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Sorting Functions of the Individual Cytoplasmic Domains of the G Protein-Coupled Vasopressin V_2 Receptor in Madin Darby Canine Kidney Epithelial Cells

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ABSTRACT

Previous studies have shown that the G protein-coupled human vasopressin V_2 receptor (V_2 receptor) is expressed predominantly in the basolateral membrane of Madin Darby canine kidney type II (MDCKII) epithelial cells at steady state. Here we have assessed the influence of the individual cytoplasmic domains of the V_2 receptor on polarized sorting in MDCKII cells. The second (ICL2) and third (ICL3) intracellular loops and the C-terminal tail were fused separately to a green fluorescent protein-tagged receptor fragment comprising the first transmembrane domain and flanking regions. We show that the ICL2 domain of the V_2 receptor alone promotes basolateral cell surface expression and thus seems to contain the basolateral sorting signal of the V_2 receptor. Fusion of the other cytoplasmic domains, however, does not lead to a randomized cell surface expression. The C-terminal tail of the V_2 receptor promotes apical targeting. Fusion of ICL3 leads to a receptor fragment that is retained in the endoplasmic reticulum (ER). The results are consistent with a model in which the V_2 receptor contains signals for both apical and basolateral cell surface expression, the latter being dominant. Furthermore, ICL3 may contain a retinoid X receptor ER retention signal, which is not accessible in the correctly folded full-length receptor but which is unmasked when ICL3 is fused alone.

The human V_2 receptor belongs to the superfamily of the G protein-coupled receptors (GPCRs) and is expressed in the principal epithelial cells of the renal collecting duct (Klussmann et al., 2000). These cells have two different plasma membrane compartments: the apical, which is in contact with the urine, and the basolateral, which is accessible to the ligand in the circulation. Activation of the V_2 receptor in the basolateral membrane leads to stimulation of the G_s/adenylyl cyclase system. The subsequent rise in intracellular cAMP induces the fusion of vesicles containing water channels (aquaporin 2) with the apical membrane, which is thus rendered water permeable. Consequently, water is reabsorbed from the lumen of the collecting duct. One of the striking features of this cascade is its polarity (i.e., the signal must be transduced from the basolateral to the apical side of the epithelial cells). The function of such a polarized signal transduction is thus dependent on the transport of the receptor to its "correct" cell surface compartment.

A growing number of the heptahelical GPCRs have been shown to sort to either the basolateral or apical membrane in epithelial cells. Stably transfected MDCKII cells grown on permeable polycarbonate filter supports have become the standard cell system in these studies. Steady-state basolateral expression was demonstrated for the α_{2A}- (Keefer and Limbird, 1993), α_{2B}-, and α_{2C}-adrenergic receptors (Wozniak and Limbird, 1996). A similar basolateral expression was observed for the receptors of the luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone (Beau et al., 1997), and $P2Y_{11}$ purinergic receptors (Zambon et al., 2001). Apical expression was demonstrated for the A_1 adenosine receptor (Saunders et al., 1996) and the light receptor rhodopsin (Chuang and Sung, 1998). Transport signals that determine polarized cell surface expression in epithelial cells have been characterized in the cytoplasmic domains of unrelated membrane proteins. In most cases, these signals were either of the tyrosine type, such as that described originally for the low density lipoprotein receptor (Matter et al., 1992), or the dileucine type, as found in the IgG Fc receptor (Hunziker and Fumey, 1994). For the heptahelical GPCRs, these signals are less well defined, but it was shown for the follicle-

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ABBREVIATIONS: V_2 receptor, human vasopressin V_2 receptor; GPCR, G protein-coupled receptor; MDCKII, Madin Darby canine kidney cells type II; ICL, intracellular loop; GFP, green fluorescent protein; RXR, retinoid X receptor; PBS-CM, phosphate-buffered saline with calcium and magnesium; PAGE, polyacrylamide gel electrophoresis; EndoH, endoglycosidase H; PNGaseF, peptide *N*-glycosidase F; LSM, confocal laser scanning microscopy; ER, endoplasmic reticulum; NDI, nephrogenic diabetes insipidus; PCR, polymerase chain reaction.

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stimulating hormone receptor that a tyrosine and a leucine residue in its intracellular C terminus contribute to a motif that directs basolateral sorting (Beau et al., 1998). In the case of the α_{2A}-adrenergic receptor, the large ICL3 domain stabilizes the receptor in the basolateral membrane, and it was proposed that basolateral localization in this instance is mediated by the actual length of the ICL3 domain rather than by a defined transport signal (Edwards and Limbird, 1999).

Immunocytochemical studies of the V_2 receptor in rat kidneys (Nonoguchi et al., 1995) as well as studies with recombinant GFP- (Schülein et al., 1998b) and *C-myc*-tagged receptors in MDCKII epithelial cells (Andersen-Beckh et al., 1999) verified its predominantly basolateral localization, which was expected, considering the direction of signal transduction in epithelial cells. Minor amounts of receptor, however, were also detected apically (Schülein et al., 1998b; Andersen-Beckh et al., 1999).

To clarify which of the cytoplasmic domains of the V_2 receptor is responsible for basolateral transport, we have assessed the sorting functions of the individual cytoplasmic receptor domains in MDCKII epithelial cells. Using fusions to a GFP-tagged receptor fragment (comprising the N terminus, first transmembrane domain, and part of ICL1; 71 residues), we show that ICL2 alone mediates basolateral sorting. In contrast, the C-terminal tail directs apical targeting, whereas the receptor fragment containing ICL3 is transport-defective and is retained via a RXR retention motif in the ER.

