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# The Splice Variant of the V2 Vasopressin Receptor Adopts Alternative Topologies

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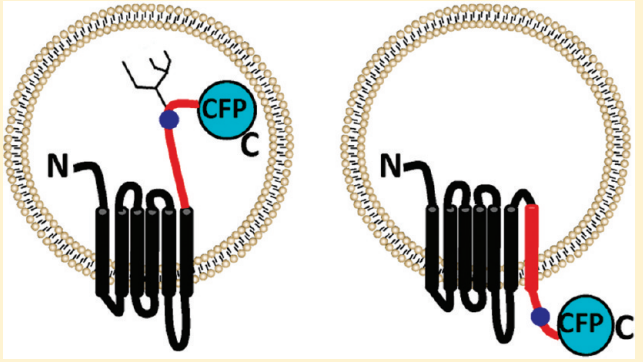
Supporting Information

**ABSTRACT:** The V2 receptor gene encodes two receptor variants by alternative splicing, the canonical V2 receptor (V2a receptor) and V2b. The V2b variant has an amino acid sequence identical to that of the V2a receptor up to the sixth transmembrane domain, but the V2b sequences corresponding to the putative seventh transmembrane domain and the carboxyl terminus are different from those of the V2a receptor. Here we investigate the topology and subcellular distribution of the V2b variant. We found that, in contrast to the V2a receptor, the V2b adopted two topologies: one with six transmembrane segments with the C-terminus on the extracellular side of the membrane and another with seven transmembrane segments with the C-terminus on the intracellular side, similar to typical G-protein-coupled receptors. Furthermore, we observed that both topological isoforms oligomerized with the V2a canonical receptor. Unlike the V2a receptor, V2b did not move to the plasma membrane, but it is retained in the ER−Golgi compartments. These findings indicate that the C-terminal sequence beyond the sixth transmembrane of the V2a is required for the stabilization of the seven-transmembrane topology of the receptor and is also essential for the trafficking of the receptor to the plasma membrane.




Alternative splicing of the RNAs encoding GPCRs promotes the functional and structural diversity of this class of receptors. Splice variants of the corticotropin-releasing hormone receptor[1] and the calcitonin receptor[2] containing diverse extracellular N-terminal sequences give rise to different ligand binding profiles. Alternative splicing also generates truncations of GPCRs, including deletions of all transmembrane domains and generation of soluble N-terminal domains of metabotropic glutamate receptors,[3] corticotropin-releasing hormone receptors,[4] and luteinizing hormone receptors.[5] Of particular importance are those splice variants such as dopamine D2,[6] histamine H3,[7] and the cholescytokinin receptors,[8] which contain diverse intracellular loop sequences, thus giving rise to changes in the coupling to G-protein subtypes. Splice variants with distinct extracellular loop sequences are less frequent.[9] The most frequent splice variants are those containing distinct C-terminal sequences, including transmembrane segment 7 (TM7) and the C-terminal tails.[10−15] Previously, we identified a splice variant (V2b) of the canonical V2a vasopressin receptor.[16] V2b has an amino acid sequence identical to that of the V2a receptor up to residue 303, close to the end of the sixth transmembrane domain, but the V2b sequences corresponding to the seventh transmembrane domain and the carboxyl terminus are distinct from those of V2a. The V2b variant is expressed in the distal tubule and collecting duct of the kidney, but in contrast to V2a, V2b is retained in the endoplasmic reticulum−Golgi complex and does not bind vasopressin. Importantly, the V2b variant forms oligomers with the V2a receptor and behaves as a dominant negative mutant by retaining the V2a inside the cell. On this basis, we have suggested that the expression of V2a on the cell surface is determined by the relative expressions of V2b to V2a receptors. Because the V2b variant contains noncanonical sequences corresponding to the putative seventh transmembrane segment and C-terminal tail we have investigated the topology adopted by V2b and its subcellular distribution. We found that in contrast to the canonical V2a, V2b adopted two topologies, one with six transmembrane segments (TM6) with the C-terminus on the extracellular side of the membrane and another with seven transmembrane segments (TM7) with the C-terminus on the intracellular side. Furthermore, we observed that both topological isoforms oligomerized with the V2a canonical receptor.

