antibody. A: Non-permeabilized cells; B: permeabilized cells (WT, R337X, P349X, D355X). Shown are focal planes of cells (upper panels) and *z*-scans at the indicated lines (lower panels) obtained by laser scanning microscopy. C: Non-permeabilized (V2β2 np) and permeabilized (V2β2 p) COS cells expressing the V2β2 chimeric receptor. Shown are focal planes of cells. Scale bar, 10 μm.
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pressed in HEK and COS.M6 cells was located mainly at the plasma membrane. Only in COS.M6 cells, which grossly overexpress, the receptor was also detected in a compartment near the nucleus (Fig. 5), which colocalized with the Golgi marker BODIPY TR ceramide (not shown). In contrast, the receptor truncated after leucine 336 (L336.EGFP) was not detected at the plasma membrane but exclusively found within the cell. The signal obtained with the L336.EGFP fusion in HEK and in COS.M6 cells colocalized with the fluorescent probe rhodamine 6G, which is enriched within the endoplasmic reticulum

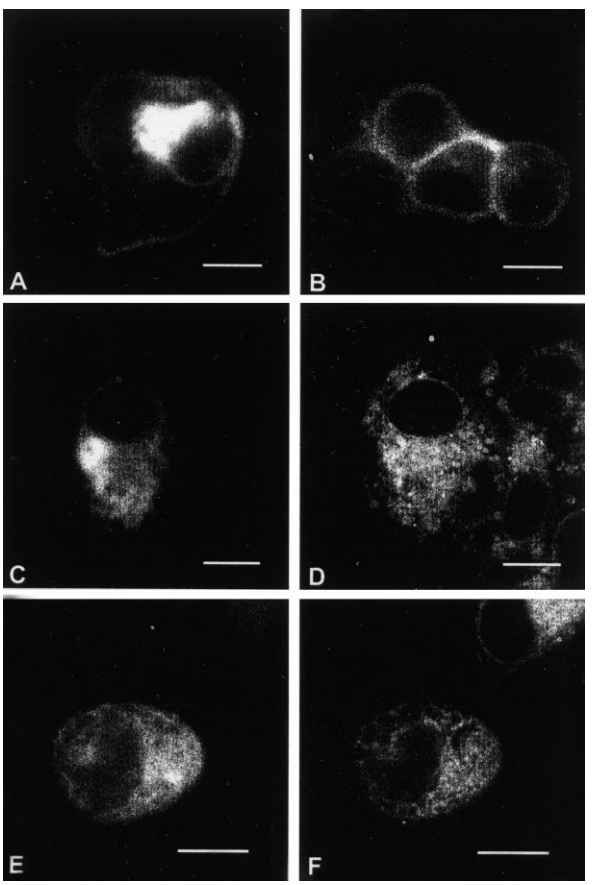

Fig. 5. Localization of wild-type and truncated V2 receptors fused to EGFP protein in COS.M6 (A,C,D) and HEK cells (B,E,F). The wild-type fusion protein (V2R.EGFP: A,B) was predominantly found in the plasma membrane. In COS.M6 cells it was also detected in the Golgi apparatus (A) as determined by colocalization with BODIPY TR ceramide staining (not shown). The truncated receptor (L336.EGFP) was only detected within the cell (C,E) and colocalized with rhodamine 6G staining (D,F) indicating its retention within the endoplasmic reticulum. Note that rhodamine 6G also stains untransfected COS.M6 cells. The analysis was performed with a laser scanning microscope. Scale bar, 10 μm.

and in mitochondria [7] (see Fig. 5). Thus the mutant R337X seems to be misfolded and is therefore retained within the ER.