Experimental Procedures

Materials. Sulfo-NHS-Biotin and immobilized NeutrAvidin were from Pierce Chemical (Rockford, IL). Benzamidine, phenylmethylsulfonyl fluoride, Trasylol and Triton X-100 were from Sigma (Munich, Germany). Permeable polycarbonate filter supports (24-mm diameter) were from Costar (no. 3412; Bodenheim, Germany), Type IV collagen from BD Biosciences (Erembodegem, Belgium), and Lipofectin from Invitrogen (Karlsruhe, Germany). DNA modifying enzymes, EndoH and PNGaseF were from New England Biolabs (Schwalbach, Germany). All other reagents were from Merck (Darmstadt, Germany). Vector plasmid pEGFP-N1, encoding the red-shifted variant of GFP, was purchased from CLONTECH Laboratories (Heidelberg, Germany). The polyclonal peptide-derived anti-GFP antibodies were described previously (Krause et al., 2000). Alkaline phosphatase antirabbit IgG was from Dianova (Braunschweig, Germany). MDCKII epithelial cells were a gift from K. Simons (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany).

DNA Manipulations. Standard DNA manipulations were carried out according to the handbooks of Sambrook et al. (1989). Nucleotide sequences were verified using the FS Dye Terminator kit from PerkinElmer (Weiterstadt, Germany). Site-directed mutagenesis was carried out with the QuikChange site-directed mutagenesis kit from Stratagene (Heidelberg, Germany).

Construction of GFP-Tagged V_2 Receptor Fragments Containing Different Cytoplasmic Domains. The plasmid pWT.GFP, encoding a C-terminal GFP fusion to residue K367 of the V_2 receptor, was described previously (Schülein et al., 1998a). Plasmid pEU71.PhoA, encoding an alkaline phosphatase fusion to residue W71 of the V_2 receptor (i.e., to a fragment consisting of the N terminus, first transmembrane domain and ICL1; Schülein et al., 1996b), was the starting plasmid for the construction of the corresponding GFP fusions. The receptor portion was cloned as a *Sac*I/*Bam*HI fragment into the vector pEGFP-N1, yielding plasmid p71.GFP. Plasmid p71CT/WT.GFP was described previously (Krause et al., 2000). Here, the C-terminal tail of the V_2 receptor is inserted between the first cytoplasmic loop and the GFP moiety of p71.GFP (see Fig. 1 for the fused sequence). To introduce the second intracellular loop (ICL2) of the V_2 receptor between ICL1 and the GFP moiety in p71.GFP, the coding sequence of ICL2 was PCR-amplified (5′ primer, 5′ CTACATGATCCTGGATCCGACGCTGGACCGCC 3′; 3′ primer, 5′ GCCCAAGCCACTGGATCCGGCCGGTTC 3′). The PCR primers introduced novel *Bam*HI sites into the flanking regions of ICL2 at positions 393 and 477 of the V_2 receptor cDNA. The PCR fragment was cut with *Bam*HI and cloned into the *Bam*HI-cut p71.GFP, yielding plasmid p71/ICL2.GFP. The same cloning strategy was used to introduce the third intracellular loop (ICL3) between ICL1 and the GFP moiety of p71.GFP, but the *Bam*HI sites were introduced at positions 675 and 813 of the V_2 receptor cDNA (5′ primer, 5′ CGCCGCCTGCCAGGATCCCATCTTCCGGG 3′; 3′ primer, 5′ CCACAATCACTAGCGGATCCCTCACAGTCTTGG 3′). The resulting plasmid was designated p71/ICL3.GFP.

The Y148A mutation was introduced into plasmids pWT.GFP and p71/ICL2.GFP using the QuikChange Mutagenesis kit yielding plasmids pY148A.GFP and p71/ICL2/Y148A.GFP, respectively. A primer with the sequence 5′ CCATGCTGG-CGGCCCGGCATGGAAGTGG 3′ and its complementary equivalent were used. The R247-R252K mutation was introduced into plasmids pWT.GFP and p71/ICL3.GFP using the same technique, yielding plasmids pR247-R252K.GFP and p71/ICL3/R247-R252K.GFP. A primer with the sequence 5′ GAGAGGCCTGGGGGGAAGAAGAAGGGAAAGAAGACAGGCAGCCCCGGTGAG 3′ and its complementary equivalent were used.

Cell Culture and Transfection. Lipofectin transfection of MDCKII epithelial cells and selection of stable cell lines were performed as described previously for L^{tk-} cells (Schülein et al., 1996a). Stable cell clones expressing the GFP fusions were identified using an inverted fluorescence microscope. Total cell lysates of the stably transfected clones were assessed fluorometrically, and clones with similar GFP fluorescence intensities were selected for the study.

Cell Surface Biotinylation Assay. To specifically label apical and basolateral membrane proteins, the method described by Okusa et al. (1997) was slightly modified. MDCKII cells (2×10^6) stably expressing the GFP-tagged V_2 receptor constructs (WT.GFP, 71.GFP, 71/ICL2.GFP, 71/ICL3.GFP, 71/CT.GFP) were plated on

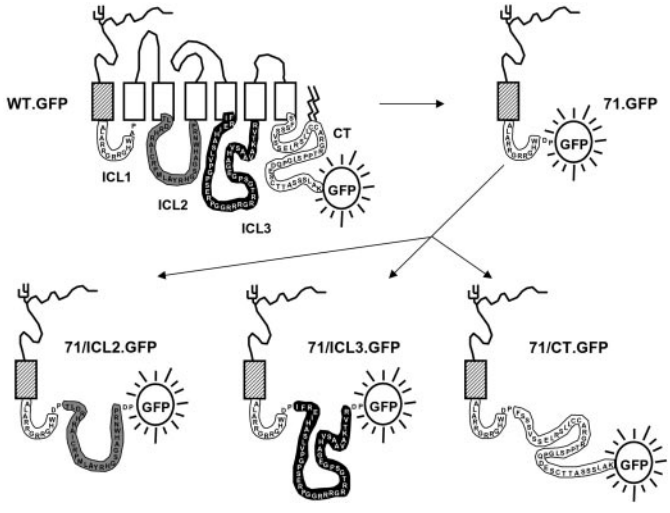

Fig. 1. Construction of GFP-tagged V_2 receptor fragments containing different cytoplasmic receptor domains. ICL2, ICL3, and the C-terminal tail were fused to a V_2 receptor fragment (71 residues) consisting of the N terminus, first transmembrane domain of ICL1. The resulting receptor fragments were designated 71/ICL2.GFP, 71/ICL3.GFP, and 71/CT.GFP, respectively. All receptor fragments were tagged at the C terminus with GFP to allow their localization in living cells. The amino acid sequences of the cytoplasmic domains are indicated in the single letter code. The *N*-glycosylation site in the N terminus of all receptor fragments is indicated.