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## EXPERIMENTAL PROCEDURES

**Plasmid Construction.** The construction of the expression vectors (pECFP-N1) encoding V2b−CFP and V2a−CFP fusion proteins has been described previously.[16,17] The V2a and V2b mutants without native glycosylation sites (N22Q and N185Q) and with an engineered glycosylation site in the linker sequence between the receptors and CFP were constructed by PCR-based mutagenesis,[18] which produced the V2b(m)−CFP and V2a(m)−CFP constructs, respectively. Similarly, we generated a C-terminally truncated V2a mutant containing only transmembrane segments 1−6 (TM1−6), lacking the native glycosylation sites and containing the engineered glycosylation site in the linker sequence as described above [V2a(m)-TM1−6−CFP]. In addition, we inserted an HA tag at the amino terminus of all mutants [HA-V2b(m)−CFP, HA-V2a(m)−CFP, and HA-V2a(m)-TM1−6−CFP].

**Cell Culture and Transfections.** CHO-K1 cells were transiently transfected with the expression plasmids encoding the receptor mutants, using Fugene 6 as described previously.[16,17]

**Cell Surface Biotinylation Assay.** CHO-K1 cells transfected with plasmids encoding the V2a−CFP or the V2b−CFP were washed three times with PBS (pH 8.0), and incubated with *N*-hydroxysulfosuccinimide esters of biotin (Sulfo-biotin) in biotinylation buffer (2 mM $CaCl_2$, 150 mM NaCl, and 10 mM triethanolamine) for 30 min at 4 °C. The biotinylation reaction was stopped with three washes of PBS containing 100 mM glycine and three more washes with PBS. Cells were lysed with RIPA buffer [50 nM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, 0.1 μg/mL PMSF, and 10 mM *N*-methylmaleimide]. Cell extracts were centrifuged at 16000*g* for 1 h. Supernatants were incubated with 50 μL of neutravidine-agarose. The precipitates were collected by centrifugation, washed with 100 and 300 mM NaCl, resuspended in Laemmli buffer, and subjected to Western blotting.

**Western Blotting.** Cell extracts were fractionated via sodium dodecyl sulfate−polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters as described previously.[16] The CFP-tagged proteins were detected with a polyclonal anti-GFP antibody (Invitrogen) that reacts against all the variants of *Aequorea victoria* such as EGFP, EYFP, and ECFP.

**Co-Immunoprecipitation.** CHO-K1 cells were cotransfected with two plasmids, one containing the cDNA encoding the V2a receptor tagged with HA and the other containing the cDNA encoding V2b tagged with CFP. Transfected cells were treated with and without 5 μg/mL tunicamycin. After being incubated for 48 h, the cells were washed with phosphate-buffered saline and homogenized with a Dounce homogenizer in a 5 mM Tris-HCl (pH 7.4) buffer containing 15 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. The homogenate was centrifuged for 10 min at 6000*g*, and the postnuclear supernatant was centrifuged for 45 min at 150000*g*. The membrane pellet was solubilized in RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 mM *N*-ethylmaleimide, 0.1 mM phenylmethanesulfonyl fluoride, 5 mg/mL soybean trypsin inhibitor, and 1 μg/mL leupeptin] and centrifuged for 45 min at 16000*g*. The supernatant was incubated with a polyclonal anti-GFP antibody (2.5 μg, Invitrogen) and 20 μL of agarose-protein A (50% slurry) at 4 °C for 16 h. The immunoabsorbent was washed three times with RIPA buffer and collected by centrifugation for 2 min at 1000*g*. The immune complexes were resuspended in sample buffer and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis and Western blotting. The V2a receptor was detected by using a mouse monoclonal anti-HA antibody (Roche) and a peroxidase-labeled goat anti-mouse antibody (Jackson Laboratories).

