Novel Down-regulatory Mechanism of the Surface Expression of the Vasopressin V2 Receptor by an Alternative Splice Receptor Variant*

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In rat kidney, two alternatively spliced transcripts are generated from the V2 vasopressin receptor gene. The large transcript (1.2 kb) encodes the canonical V2 receptor, whereas the small transcript encodes a splice variant displaying a distinct sequence corresponding to the putative seventh transmembrane domain and the intracellular C terminus of the V2 receptor. This work showed that the small spliced transcript is translated in the rat kidney collecting tubules. However, the protein encoded by the small transcript (here called the V2b splice variant) is retained inside the cell, in contrast to the preferential surface distribution of the V2 receptor (here called the V2a receptor). Cells expressing the V2b splice variant do not exhibit binding to 3H-labeled vasopressin. Importantly, we showed that expression of the splice variant displays a distinct sequence corresponding to the putative seventh transmembrane domain and the C-terminal domain (10). Reverse transcription PCR of RNA from isolated kidney tubules showed two transcripts, a major 1.2-kb transcript corresponding to the sequence encoding the V2a receptor and a splice variant of 1.1 kb. (Fig 1B) This variant encodes an identical amino acid sequence to the V2 receptor up to residue 303; however, the downstream sequence of the splice variant encodes a distinct amino acid sequence from the seventh transmembrane domain to the C terminus of the V2 receptor (Fig 1C). The splice variant was generated by an alternative splicing at a site 76 bp downstream of the V2 receptor splice site, resulting in a frameshift in the 3′-end coding region. The mRNA of the splice variant is expressed in the collecting tubules at ~15% of the major V2 receptor (11). In this work, we showed that the splice variant mRNA is translated into a protein (here called V2b) but is retained inside the cell. Most importantly, we showed that the V2b protein forms heterodimers.
with the wild-type V2 receptor (here called V2a) and acts as a dominant negative by sequestering V2a receptors inside the cells.

**EXPERIMENTAL PROCEDURES**

**Reagents—**AVP was purchased from Bachem (Torrance, CA), [3H]AVP came from PerkinElmer Life Sciences, and the monoclonal anti-GFP antibody was from Molecular Probes (Eugene, OR). Rat monoclonal anti-HA (3F10) and FuGENE 6 were from Roche Diagnostics, and Ultraspec RNA came from Biotec (Houston, TX). Enhanced chemiluminescence solutions and the secondary anti-rabbit peroxidase-labeled antibody were from Pierce. The reporter vectors ECFP-N1, EGF-N1, and EYFP-N1 were from BD Biosciences Clontech. Dulbecco's modified Eagle's medium, Ham's medium, penicillin G, streptomycin sulfate, and Fungizone were from Invitrogen.

The V2A deletion mutant MDCK cells stably transfected with the wild-type V2a vasopressin receptor and the splice variant were synthesized by reverse transcription PCR using rat kidney RNA along with a sense primer (5'-ggattgggtggttagctatatca-3') and an antisense primer (5'-gctctagacagctgctggagggtt-3'). Both cDNAs were cloned into the expression vector pcDNA 3. To create expression plasmids encoding fusion proteins, the wild-type V2a receptor cDNA was amplified with a sense primer (5'-ggattgggtggttagctatatca-3') and an antisense primer (5'-gctctagacagctgctggagggtt-3'); the splice variant cDNA was amplified with the same sense primer and the antisense primer 5'-gctctagacagctgctggagggtt-3'. These amplified products were subcloned in-frame to the cDNAs encoding the enhanced cyan fluorescent protein or the enhanced yellow fluorescent protein of the reporter vectors.