24-mm diameter polycarbonate filter supports and grown for 5 days to allow formation of a tight epithelial monolayer. Cells were washed twice with ice-cold PBS-CM buffer (PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). Apical or basolateral membrane proteins were labeled with a solution of 0.5 mg/ml Sulfo-NHS-Biotin in PBS-CM buffer for 30 min at 4°C. Epithelial integrity was assessed as described previously by the direct determination of biotin leakage to the opposite side using an enzyme-linked immunosorbent assay (filters were only used if the biotin leakage was less than 0.5%) (Schülein et al., 1998b). Labeling reactions were quenched by replacing the biotin solution with 1 ml of NH_4Cl solution (50 mM in PBS-CM). After a 10-min incubation, the cells were washed 3 times with ice-cold PBS-CM, and filters were excised and transferred into a reaction tube. Labeled proteins were extracted for 1 h with 1 ml of ice-cold buffer (1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM Na-EDTA, 40 mM phenylmethylsulphonyl fluoride, 1 μg/ml Trasylol, and 100 mM benzamidine, pH 8.0). Insoluble debris was removed by centrifugation (20 min, 4°C, 47,000*g*), and biotinylated proteins were recovered from the supernatant by 1.5 h incubation at 4°C with NeutrAvidin-agarose beads. Beads were sedimented (3 min, 4°C, 17,000*g*), washed twice with buffer (0.5% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 50 mM NaCl, and 1 mM Na-EDTA, pH 7.4) and once with NaCl free buffer (0.5% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, 1 mM Na-EDTA, pH 7.4). Proteins were solubilized in 50 μl of Laemmli buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8), separated by SDS-PAGE (10% acrylamide), and blotted onto nitrocellulose as described previously (Khyse-Andersen, 1984). Biotinylated proteins were detected with a polyclonal anti-GFP antiserum and alkaline phosphatase-conjugated antirabbit IgG. The method was essentially the same as described previously for monoclonal anti-GFP antibodies and alkaline phosphatase-conjugated antimouse IgG (Schülein et al., 1998a).

Isolation of Crude Membrane Fractions from Stably Transfected MDCKII Cells Containing GFP-Tagged V_2 Receptor Fragments and EndoH/PNGaseF Treatment. Crude membranes of stably transfected MDCKII cells were isolated from confluent cells grown on a 60-mm diameter dish as described previously for COS.M6 cells (Schülein et al., 1996b). Membranes (60 μg of total protein) were incubated with or without EndoH or PNGaseF according to the supplier's recommendations. For the detection of the GFP-tagged receptor fragments, proteins were separated by SDS-PAGE (10% acrylamide) and blotted onto nitrocellulose as described previously (Khyse-Andersen, 1984). Receptor fragments were detected with polyclonal anti-GFP antibodies and alkaline phosphatase-conjugated antirabbit IgG as described previously (Schülein et al., 1998a).

Localization of GFP Fusion Proteins by Confocal Laser Scanning Microscopy (LSM). To visualize GFP fusions in stably transfected MDCKII cells grown on permeable filter supports, 2×10^6 cells were spread on Type IV collagen coated filters and grown for 3 days to allow the formation of epithelial monolayers. Filters were washed three times with PBS, excised, and transferred cell side-down to a slide. A drop of PBS was added, and filters were covered with a coverslip. GFP fluorescence was visualized with a inverted laser scanning microscope (l_{exc} = 488 nm, l_{em} = >515 nm, Zeiss 410, Zeiss, Göttingen, Germany). Horizontal *xy*- and vertical *z*-scans were recorded.

Results

Construction of GFP-Tagged V_2 Receptor Fragments Containing Individual Cytoplasmic Domains. The sorting functions of the cytoplasmic domains of the V_2 receptor in polarized epithelial cells are difficult to assess using full-length receptor chimeras, because exchange of the cytoplasmic domains easily causes folding defects. For example, replacement of the intracellular C terminus of the V_2 receptor by that of the β_2 adrenergic receptor led to nonfunctional receptors, which were retained in the ER (Oksche et al., 1998). The use of full-length receptors for the identification of transport signals may also be disadvantageous if more than one signal is present because signal superimposition may occur. We therefore decided to assess the sorting functions of the cytoplasmic domains of the V_2 receptor with an experimental system that allows transport studies that are independent both of the other cytoplasmic domains and of full-length receptor folding. ICL2 and ICL3 were fused separately to a V_2 receptor fragment (71 residues) consisting of the N terminus, first transmembrane domain, and ICL1 (71.GFP; Fig. 1). The resulting constructs were designated 71/ICL2.GFP and 71/ICL3.GFP. The short ICL1 domain of the V_2 receptor, which contains an excess of positively charged residues, was retained throughout to ensure correct and comparable membrane orientations according to the "charge difference" rule (Hartmann et al., 1989). This rule postulates that the orientation of a membrane protein in the ER membrane is mediated by the charge difference between the sequences flanking a transmembrane domain and that a positive charge difference determines a cytoplasmic domain. The construction of an equivalent receptor fragment containing the C-terminal tail (71/CT.GFP) and of a GFP fusion to the V_2 receptor consisting of 367 residues (i.e., to the entire receptor lacking only the four C-terminal residues) (WT.GFP) were described previously (Schülein et al., 1998a; Krause et al., 2000).