**Immunocytochemistry.** CHO-K1 cells cultured on coverslips were transfected with expression plasmids encoding HA-V2b(m)−CFP, HA-V2a(m)−CFP, or HA-V2a(m)-TM1−6−CFP. After 48 h, the cells were fixed with methanol or 4% paraformaldehyde and permeabilized with digitonin as described previously.[19,20] Cells were stained with anti-HA monoclonal or polyclonal anti-GFP, anti-calnexin, anti-ERGIC-53, or anti-58K antibodies using Alexa 488- and Alexa 633-labeled secondary antibodies and visualized with an Olympus Fluoview 1000 confocal microscope. To detect CFP, cells were excited with a 440 nm diode laser and the emitted light was detected using a BP 465−495 nm filter. To detect the Alexa 488-labeled antibody, the cells were excited with a 488 nm argon laser and the emitted light was detected by using a BP 505−525 nm filter, whereas the Alexa 633-labeled antibody was detected via excitation with a 633 nm He−Ne laser and the emitted light detected using a LP 650 nm filter.

## RESULTS

**V2b Adopted TM6 and TM7 Topologies.** Hydropathy analysis of the V2b sequence using the Kyte−Doolittle, Membrain, and Octopus algorithms (Figure 1SA−C of the Supporting Information) predicted a seven-transmembrane protein with the C-terminal tail exposed to the cytoplasmic side of the membrane. On the other hand, the TMHMM algorithm predicted a six-transmembrane domain with the C-terminus toward the extracellular side of the membrane (Figure 1SD of the Supporting Information). To determine the actual topology of the V2b variant, we employed a glycosylation assay, which has been previously used for determining the topology of integral membrane proteins.[21,22] CHO-K1 cells were transfected with a cDNA encoding V2b fused to CFP[16] and lacking the native glycosylation sites, but containing an engineered glycosylation site at the carboxyl terminus [V2b(m)−CFP]. The transfer of an oligosacharyl chain to this new site would indicate that the C-terminus of V2b(m)−CFP is now facing the lumen of the rough endoplasmic reticulum and therefore is folded into a six-transmembrane protein (Figure 1A,B). On the other hand, the lack of glycosylation of V2b(m)−CFP will indicate that the mutant is folded into a seven-transmembrane protein (Figure 1A,C). We found that in contrast to the V2b−CFP protein containing the native glycosylation sites (Figure 1A, lanes 1 and 2), Western blots of extracts of cells expressing the newly engineered V2b(m)−CFP protein showed two bands with similar intensities (Figure 1A, lane 3). The low-molecular weight band ($M_r$ = 55000) corresponded to a nonglycosylated protein (Figure 1A,C, lanes 2 and 3), whereas the high-molecular weight band ($M_r$ = 58000) corresponded to a glycosylated protein, as this large band was not detected in extracts of cells pretreated with tunicamycin, an antibiotic that blocks the N-glycosylation of newly synthesized proteins (Figure 1A,B, lane 4). To validate the glycosylation assay as a means of probing membrane topology, we used a cDNA encoding a truncated V2 receptor TM1−6 lacking the native glycosylation sites and containing an engineered glycosylation site at the carboxyl terminus [V2a(m)-TM1−6−CFP (Figure 2SA of the Supporting Information)]. As predicted,