**Transient and Stable Transfections—**COS-1 and MDCK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. CHO-K1 cells were grown in F12 Ham's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone. CHO-K1 cells seeded in 24-well plates at 6 x 10⁴ cells/well were transiently transfected with 0.2 μg of V2a-GFP cDNA and/or V2b-GFP cDNA per well, 0.4 μg of CXC1R cDNA, 0.4 μg of CXC1R plus V2a cDNA (0.1 ratio), 0.4 μg of V2a or the V2b cDNA encoding the wild-type C-terminal peptide (HTAWVLKMNPVPQP). Bound antibodies were detected with 0.1% (w/v) 3–3'-diaminobenzidine and 0.03% (v/v) hydrogen peroxide for 5 min at room temperature. Immunofluorescence studies, cells were transfected with cDNAs encoding V2a, tagged with an HA epitope at its N terminus and V2b tagged with cyan fluorescent protein (CFP) at its C terminus. After 48 h of transfection, cells were fixed with 4% paraformaldehyde and permeabilized with cold methanol. Cells were stained with anti-HA monoclonal antibody to detect the expression of V2a. Fluorescence in the cells was analyzed in a Zeiss fluorescence microscope.

**Subcellular Fractionation—**Stably transfected MDCK cells were washed with cold phosphate-buffered saline, harvested, and disrupted with a tight fitting Dounce homogenizer in 10 mM HEPS (pH 7.5) buffer containing 0.25 μM sucrose and the protease inhibitors leupeptin and aprotonin (10 μg/ml each). The cell homogenates were centrifuged for 10 min at 6,000 x g. The post-nuclear supernatant was adjusted to 1.3 μM sucrose and overlaid on a discontinuous sucrose gradient (2 ml each of 1.2, 1.15, 0.86, and 0.25 μM sucrose) in 10 mM HEPS (pH 7.5) and centrifuged for 18 h at 24,000 rpm in a SW 40 Ti rotor. One-milliliter fractions were collected from the bottom, diluted four times with ice, and centrifuged for 45 min at 150,000 x g. Subcellular distribution of each fraction was analyzed by Western blot analysis using rabbit polyclonal anti-Na+/K+ -ATPase (Rockland Immunocochromes), anti-calnexin (Abcam, Cambridge, United Kingdom), and anti Golgi-97 (Molecular Probes) antibodies to identify plasma membrane, ER, and Golgi-enriched membranes, respectively.

**Co-immunoprecipitation—**COS-1 cells were co-transfected with two plasmids, one containing the cDNA encoding the V2a receptor tagged with GFP and the other containing the cDNA encoding the V2b splice variant tagged with HA. After 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 phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotonin (each). The homogenate was centrifuged for 10 min at 6,000 x g, and the post-nuclear supernatant was centrifuged for 45 min at 150,000 x g. The membrane pellet was solubilized in radioimmuno precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P40, 10 mM N-ethy lamides, 0.1 mM phenylmethylsulfonyl fluoride, 5 mg/ml soybean trypsin inhibitor, and 1 mg/ml leupeptin) and centrifuged for 45 min at 16,000 x g. The supernatant was incubated with a polyclonal anti-GFP (2.5 μg) and 20 μl of agarose-protein A slurry at 4°C for 16 h. The immunocomplex was washed three times with radioimmuno precipitation assay buffer and collected by centrifugation for 2 min at 1,000 x g. The immune complexes were resuspended in sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and Western blot. Co-immunoprecipitation of HAV2b was detected using a rat monoclonal anti-HA antibody and a peroxidase-labeled donkey anti-rabbit antibody (Jackson Laboratories).

**Confocal Microscopy—**Stably transfected MDCK cells were grown on coverslips and used to achieve cell polarization. After washing the cells twice with Krebs-Ringer HEPES (136 mM NaCl, 10 mM HEPES, 4.7 mM KCl, 1.25 mM CaCl2, and 1.25 mM glucose), the coverslips were mounted in a camera to examine the cells in an LSM 510 Meta confocal laser-scanning microscope (Zeiss) in the Optical Imaging Laboratory at the University of Texas Medical Branch. The cells were excited with the laser at 488 nm, and the light emitted was detected using an LP 514 filter.