The ICL2 Domain and the C-Terminal Tail of the V_2 Receptor Mediate Basolateral and Apical Sorting, Respectively, in MDCKII Cells. To assess the polarized sorting functions of the different cytoplasmic domains, stably transfected MDCKII cell clones expressing the GFP-tagged receptor fragments were grown on polycarbonate filter supports, and the GFP fluorescence signals in the cells were localized by LSM (Fig. 2). The wild-type C-terminally GFP-tagged V_2 receptor (WT.GFP) was used as a control. The results described previously (Schülein et al., 1998b) for WT.GFP were confirmed. The *xy*-scans revealed a honeycomb pattern, indicative of a basolateral cell surface localization. The *z*-scans demonstrated that the bulk of the receptor is confined to the lateral subdomains within the basolateral compartment; only minor amounts are located apically. For the receptor fragment 71.GFP, a substantial part of the GFP fluorescence signals was detected in the cell's interior, indicating a partial transport defect of this fusion. The weaker cell surface signals together with the intracellular signals prevent resolution by LSM of whether this fusion is sorted apically or basolaterally [a nonpolarized expression, however, was demonstrated by selective cell surface biotinylation (see below)]. The additional fusion of ICL2 (71/ICL2.GFP) led to receptor fragments with a similar localization to that of the wild type (i.e., a honeycomb pattern of fluorescence signals in *xy*-scans and a lateral location in *z*-scans). In contrast, the receptor fragment containing the C-terminal tail (71/CT.GFP) was mainly located apically, as evidenced from the *z*-scans. The *xy*-scans consistently revealed a less sharp and weaker honeycomb pattern, superimposed by extensive GFP fluorescence signals from those cells scanned horizontally at the level of their apical membranes. Scans of cells expressing the receptor fragment containing ICL3 (71/ICL3.GFP) revealed diffuse GFP fluorescence signals filling the cells inte-

rior, although not the nucleus, demonstrating a complete transport defect of this fragment. These results show that the individual cytoplasmic domains of the V_2 receptor mediate differently polarized sorting in MDCKII cells. Furthermore, they indicate that the basolateral transport signal of the V_2 receptor lies within its ICL2 domain.

To confirm the results of the LSM localization study, biotin targeting assays were performed with MDCKII cells stably transfected with the GFP-tagged V_2 receptor constructs. Cells were grown on permeable filter supports, and the basolateral or apical membrane proteins were labeled with biotin. Tightness of the epithelial monolayer was verified by the direct quantitation of biotin leakage to the opposite side of the filters. Biotinylated proteins were precipitated with NeutrAvidin beads, and the receptor fragments were detected on immunoblots in the apically or basolaterally labeled samples using anti-GFP antibodies (Fig. 3). In all samples, an immunoreactive protein band with an apparent molecular mass of 68 kDa was detected (marked by ∗). This protein is unrelated to the fusion proteins because it was also detected in the membranes of untransfected MDCKII cells. For the WT.GFP, a 75- to 85-kDa immunoreactive protein band (marked by «) representing the complex-glycosylated fusion protein was detected predominantly in the basolateral sample (see Fig. 5 below for the verification of complex glycosylation of the fusion proteins). Minor amounts were detected apically as described previously (Schülein et al., 1998b). In the case of the receptor fragment 71.GFP, only small amounts of the complex-glycosylated forms (48–52 kDa; marked by «) were detectable by cell surface biotinylation, consistent with the partial transport defect of this fusion. The protein bands were present in roughly equivalent amounts in the apical and basolateral samples demonstrating that no signal for polarized sorting is present in 71.GFP. The strong signals obtained for 71/ICL2.GFP and 71/CT.GFP demonstrate that these two receptor fragments are efficiently transported to the cell surface. The complex-glycosylated 50- to 55-kDa form of receptor fragment 71/ICL2.GFP (marked by «) was found mainly in the basolateral sample, whereas the complex-glycosylated 55- to 60-kDa form of receptor fragment 71/CT.GFP (marked by «) was detected apically. For receptor fragment 71/ICL3.GFP, no specific immunoreactive protein band was detectable, consistent with its complete intracellular retention. The results of the biotin targeting assays were thus entirely consistent with those from the LSM localization study.

Residue Y148 of the ICL2 Domain is Not Involved in Basolateral Sorting of the V_2 Receptor. Tyrosine-based motifs were previously shown to determine the basolateral localization of various membrane proteins (Matter et al., 1992). The ICL2 domain of the V_2 receptor contains a tyrosine residue at position 148 (Y148), the only tyrosine residue throughout the cytoplasmic domains. To address the question of whether this residue might contribute to a basolateral sorting signal of the V_2 receptor, alanine substitutions were constructed for both the full-length GFP-tagged receptor (WT.GFP) and for the receptor fragment 71/ICL2.GFP (mutants Y148A.GFP and 71/ICL2/Y148A.GFP, respectively). Stably transfected MDCKII cell clones expressing the GFP fusions were grown on polycarbonate filter supports, and the GFP fluorescence signals were localized by LSM (Fig. 4). For both the wild-type and mutant full-length V_2 receptors, the *z*-scans revealed a predominantly basolateral localization of the GFP fluorescence signals. The same was true for the wild-type and mutant receptor fragments. These results demonstrate that Y148 is not part of a basolateral sorting motif in the ICL2 domain of the V_2 receptor.