To determine whether a specific sequence in the C-terminus is required for folding and cell surface expression, we constructed a chimera consisting of the amino-terminal part of the V2 receptor including the seventh transmembrane domain (amino acids 1–326 including the highly conserved NPXXY motif) and the cytoplasmic C-terminus (downstream of the NPXXY motif) of the β_2-adrenergic receptor (amino acids 327–413). Transient expression of this chimera in COS.M6 cells or stable expression in CHO cells did not confer specific binding of [^{3}H]AVP to intact cells or to membrane preparations (see Fig. 3 and Table 1). In addition, membrane preparations of CHO cells expressing the chimera showed no activation of adenylyl cyclase in response to vasopressin (up to 10 μM, not shown). Immunofluorescence microscopy of the N-terminally c-*myc*-tagged chimera revealed that this mutant is retained within the cell, similarly to the R337X and the L339X mutants (Fig. 4C). The results suggest that the proximal part of the C-terminus harbors specific residues required for a transport-competent and functional receptor.

As basic residues are frequently found in the C-terminus of GPCRs we analyzed the role of positively charged residues within the C-terminus of the V2 receptor for cell surface expression. To this end we replaced arginine residues 337, 344, 346 and the lysine residue at position 367 by glutamine residues (ΔposV2R; see Fig. 1). Saturation binding to intact COS.M6 cells expressing the ΔposV2R mutant revealed a lowered expression at the cell surface of about one third of that of the wild-type (Table 1). Dose-response curves obtained with membrane preparations from HEK cells transiently expressing the mutant ΔposV2R stimulated adenylyl cyclase to the same extent as the wild-type (Fig. 2B); the EC_{50} value was about 3-fold higher than that of the wild-type receptor expressed in HEK cells (EC_{50} values for WT and mutant receptors were 0.85 nM and 3 nM respectively). Single substitutions of arginine 337 by glutamine (R337Q) and of lysine 367 by glutamine (K367Q) reduced the level of cell surface expression to about 50 and 75% of that of the wild-type respectively (see Table 1). The K_D values of the R337Q, K367Q and ΔposV2R were similar to that of the wild-type receptor.

4. Discussion

The R337X mutation found in patients with nephrogenic diabetes insipidus produces a full clinical phenotype [3], indicating a substantial defect of the mutant V2 receptor. This is of particular interest as the receptor, truncated within the intracellular C-terminus nine amino acids after the seventh transmembrane domain, should be correctly folded and be functional. Sadeghi et al. [4] recently showed that the R337X receptor is only core-glycosylated (endoglycosidase H-sensitive), whereas the wild-type receptor is complex glycosylated (endoglycosidase H-resistant). It was suggested that the R337X mutant lacks signals required for exit from the ER. We show here that the R337X mutant is misfolded (no [^{3}H]AVP binding in membrane preparations) and retained within the ER. As positively charged residues are frequently found in cytoplasmic sequences following hydrophobic stop/transfer domains [8], it is possible that the absence of the arginine 337, close to the seventh transmembrane domain, causes misfolding. This is, however, opposed by the finding that the L339X mutant was also non-functional. Moreover, the replacement of arginine 337 by glutamine in the full-length receptor had no influence on functional activity.