**Fluorescence Resonance Energy Transfer (FRET)—**COS-1 cells seeded at 5 x 10⁴ cells per 60-mm dish were transiently co-transfected with a pair of plasmids (2 μg of each plasmid), V2a-CFP or V2b-CFP and either V2a-GFP or V2b-GFP. After 48 h, the coverslips were washed twice with Krebs-Ringer HEPES (136 mM NaCl, 10 mM HEPES, 4.7 mM KCl, 1.25 mM CaCl2, and 1.25 mM glucose) and mounted in the camera of a Leica TCS 4D confocal laser-scanning microscope. FRET was monitored by the three filter set procedure described by Gordon et al. (12). The donor filter set (D) is composed of a laser for excitation (458 nm), a main beam splitter (HFT 485/514), and secondary beam splitters (NFT 545 and NFT 515). The light emitted by the donor was detected with a 475–525-nm band-pass filter. The acceptor filter set (A) consisted of a laser for excitation (458 nm), corresponding to the absorption spectrum of the donor and the beam splitters described above. The FRET signal was recorded using an LP 530 filter.

To normalize FRET (FRETN), the background given by the images from non-transfected cells was subtracted. For each fluorescence image, the pixel values of the FRET filter set (F0) were divided by the pixel values of the donor filter set (D0) and then corrected by a factor (Ff/Df) which is a factor that relates the loss of donor emission due to FRET to the gain of acceptor emission due to FRET. This factor is determined as

\[ FRETN = \frac{Ff - Df}{Af - Fd} \times \frac{Fd}{Df} \times \frac{Af}{Ff} \]  

(1)

in which we use the two-letter symbols proposed by Gordon et al. in 1998 (12). Aa and Fd represent images obtained with the Acceptor and FRET filter sets, respectively, when only the acceptor fluorophore is present in the sample, whereas Dd and Fd represent images obtained with the Donor and FRET filter sets, respectively, with the donor fluorophore. Ff, Af, and Df represent images obtained with the FRET, Acceptor, and Donor filter sets, respectively, when both fluorophores are present. G is a factor that relates the loss of donor emission due to FRET in the Donor filter set to the gain of acceptor emission due to FRET in the FRET filter set.

**Fluorescence Microscopy—**Stably transfected CHO-K1 cells were grown on coverslips and used to achieve cell polarization. The cells were washed twice with Krebs-Ringer HEPES (136 mM NaCl, 10 mM HEPES, 4.7 mM KCl, 1.25 mM CaCl2, and 1.25 mM glucose), the coverslips were mounted in the camera of a Leica TCS 4D confocal laser-scanning microscope. FRET was monitored by the three filter set procedure described by Gordon et al. (12). The donor filter set (D) is composed of a laser for excitation (458 nm), a main beam splitter (HFT 485/514), and secondary beam splitters (NFT 570 and NFT 515). The light emitted by the acceptor was detected with a LP 530 filter. The FRET filter set (F) consisted of a laser for excitation (458 nm), corresponding to the absorption spectrum of the donor and the beam splitters described above.

The FRET signal was recorded using an LP 530 filter.

To normalize FRET (FRETN), the background given by the images from non-transfected cells was subtracted. For each fluorescence image, the pixel values of the FRET filter set (F0) were divided by the pixel values of the donor filter set (D0) and then corrected by a factor (Ff/Df) which is a factor that relates the loss of donor emission due to FRET in the Donor filter set to the gain of acceptor emission due to FRET in the FRET filter set.
Regulation of V2 Vasopressin Receptor Surface Expression