The ICL3 Domain of the V_2 Receptor Mediates ER Retention. The receptor fragment 71/ICL3.GFP is retained within the cell. The diffusely distributed GFP fluorescence signals obtained for this fusion (see Fig. 2) point to the ER as the retention site. To verify this assumption, glycosylation state analyses were performed with crude membranes of stably transfected MDCKII cells. EndoH was used to remove high-mannose glycosylations from ER forms of the receptor fragments and PNGaseF to remove all *N*-glycosylations (i.e.,

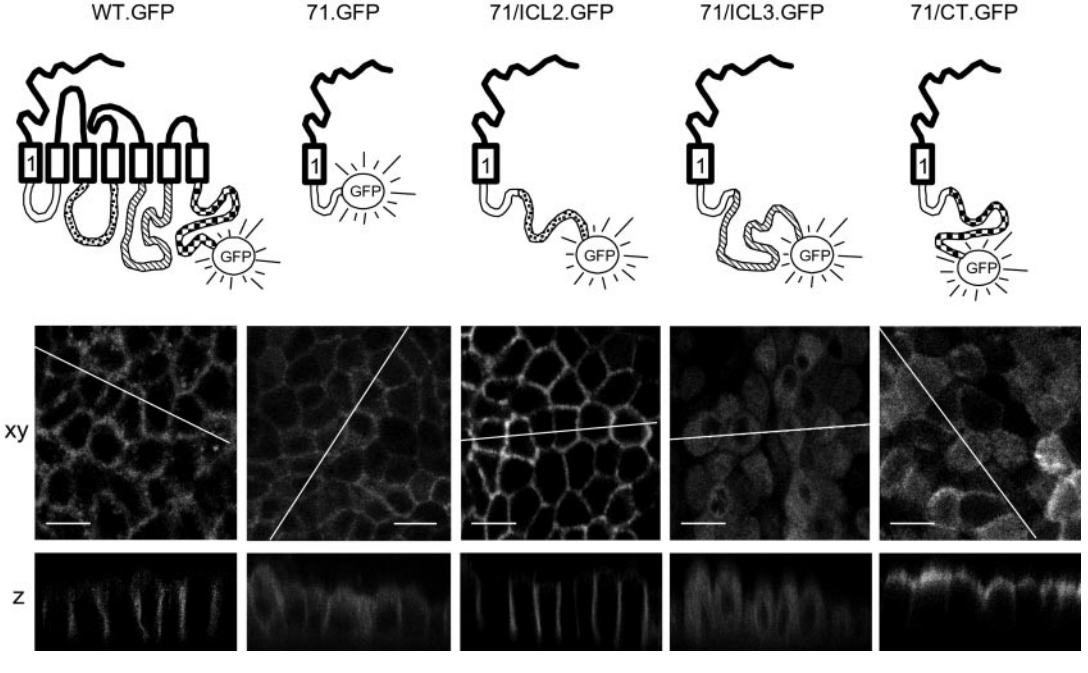

Fig. 2. Localization of GFP-tagged V_2 receptor fragments containing different cytoplasmic domains in stably transfected MDCKII cells. Cells were grown to confluence on permeable filter supports. The GFP fluorescence signals of receptor fragments (71.GFP, 71/ICL2.GFP, 71/ICL3.GFP, and 71/CT.GFP) and the intact V_2 receptor (WT-.GFP) were analyzed in living cells by confocal laser scanning microscopy with horizontal *xy*-scans (top) and with vertical *z*-scans (bottom) along the indicated lines. The apical membranes lie uppermost in the *z*-scans. Each photograph shows a representative scan ($n \geq 10$). Scale bar, 10 μm.

high-mannose and complex glycosylations from ER and post ER forms, respectively). Receptor fragments 71.GFP, 71/ICL2.GFP, and 71/CT.GFP were also analyzed to prove that the protein bands identified by the biotin targeting assay (see Fig. 3) did indeed represent complex-glycosylated forms. WT.GFP was used as a positive control for complex glycosylations. All fusions were analyzed in Western blot experiments using polyclonal anti-GFP antibodies (Fig. 5). In the case of WT.GFP, the complex (75–85 kDa; marked by ∗) and high-mannose (60–65 kDa marked by «) glycosylated forms previously described for human embryonic kidney 293 cells (Krause et al., 2000) were detected in the untreated membranes.

In the untreated membranes containing the transport-competent receptor fragments 71.GFP, 71/ICL2.GFP, and 71/CT.GFP, three groups of immunoreactive protein bands were detected:

1. Broad bands (marked by ∗) with apparent molecular masses of 48 to 52 kDa (71.GFP), 50 to 55 kDa (71/ICL2.GFP), and 55 to 60 kDa (71/CT.GFP). These protein bands were resistant to EndoH but sensitive to PNGaseF and thus represent the complex-glycosylated forms that had left the ER. Protein bands with equivalent molecular masses were consistently detected at the cell surface by the biotin-targeting assay (see Fig. 3). For receptor fragment 71.GFP, the intensity of this complex-glycosylated protein band was much weaker than for 71/ICL2.GFP and 71/CT.GFP, consistent with the partial transport defect of this receptor fragment.

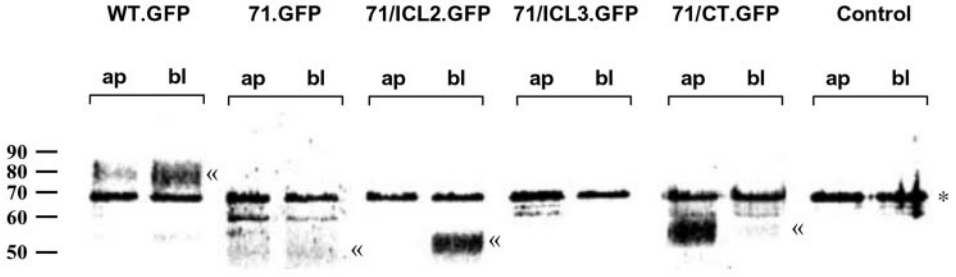

Fig. 3. Selective biotinylation of apically or basolaterally expressed GFP-tagged V_2 receptor fragments containing different cytoplasmic domains in stably transfected MDCKII cells. The apical or basolateral membrane proteins of confluent filter grown cells were labeled with biotin. Biotinylated proteins were precipitated with NeutrAvidin-agarose beads. The receptor fragments (71.GFP, 71/ICL2.GFP, 71/ICL3.GFP, and 71/CT.GFP) and the full-length V_2 receptor (WT.GFP) were detected in the apically (ap) or basolaterally (bs) biotinylated samples by SDS/PAGE-immunoblot analysis using polyclonal anti-GFP antibodies. Untransfected cells were used as control (Control). Each sample was prepared from confluent cells grown on a 24-mm diameter filter support. Immunoreactive protein bands are indicated by symbols (∗, nonspecific band; «, relevant, complex glycosylated bands). The immunoblot is representative of three independent experiments.