Truncations of the C-termini of other GPCRs also resulted in non-functional receptors. For example, truncation of the C-terminus close (1–7 residues) to the seventh transmembrane domain yielded non-functional receptors in case of the human PTH receptor [9], the rat substance P receptor [10], the canine histamine H2 receptor [11], the human endothelin A receptor and the human neurokinin A receptor [12]. The removal of the complete C-terminus of the TSH receptor caused a 100-fold decrease of the K_D and abolished G_s coupling [13]. For bovine rhodopsin it was found that truncation of the receptor one residue after the seventh transmembrane domain abolished retinal binding; the mere presence of five additional residues, but not their identity was essential for correct folding [2]. Our findings suggest that specific residues within the C-terminus of the V2 receptor are required for correct folding since the replacement of the C-terminus by that of the β_2-adrenergic receptor yielded a non-functional receptor. Interestingly, a chimeric receptor protein consisting of the N-terminal part of the human V2 receptor (amino acids 1–331) and of the rat V1a receptor (amino acids 359–395) was found to be functional with properties similar to that of the wild-type V2 receptor [14]. The discrepancy of the effects observed for the chimeric receptor proteins, the V2β2 and the V2V1a chimeras, may be caused by the different fusion strategies. In the V2V1a chimera the rat V1a C-terminus was fused to the human V2 receptor after serine 330, whereas in the V2β2 chimera, the C-terminus of the human β_2-adrenergic receptor was fused after alanine 326. Alternatively, there may be a requirement for specific amino acids in the C-terminus for proper folding. The amino acid sequence of the rat V1a receptor, although different from that of the human V2 receptor, may harbor in some positions residues which allow correct folding, whereas the human β_2-adrenergic receptor does not possess these residues. The significance of cysteine residues 341 and 342 (shown to be palmitoylated in the wild-type receptor [15]) for folding also has to be considered. Both cysteine residues are lacking in the misfolded R337X and L339X mutants. Replacement of both cysteine residues by serines did not alter the V2 receptor's functional properties for [^{3}H]AVP binding and AVP-induced stimulation of the adenylyl cyclase [5]. We show here that the V2β2 chimera is misfolded, despite the presence of a presumably palmitoylated cysteine residue (cysteine 341 within the β_2-adrenergic C-terminus). Thus the palmitoylation of C-terminal cysteines is apparently not required for proper folding.

The mechanisms underlying our findings remain speculative. It is possible that specific interactions (hydrogen bonds, interactions of charged residues) between the residues of the C-terminus and the three intracellular loops contribute to the correct positioning of all seven helices. In fact, the solution structure of a complex of the three intracellular loops and the C-terminus of rhodopsin provided evidence for seven intramolecular bonds, five of them involving the N-terminal part of the C-terminus [16]. Alternatively, the premature termination may disturb the correct positioning of transmembrane domains in the ER membrane. Support for this hypothesis comes from the finding that peptides corresponding to the first and second intracellular loops of bovine rhodopsin produce well-ordered structures (in the absence of transmem-

brane helices) [17]. It was suggested that folding of the loops enables correct positioning of the transmembrane domains. Whether ordered structures of the C-terminus of GPCRs are also required for the correct positioning of the transmembrane domain has not been investigated so far.

Besides the finding that N-terminal residues of the V2 receptor are needed for proper folding, we show that the distal C-terminus is required for efficient transport to the cell surface. While the truncations after residues 348, 354 and 356 did not affect ligand binding and G protein activation, they caused substantially lowered expression of the receptor at the cell surface. The length of the C-terminus of the rat PTH receptor also influences cell surface expression. A deletion of 78 or 111 residues decreased cell surface expression and, in addition, decreased ligand affinity 1.5- and 4-fold respectively [18]. For rhodopsin it was shown that the C-terminus harbours motifs important for transport. A monoclonal antibody directed against the C-terminus inhibited vesicle formation at the trans-Golgi network, resulting in accumulation of completely processed receptors within this compartment [19]. Not only the length of the C-terminus, but also the substitution of positively charged residues by glutamines within the C-terminus of the V2 receptor caused reduction of cell surface expression. Interestingly, of the 35 reported missense mutations of the V2 receptor causing nephrogenic diabetes insipidus, only two reside within the intracellular loops (specifically the second intracellular loop, for review see [20]). The mutations (R137H, R143P) are predictive for a replacement of arginine in each case, either by histidine (which is usually not protonated in the cytoplasmic environment) or proline; for both, a reduced surface expression to 10% or less of the wild-type was demonstrated [21,22]. As basic residues are frequently found in the C-termini of many GPCRs it would be of interest to determine whether removal of basic residues affect transport of other receptors in a similar way. It also remains to be shown whether the reduced cell surface expression results from a hampered transport, e.g. from the ER or Golgi to the plasma membrane or less likely misrouting, e.g. to lysosomes.

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