RESULTS

Subcellular Localization of V2a Receptor and the Splice Variant V2b—As described previously (11), reverse transcription PCR with total RNA from rat kidney showed co-expression of two transcripts encoding two potential V2 receptor subtypes (Fig. 1B). The large transcript of 1.2 kb encodes the classical V2 receptor (V2a), whereas the small transcript of 1.1 kb encodes the splice variant V2b. The latter displays an identical sequence to the V2a receptor up to residue 303. However, because of the frameshift, the splice variant displays a short and distinct sequence from the corresponding seventh transmembrane and C terminus domains of the wild-type V2a receptor (Fig. 1C). Here, we showed by the immunostaining of rat kidney cells with subtype-specific antibodies that V2a receptors are preferentially expressed in the plasma membrane (Fig. 2A), whereas the splice variant V2b is broadly distributed in the cell (Fig. 2B). Interestingly, MDCK cells expressing the V2b receptor-GFP fusion protein exhibited significant fluorescence in both the perinuclear region and the plasma membrane (Fig. 3A). In contrast, cells expressing the splice variant V2b-GFP fusion protein revealed fluorescence only in the cytoplasmic region without any significant labeling in the plasma membrane (Fig. 3B). Consistent with these findings, CHO cells expressing V2a receptors exhibited cell surface [3H]AVP high affinity binding, whereas cells expressing the splice variant V2b displayed negligible high affinity binding to [3H]AVP (Fig. 3C). This finding is in good agreement with the intense fluorescence displayed by permeabilized cells expressing the V2b splice variant tagged with HA at its N terminus (Fig 4A), which is in contrast to the negligible fluorescence displayed by the corresponding non-permeabilized cells (Fig 4B). As expected, non-permeabilized cells expressing the HA-tagged V2a receptor showed intense fluorescence (Fig. 4C). This result indicates that the splice variant is retained inside the cells. To identify the location of the splice variant inside the cell, we performed subcellular fractionation. The V2b splice variant preferentially localizes in the ER/Golgi enriched fractions, as monitored with the specific markers for ER (calnexin) and Golgi (Golgin-97). On the other hand, V2a localized in all the fractions, including the plasma membrane-enriched fraction, as monitored by the ligand binding plug-in. Binding is reported as the average of triplicates. Each experiment was performed at least three times. For binding in crude membranes, transfected cells were disrupted with a tight fitting Dounce homogenizer in 250 mM Tris- HCl buffer containing 5 mM MgCl2 and 500 mM EDTA. The homogenate was centrifuged for 3 min at 37°C with 4 µM [3H]AVP in 50 mM Tris-HCl buffer containing 5 mM MgCl2 and 1 mg/ml bovine serum albumin in a 250-µl final volume. The reaction was terminated by rapid filtration through a Whatman GF/C glass fiber filter.

Fig. 2. Subcellular localization of the V2 splice variant in rat kidney. A, immunostaining of a rat kidney section with the anti-V2a receptor antibody raised against a peptide corresponding to the C-terminal domain of the receptor. Plasma membranes of collecting tubule cells are preferentially stained with anti-V2a receptor antibodies. B, immunostaining of a rat kidney section with an anti-V2 splice variant antibody raised against a peptide corresponding to C-terminal domain of V2a. Collecting tubule cells are broadly stained with anti-V2b splice variant antibodies.

FIG. 1. Expression of the V2 vasopressin receptor and its splice variant mRNA. A, structure of the V2 vasopressin receptor gene. The V2a splice variant is created by splicing at a site 76 bp downstream of the splice site that produces the V2a receptor mRNA. B, reverse transcription PCR from total RNA from rat kidney showing the two transcripts. Left lane, molecular markers on a 100-bp ladder; right lane, 1.1 and 1.2 kb. C, comparison of partial amino acid sequence between the V2a receptor and V2b splice variant; the divergent sequence is shown in boldface. The putative transmembrane domains VI and VII are underlined.
and V2b-YFP). We found FRET signals in cells expressing the V2a-CFP/V2a-YFP and V2a-CFP/V2b-YFP pairs as displayed in pseudo color mode intensity, where white areas indicate high values of FRET and black areas indicate low values of FRET. In agreement with previous studies, V2a receptors form homodimers inside the cell (Fig. 7A). Interestingly, the V2a receptor and the splice variant form heterodimers inside the cell (Fig. 7B). Only a low FRET signal was detected in cells expressing the unfused (control) fluorescence proteins CFP and YFP (Fig. 7C), indicating little interaction between these proteins. Quantitative fluorescence analysis showed that cells expressing V2a-CFP/

V2b-YFP and V2a-CFP/V2b-YFP pairs had a ~6-fold higher FRET signal than cells expressing CFP and YFP (Fig. 7D). We also demonstrated by co-immunoprecipitation that the V2a receptor and the V2b splice isoform oligomerize. As shown in Fig. 8, a HA-tagged V2b splice variant can be immunoprecipitated with anti-GFP antibodies from extracts of cells expressing both the splice variant and the GFP-tagged V2a receptor.