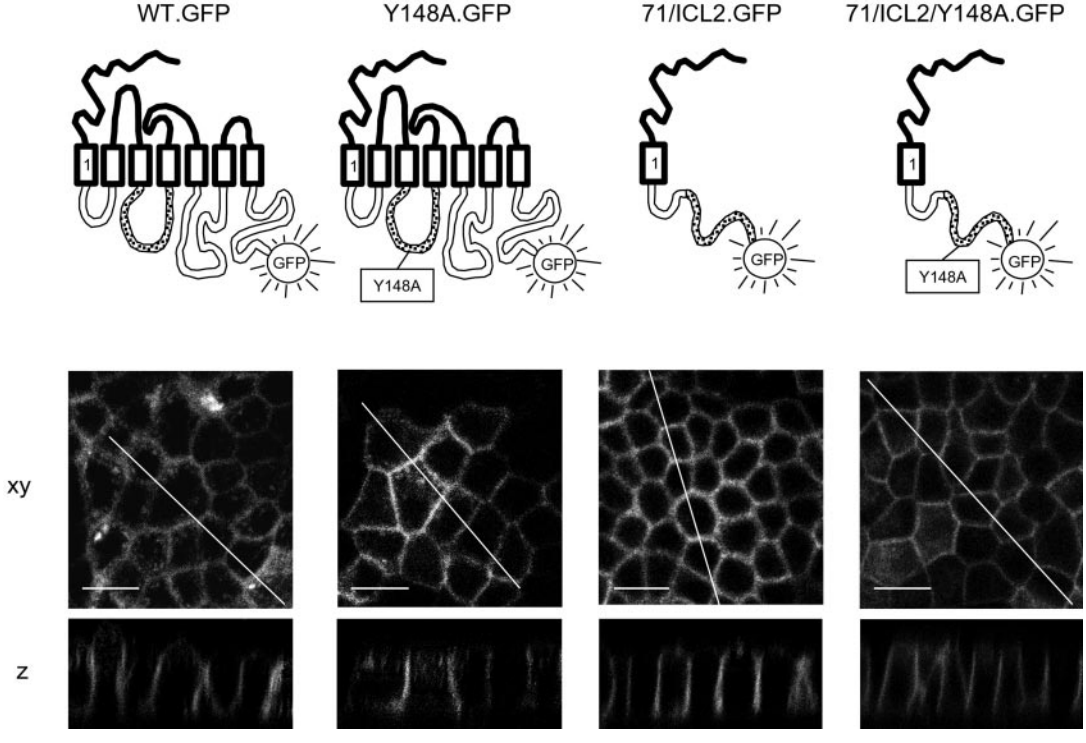

Fig. 4. Localization of Y184A mutants of the GFP-tagged full-length V_2 receptor and receptor fragment 71/ICL2.GFP in stably transfected MDCKII cells. Cells expressing wild-type and mutant full-length V_2 receptors (WT.GFP and Y148A.GFP) and wild-type and mutant receptor fragments (71/ICL2.GFP and 71/ICL2/Y148A.GFP) were grown to confluence on permeable filter supports. The GFP fluorescence signals were analyzed in living cells by confocal laser scanning microscopy with horizontal *xy*-scans (top) and with *z*-scans (bottom) along the indicated lines. The apical membranes lie uppermost in the *z*-scans. Each photograph shows a representative scan ($n \geq 10$). Scale bar, 10 μm.

2. Protein bands (marked by «) with apparent molecular masses of 38 kDa (71.GFP), 40 kDa (71/ICL2.GFP), and 42 kDa (71/CT.GFP). These protein bands were sensitive to both EndoH and PNGaseF and thus represent the high-mannose glycosylated forms of the fusions.
3. Sharp protein bands (marked by #) with apparent molecular masses of 34 kDa (71.GFP), 38 kDa (71/ICL2.GFP), and 40 kDa (71/CT.GFP), representing the nonglycosylated forms of the fusions, because the high-mannose glycosylated forms shifted to these sizes upon EndoH and PNGaseF treatment (calculated molecular mass, 36.09, 39.05, and 39.88 kDa, respectively). The complex-glycosylated forms shifted to larger sizes (marked by •) upon PNGaseF treatment because additional PNGaseF-resistant *O*-glycosylations are added to the N terminus of the V_2 receptor in the Golgi apparatus, increasing the molecular mass (Sadeghi and Birnbaumer, 1999). The presence of nonglycosylated forms of 71.GFP, 71/ICL2.GFP, and 71/CT.GFP (not observed for WT.GFP) may be caused by overexpression and subsequent saturation of the glycosylation machinery. Deglycosylation of overexpressed forms before transport to the proteasome (Petäjä-Repo et al., 2001) may also explain the presence of the nonglycosylated forms.

In untreated membranes expressing the transport deficient receptor fragment 71/ICL3.GFP, only a single immunoreactive protein band with an apparent molecular mass of 42 kDa was detected (marked by «); this shifted upon EndoH treatment to the 40-kDa nonglycosylated form (marked by #; calculated molecular mass, 40.34 kDa). Thus only EndoH-sensitive, high-mannose glycosylated forms are present in the case of 71/ICL3.GFP, demonstrating that this fusion is indeed trapped in the ER. In contrast to the transport-competent receptor fragments, the nonglycosylated form of this fusion was not detected in the untreated membranes at steady state. ER accumulation may induce a rapid proteolytic degradation of this receptor fragment and thus prevent saturation of the glycosylation machinery. Compared with the other GFP fusions, the total amount of immunoreactive 71/ICL3.GFP protein is substantially lower. This is consistent with an increased proteolytic degradation. The nevertheless strong GFP fluorescence signals detectable for this receptor fragment in Fig. 2 (the cell clones were selected for similar GFP fluorescence intensities; see also *Experimental Procedures*) are explicable if one assumes a C-terminal degradation of the receptor fragment. The peptide derived anti-GFP antibodies are directed against the extreme C terminus of the protein (Krause et al., 2000), and in the case of such C-terminal degradation, the GFP fluorescence of 71/ICL3.GFP might still be detectable even though immunoreactivity is abolished.