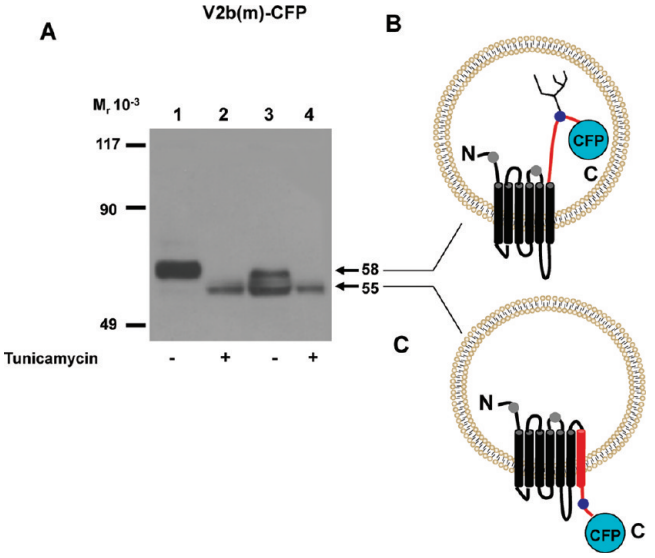


**Figure 1.** Alternative topologies of the V2b variant. (A) Western blot analysis of extracts from cells expressing the V2b mutants. Lanes 1 and 2 show the immunodetection of wild-type V2b fused to CFP from untreated cells and cells treated with tunicamycin, respectively. Lanes 3 and 4 show the immunodetection of V2b(m)−CFP from untreated cells and cells treated with tunicamycin, respectively. The upper band in lane 3 corresponds to the $M_r$ = 58000 glycosylated protein, whereas the lower band corresponds to the $M_r$ = 55000 nonglycosylated protein. (B) Representation of the six-transmembrane (top) and seven-transmembrane (bottom) topologies of V2b(m)−CFP in the endoplasmic reticulum. Gray spheres denote the deleted native glycosylation sites; blue spheres denote the engineered glycosylation sites, and the C-terminal sequence of V2b, distinct from the corresponding sequence of V2a, is colored red.

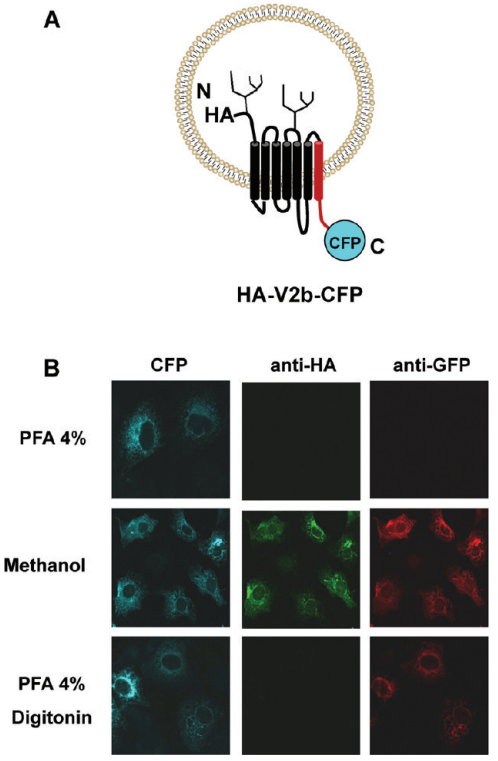


**Figure 2.** Seven-transmembrane topology of V2b, probed by confocal microscopy. (A) Representation of the seven-transmembrane topology of HA-V2b−CFP in intracellular vesicles. (B) Images of CHO cells expressing HA-V2b−CFP treated with 4% paraformaldehyde (PFA, top row), methanol (middle row), or 4% PFA/digitonin (bottom row). The left column shows CFP fluorescence, the middle column staining with the anti-HA antibody, and the right column staining with the anti-GFP antibody (which reacts against all the variants of *A. victoria* such as EGFP, EYFP, and ECFP).

the Western blots showed a single glycosylated protein, as demonstrated by the faster migration of the protein from cells treated with tunicamycin (Figure 2SB of the Supporting Information, lanes 1 and 2). Furthermore, extracts of cells expressing the wild-type V2a receptor lacking the native glycosylation sites and containing an engineered glycosylation site at the carboxyl terminus [V2a(m)−CFP (Figure 2SC of the Supporting Information)] showed a single nonglycosylated protein, as its electrophoretic migration was the same regardless of whether the cells were treated with tunicamycin (Figure 2SD of the Supporting Information, lanes 1 and 2). To further probe the folding of V2b into a TM7 protein, we detected by immunocytochemistry and selective cell permeabilization the orientation of two artificial epitopes inserted at the N- and C-termini of wild-type V2b. The HA and CFP epitopes were tagged at the N- and C-termini of V2b, respectively (Figure 2A). We found that the HA and CFP epitopes were not stained with their corresponding antibodies in transfected cells fixed with the nonpermeabilizing agent paraformaldehyde, consistent with our previous studies[16] showing that V2b is retained inside the cell and with the intracellular fluorescence of CFP (Figure 2B, top row). On the other hand, both HA and CFP epitopes were immunodetected in methanol-induced permeabilization of the plasma membrane and intracellular organelles of transfected cells (Figure 2B, middle row). Interestingly, the selective permeabilization of the plasma membrane with digitonin allowed only the immunodetection of the CFP but not the HA epitope (Figure 2B, bottom row). These findings further validate the TM7 topology adopted by V2b.