**Splice Variant V2b Down-regulates the Surface Expression of the V2a Receptor**—Because the splice variant V2b forms heterodimers with V2a receptors inside the cell, we investigated whether this splice variant regulates the trafficking of V2a receptors. The surface expression of V2a receptors was monitored by measuring [3H]AVP binding in CHO cells transfected with cDNAs encoding the V2a receptor and the splice variant V2b. We found that the surface expression of V2a receptors was abrogated by increased expression of the splice variant V2b (Fig. 9). Interestingly, the total cell expression of the V2a receptors was unaffected by the expression of the splice variant as demonstrated by Western blotting of the V2a-GFP fusion

**FIG. 3.** Subcellular distribution of the V2a receptor and the V2b splice variant and [3H]AVP binding in transfected cells. A, polarized MDCK cells expressing the GFP-tagged V2a receptor. The V2a-GFP fusion protein is localized preferentially in the plasma membrane and perinuclear region. B, polarized MDCK cells stably expressing the GFP-tagged V2b splice variant. The V2b-GFP fusion protein is diffusely distributed in the cell. C, surface binding of [3H]AVP to CHO cells expressing either V2a receptors (●) or the V2b splice variant (○). Binding assays were carried out three times, each time in triplicate; the figure shows a representative experiment.

**FIG. 4.** Expression of the V2a splice variant inside the cell. HAV2aCFP (A and B) HAV2aCFP (C) were probed with an anti-HA antibody in permeabilized (A) and intact (B and C) transfected CHO cells. The expression of the isoforms in COS-1 cells was independently monitored by CFP fluorescence (data not shown).

**FIG. 5.** The V2b splice variant is retained in the ER/Golgi compartments. MDCK cells stably expressing the V2a, or the V2b isoforms were lysed and the post-nuclear supernatant fractionated on a discontinuous sucrose gradient. The GFP-tagged V2a and V2b receptors were localized by Western blot using an anti-GFP antibody. Subfractions enriched of ER, Golgi, and plasma membranes were identified using antibodies against calnexin, Golgin-97, and Na+/K+-ATPase, respectively.

**FIG. 6.** The V2b splice variant does not display high affinity binding to [3H]AVP. Binding of [3H]AVP to homogenates from CHO cells expressing either V2a receptors (●) or V2b splice variant (○). Binding assays were carried out three times; this result is from a representative experiment.
protein using anti-GFP antibodies (Fig. 9, inset). Further analysis of the binding data indicates that the dissociation constant (K_d) of the binding of [3H]AVP to the V_2a receptor was unaffected by the expression of the splice variant; however, B_max decreased by almost 3-fold (Fig 10A). These findings indicate that the splice variant V_2b down-regulates the surface expression of the V_2a receptor, which is retained inside the cells, probably as V_2a-V_2b receptor heterodimers. We also tested whether the receptor C-terminal domain also regulates V_2 receptor trafficking, as that domain rescued V_2 receptor mutants (14, 15). We found that the sequence encompassing residues 242–339 of the splice variant V_2b down-regulates the surface expression of the V_2a receptor.
expression of the V2a receptor (Fig. 10B), but not with the corresponding sequence 242–371 of the V2a receptor (Fig. 10C). These results suggest that the region comprising the sixth transmembrane domain is part of the dimer interface, whereas the seventh transmembrane domain and the C terminus contain the sorting motifs for translocation of the receptor to the plasma membrane. We also demonstrate that the down-regulation of the V2a receptor by the splice variant is specific, as the surface expression of the G protein-coupled receptor CXCR1 (interleukin-8 receptor A, a chemokine receptor) was unaffected on CHO cells co-transfected with cDNAs encoding CXCR1 and the splice variant V2b (Fig. 10D).