The Sequence ^{247}RRRGRR^{252} of the ICL3 Domain May Contain an "RXR" ER Retention Signal. Two interpretations are plausible for the ER retention of fragment 71/ICL3.GFP. (1) in contrast to the ICL2 domain and the C-terminal tail, the ICL3 domain contains no signals that may facilitate transport from the ER to the Golgi apparatus. "ER to Golgi" transport signals are not well understood at present. Diphenylalanine and diacidic motifs, however, which were described as serving to concentrate membrane proteins in ER to Golgi vesicles (Fiedler et al., 1996; Nishimura and Balch, 1997), are present in neither the ICL2 domain nor the C-terminal tail. (2) receptor fragment fusions containing ICL3 may be recognized and retained by the quality control system of the ER, whereas those containing ICL2 and the C-terminal tail are not. It was recently shown that membrane proteins may contain arginine framed (RXR) ER retention signals in their cytoplasmic domains (Zerangue et al., 1999). It was suggested that these signals may be masked when subunits of multimeric proteins assemble correctly [ATP-sensitive K^+ channels (Zerangue et al., 1999); γ-aminobutyric acid receptor subtype B receptor (Margeta-Mitrovic et al., 2000)], or if a monomer folds correctly [cystic fibrosis transmembrane conductance regulator (Chang et al., 1999)], but remain unmasked in unassembled or misfolded proteins. Unmasking leads to ER retention via unknown receptors. The ICL3 domain of the V_2 receptor contains an arginine rich sequence (^{247}RRRGRR^{252}; see Fig. 1) with two putative RXR motifs, which may thus function in a similar manner; i.e., they may be masked in the full-length wild-type receptor but exposed in the 71/ICL3.GFP receptor fusion in the absence of the normal folded receptor structure.

To address the question of whether an arginine framed retention signal is contained within this sequence, the arginine residues were replaced by likewise positively charged lysine residues in the receptor fragment 71/ICL3.GFP (yield-

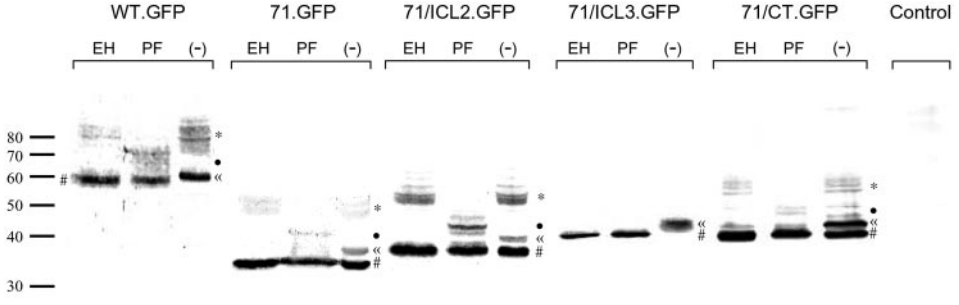

Fig. 5. Glycosylation state analysis of GFP-tagged V_2 receptor fragments containing different cytoplasmic domains. Crude membranes (60 μg of protein) of stably transfected MDCKII cells expressing receptor fragments 71.GFP, 71/ICL2.GFP, 71/ICL3.GFP, and 71/CT.GFP were isolated and treated with EndoH (EH, to remove high-mannose glycosylations) or PNGaseF (PF, to remove both high-mannose and complex glycosylations); untreated controls (−). Immunoreactive proteins were detected by SDS/PAGE-immunoblotting using a polyclonal anti-GFP antiserum and alkaline phosphatase-conjugated antirabbit IgG. Membranes from cells expressing the wild-type GFP-tagged V_2 receptor (WT.GFP) were used as a control for the presence of complex glycosylations. Untransfected MDCKII cells (Control) were used as a control for antibody specificity. Relevant immunoreactive protein bands are indicated by symbols (#, unmodified forms; «, high-mannose forms; •, *O*-glycosylated forms; ∗, complex glycosylated forms). The immunoblots are representative of three independent experiments.

ing mutant 71/ICL3/R247-R252K.GFP) and in the full-length receptor (yielding mutant R247-R252K.GFP). Stably transfected MDCKII cell clones expressing the GFP fusions were grown on polycarbonate filter supports, and the GFP fluorescence signals were localized by LSM (Fig. 6A). For the full-length R247-R252K.GFP mutant, a distribution of the GFP signals similar to those for WT.GFP was observed; i.e., fluorescence was located predominantly basolaterally (data not shown). The mutation of the RXR motif of ICL3 thus has no influence on the transport of the full-length V_2 receptor. The GFP signals of the receptor fragment 71/ICL3.GFP, which contains the wild-type ICL3 sequence as a control, were again located in the cell's interior (see also Fig. 2). The signals of the corresponding mutant receptor fragment 71/ICL3/R247-R252K.GFP, however, were detected without polarization at the plasma membrane. To confirm these results, a cell surface biotinylation assay with filter-grown cells was performed (Fig. 6B). A broad 52- to 58-kDa protein band (marked by «) was detected in roughly equal amounts in the apical and basolateral samples of receptor fragment 71/ICL3/R247-R252K.GFP consistent with the results obtained from LSM. These results demonstrate that the transport defect of receptor fragment 71/ICL3.GFP can be rescued by the lysine substitutions and that the ^{247}RRRGRR^{252} sequence can function as an ER retention signal. Furthermore, the nonpolarized expression of the rescued receptor fragment fashion demonstrates that the ICL3 has no further apical or basolateral sorting signal.