**Trafficking of the V2b TM7 Isoform to the Golgi Apparatus.** Although the V2b−CFP isoform does not reach the plasma membrane, as shown by using a surface biotinylation assay (Figure 3S of the Supporting Information), we investigated the intracellular trafficking of V2b. We found that CHO cells expressing HA-V2b−CFP colocalized with calnexin, an ER marker, with ERGIC-53, an endoplasmic reticulum intermediate compartment (ERGIC) marker, and with 58K protein, a Golgi marker (Figure 3). Further, we found that, at least, the V2b that reaches the Golgi complex is the TM7 variant, as demonstrated by immunofluorescence microscopy of cells fixed with paraformaldehyde, permeabilized with digitonin, and stained with anti-GFP and anti-58K antibodies (Figure 4).

**Oligomerization of V2a with the Two Topological Isoforms of V2b.** Our previous studies showed that V2b oligomerized with V2a, leading to the intracellular retention of the canonical V2a.[16] To determine whether the TM6 or TM7 topological isoform of V2b forms oligomers with V2a, we conducted co-immunoprecipitation assays of lysates prepared from CHO cells expressing HA-V2a and V2b(m)−CFP. Using anti-HA antibodies for immunoprecipitations and staining of Western blots with anti-GFP antibodies, we detected two protein bands [$M_r$ = 55000, and $M_r$ = 58000 (Figure 5, lane 1)]. The latter was sensitive to the treatment with tunicamycin (Figure 5, lane 2), indicating that

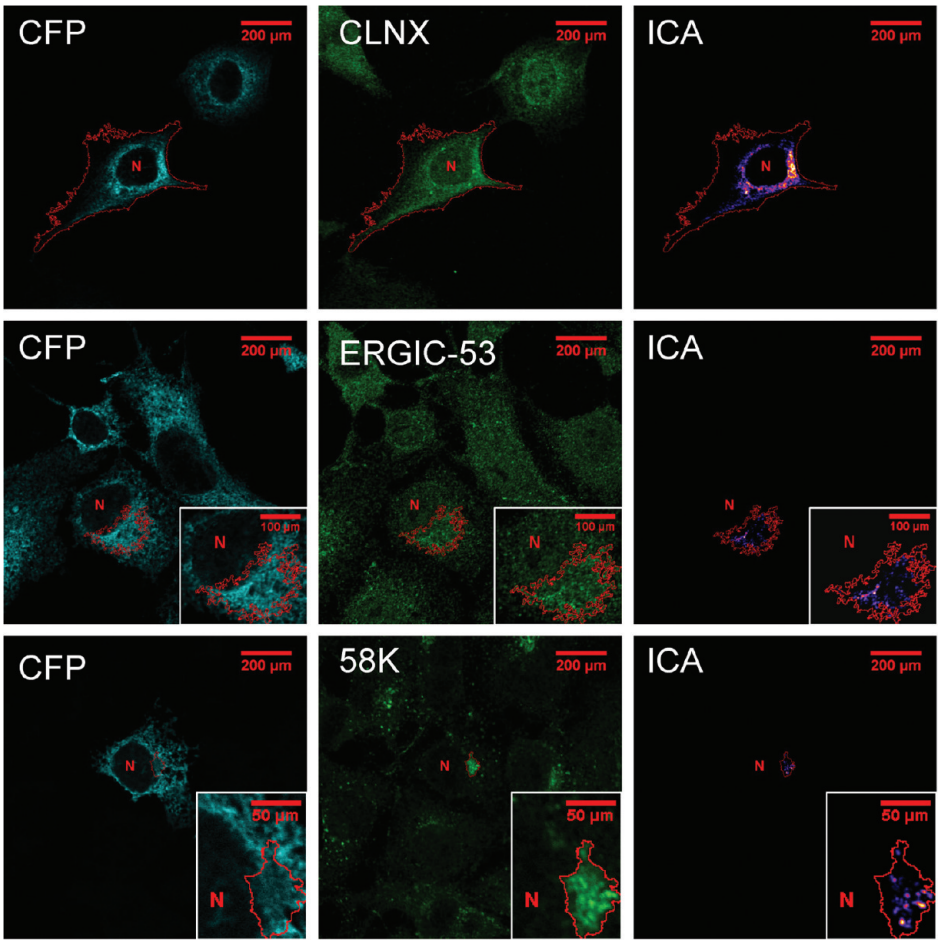


**Figure 3.** Colocalization of V2b−CFP with ER, ERGIC, and Golgi markers. Images of CHO cells expressing HA-V2b−CFP were fixed with methanol and incubated with anti-calnexin (CLNX), anti-ERGIC-53, or anti-58K antibodies. The left column shows CFP fluorescence, the middle column staining with organelle markers, and the right column colocalization of CFP and the markers by intensity correlation analysis (ICA).