DISCUSSION

In contrast to the preferential distribution of the V2a receptor to the cell surface, we demonstrate that the translated splice variant V2b transcript is retained in ER/Golgi compartments, as shown by subcellular fractionation analysis. Although the V2b splice variant is normally expressed in the kidney, its functional significance does not appear to be related to its signaling or binding to AVP, as this splice variant did not exhibit high affinity binding to [3H]AVP. However, we found that the expression of the splice variant down-regulates the surface expression of the V2a receptor. V2a receptors form both homodimers and heterodimers with V2b as demonstrated by FRET experiments and co-immunoprecipitation studies between V2a receptor and the V2b splice variant. These findings are consistent with the view that the V2b splice variant down-regulates the surface expression of the V2a receptor by forming V2a/V2b heterodimers, which are then retained inside the cells. The splice variant V2b would be then acting as a dominant negative. The effect of V2b is specific, as it does not affect the surface expression of CXCR1. Furthermore, the sequence encompassing residues 242–339 of the V2b receptor mimics the down-regulation of the V2a receptor by the full-length splice variant, suggesting that the sixth transmembrane segment of these receptors is part of the dimerization interface. Similar effects have been reported in other G protein-coupled receptors, e.g. a splice variant of the calcitonin receptor lacking 14-residues of the seventh transmembrane segment prevented the surface expression of the wild-type receptor (16). The precise mechanism underlying the retention of the V2a receptor by the splice variant V2b is unknown. On the basis of current information about the folding and trafficking of proteins, we reasoned that the V2a/V2b heterodimer complex may be improperly folded to be processed and transported to the plasma membrane. Indeed, misfolded mutants of V2 receptors that cause nephrogenic diabetes insipidus accumulate inside the cells but can be rescued by non-peptide antagonists of the V2 receptor (17). It is argued that the binding of the V2 receptor antagonist stabilizes the folded conformation of the receptor and primes it for processing and transport to the cell surface. Also, misfolded rhodopsin mutants causing retinal degeneration interfere with the processing and transport of the wild-type rhodopsin (18). The best-studied system is the improperly folded ΔF508 mutant of the cystic fibrosis transmembrane conductance regula-

![Fig. 10. The V2b splice variant and the C-terminal partial sequence of V2a reduced specifically the number of [3H]AVP binding sites of CHO cells expressing the V2a receptor. A, saturation [3H]AVP binding to CHO cells expressing V2a receptors (●) or V2a receptors plus the V2b splice variant (○). B, [3H]AVP binding to CHO cells expressing the V2a receptor (●), the V2a receptor plus the V2b splice variant (○), or the V2a receptor and a V2a 242 tail (■). C, [3H]AVP binding to CHO cells expressing the V2a receptor (●) or the V2a receptor and a V2a 242 tail (■). D, [125I] interleukin-8 binding to CHO cells expressing a V2a splice variant (●), an interleukin-8 receptor (CXCR1; ■), or CXCR1 plus the V2b splice variant (▼).]
tor, which is retained inside the cell (19). However this misfolded mutant can be rescued by organic solutes that stabilize its folded conformation (20). All of these disorders belong to a group of misfolding diseases that include Alzheimer’s disease, prion encephalopathies, Parkinson’s disease, and some cancers (21–26). This model of retention of the misfolded receptor complex inside the cell would be consistent with the down-regulation of the wild-type V2a receptor by a misfolded splice variant V2b (27–29). We suggest that the fragment encompassing residues 242–339 of the splice variant is misfolded, as expression of this fragment is sufficient to down-regulate the surface expression of the V2a receptor. Our findings support the idea that dimerization of V2a receptors inside the cell stabilizes the folded structure of the receptor for processing and transport to the plasma membrane, whereas heterodimerization of the splice variant V2b with the wild-type V2a receptor gives rise to misfolded V2aV2b complex, which is retained inside the cell. It is possible that some forms of nephrogenic diabetes insipidus may be due to the overexpression of the splice variant V2b, which retains the wild-type V2a receptor inside the cell via the formation of V2aV2b heterodimers.

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