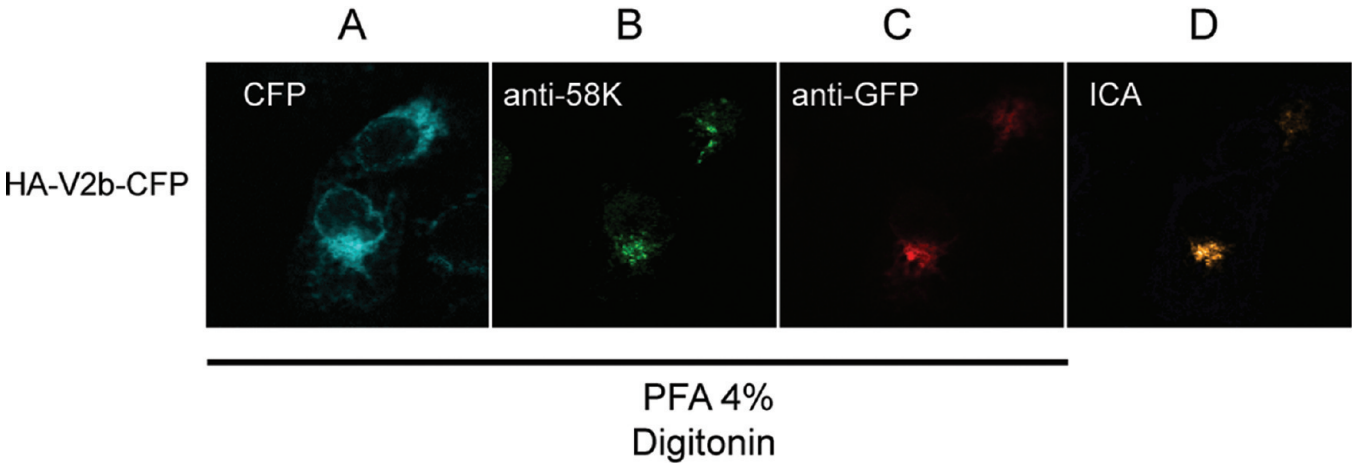


**Figure 4.** Localization of HA-V2b−CFP in the Golgi apparatus. Images of CHO cells expressing HA-V2b−CFP were fixed with 4% PFA and permeabilized with digitonin. (A) CFP fluorescence due to the expression of HA-V2b−CFP. (B) Detection of the Golgi apparatus marker 58K protein using an anti-58K antibody. (C) Staining of HA-V2b−CFP with an anti-GFP antibody. (D) Colocalization of the fluorescence signals of panels B and C by intensity correlation analysis (ICA).

the $M_r$ values of 55000 and 58000 corresponded to the TM7 and TM6 V2b variants, respectively. On this basis, we conclude that both TM6 and TM7 V2b variants oligomerize with the canonical V2a receptor.


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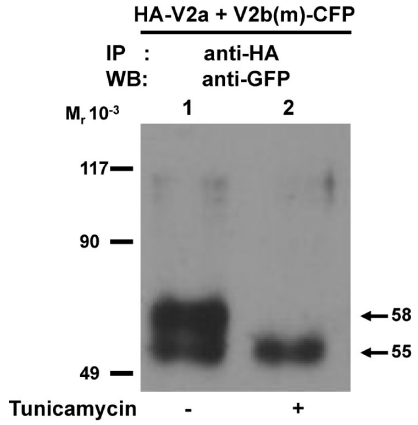


**Figure 5.** Oligomerization of V2a with both V2b topological isoforms. CHO cells coexpressing HA-V2a and V2b(m)−CFP were subjected to immunoprecipitation (IP) using an anti-HA antibody and protein A-agarose. Western blots (WB) of the immunoprecipitates were detected with anti-GFP antibody. Lanes 1 and 2 show the immunodetection of V2b(m)−CFP of the immunoprecipitate from untreated cells or cells treated with tunicamycin.

## DISCUSSION

On the basis of glycosylation assays and epitope mapping of V2b, we have demonstrated that this splice variant, in contrast to the canonical V2a receptor, is assembled into TM6 and TM7 topologies. The mechanisms underlying the generation of TM6 or TM7 are unknown. Previous studies have suggested that the assembly of membrane domains into the membrane is determined by partitioning of the protein domains between the traslocon (Sec61) and the surrounding lipid bilayer.[23,24] The efficiency of insertion of a protein domain into the membrane seems to depend on the number of hydrophobic residues and the length of the helix but also on the relative position of the amino acid within the helix.[25,26] Consequently, short hydrophobic segments or the presence of charged residues may affect the probability of the insertion. We suggest that the two topologies adopted by V2b are due to the fact that the noncanonical seventh transmembrane segment of V2b is not efficiently inserted into the membrane. Alternatively, it is possible that the seventh transmembrane segment and the C-terminus of V2a stabilize the receptor into a seven-transmembrane protein topology, whereas the noncanonical seventh transmembrane and the short C-terminus in V2b most likely destabilize the seven-transmembrane topology. This idea is supported by the analysis of the recent crystal structures of GPCRs indicating multiple interactions between residues of the seventh transmembrane helix and residues from helices 1−6,[27] and with the poor assembly of receptor chimeras derived from heterologous GPCRs,[28] which exhibit sequence diversity in their transmembrane segments, including the seventh transmembrane segment. Furthermore, helix 8 in the C-terminus of the receptor, identified in the crystal structures of GPCRs, may further stabilize the seven-transmembrane protein topology, via interaction with lipids on the cytoplasmic side of the plasma membrane. Indeed, recent studies have shown that helix 8 of GPCRs binds to the plasma membrane.[29] This is consistent with previous observations indicating that the interaction of specific lipids with membrane proteins dictates the transmembrane topology.[30]

We also demonstrated that the V2b isoform reaches the Golgi apparatus but fails to traffic to the plasma membrane, as suggested by our previous binding studies.[16] This is most likely due to the lack of trafficking motifs in the C-terminal domains, as V2a receptor mutant R337X, a truncated V2 mutant lacking the cytoplasmic C-terminus, failed to traffic to the plasma membrane.[31] Alternatively, the C-terminus could confer stability to the V2a receptor, an important requirement for trafficking to the plasma membrane.[32,33] In fact, unstable mutants of CFTR failed to traffic to the plasma membrane.[34,35]

Interestingly, both the TM6 and the TM7 V2b topological isoforms form oligomers with the V2a receptor, indicating that the first six transmembrane segments of the receptor are sufficient to drive the oligomerization between V2a and V2b, which is consistent with our findings that the truncated V2a mutant containing only transmembrane segments 1−6 is also retained inside the cell and also oligomerizes with the canonical V2a receptor (unpublished observations).

In conclusion, we have demonstrated that the V2b splice variant is folded into two alternative topological isoforms of six and seven transmembrane segments, and that the seven-transmembrane segment isoform traffics to the Golgi apparatus.

## ASSOCIATED CONTENT

**Supporting Information.** Hydrophobicity profiles for the V2 receptor isoform and seven-transmembrane topology of the V2a receptor. This material is available free of charge via the Internet at http://pubs.acs.org.

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## ABBREVIATIONS

ECFP, enhanced cyan fluorescence protein; V2a, vasopressin receptor type 2a isoform; V2b, vasopressin receptor type 2b isoform; GPCR, G-protein-coupled receptor; TM, transmembrane segment; RIPA, radio-immunoprecipitation assay; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TMHMM, transmembrane hidden Markov model; V2b(m)−CFP, CFP fused to the V2b mutant; V2a(m)−CFP, CFP fused to the V2a mutant; CHO-K1, Chinese hamster ovary; PFA, paraformaldehyde; HA, hemagglutinin antigen; TM6, six-transmembrane segments; TM7, seven-transmembrane segments; PCR, polymerase chain reaction.

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