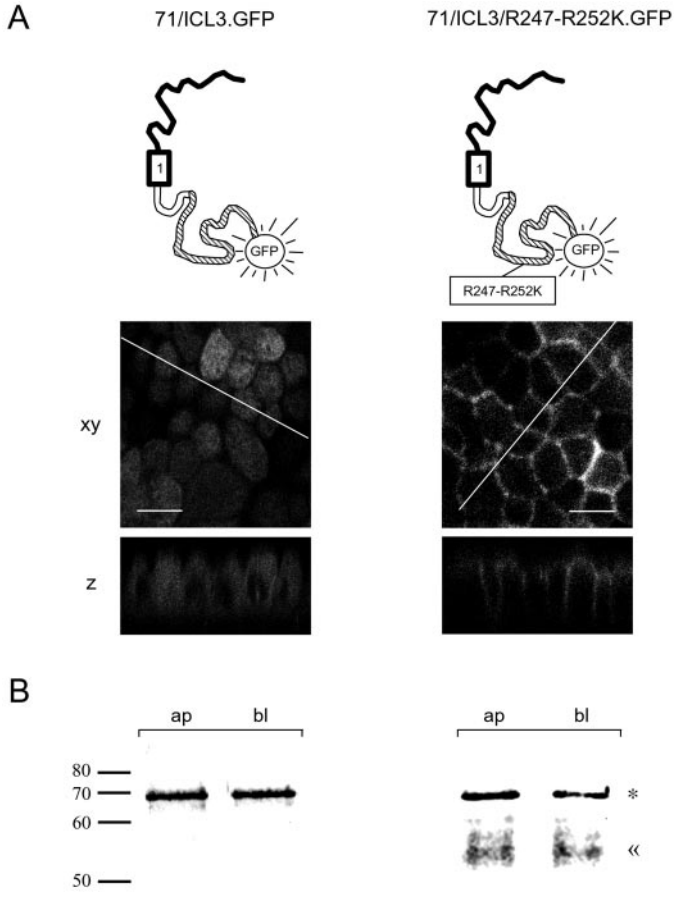

Fig. 6. Localization of the GFP-tagged V_2 receptor fragments 71/ICL3.GFP and 71/ICL3/R247-R252K.GFP in stably transfected MDCKII cells. A, LSM localization study. Cells expressing wild-type and mutant receptor fragments (71/ICL3.GFP and 71/ICL3/R247-R252K.GFP) were grown to confluence on permeable filter supports. GFP fluorescence signals were analyzed in living cells with *xy*-scans (top) and with *z*-scans (bottom) along the indicated lines. The apical membranes lie uppermost in the *z*-scans. Each photograph shows a representative scan ($n \geq 10$). Scale bar, 10 μm. B, selective cell surface biotinylation assay. The apical or basolateral membrane proteins of filter grown cells expressing the wild-type and mutant receptor fragments (71/ICL3.GFP and 71/ICL3/R247-R252R.GFP) were labeled with biotin. Biotinylated proteins were precipitated with NeutrAvidin-agarose beads. The receptor fragments were detected in the apically (ap) or basolaterally (bs) biotinylated samples by SDS/PAGE-immunoblot analysis using polyclonal anti-GFP antibodies. Each sample was prepared from confluent cells grown on a 24-mm diameter filter support. Immunoreactive protein bands are indicated by symbols (*, nonspecific band; «, relevant, complex glycosylated bands). The immunoblot is representative of three independent experiments.

These data also raise the possibility that the arginine frame sequence of ICL3 could function as a retention signal in full-length V_2 receptor mutants when misfolding leads to an exposure of the ^{247}RRRGRR^{252} sequence. This may have clinical implications because naturally occurring V_2 receptor mutations often lead to ER-retained forms and cause X-linked nephrogenic diabetes insipidus (NDI) (Oksche and Rosenthal, 1998). Mutation of the sequence may help to rescue the transport of at least some of these NDI-causing mutants. To address this question, we have also introduced the R247-R252K mutation into the NDI-causing mutant ΔL62-R64 (deletion of residues ^{62}LAR^{64} at the junction of transmembrane domain 1 and ICL1), which was previously shown to be retained in the ER (Krause et al., 2000; Morello et al., 2000). However, this additional mutation alone was not sufficient to rescue cell surface expression of this mutant receptor (data not shown).

Discussion

We have assessed the functions of the cytoplasmic domains of the V_2 receptor for intracellular transport in MDCKII epithelial cells. We show that the C-terminal tail of the V_2 receptor mediates apical and the second intracellular loop basolateral plasma membrane location, respectively. Fusion of the third intracellular loop led to a receptor fragment that was not transported to the cell surface but was retained in the ER.

We have shown previously that the V_2 receptor is expressed in MDCKII cells predominantly basolaterally at steady state, although minor amounts are located apically (Schülein et al., 1998b; Andersen-Beckh et al., 1999). The data presented here may explain these results and are consistent with a model in which the V_2 receptor contains signals for both apical and basolateral membrane expression, the latter being dominant. The identity of the basolateral sorting signal in ICL2 remains elusive. Previously described basolateral sorting signals found in unrelated membrane proteins were in most cases either of the tyrosine or the dileucine type (Matter et al., 1992; Hunziker and Fumey, 1994). A tyrosine residue in the C-terminal tail of the G protein-coupled follicle-stimulating hormone receptor was also shown to be important for its basolateral localization (Beau et al., 1998). Tyrosine residue Y148 of ICL2 (the only tyrosine residue in the whole cytoplasmic face of the V_2 receptor) does not, however, contribute to such a signal. Basolateral transport of the corresponding alanine mutants of the full-length receptor and of 71/ICL2.GFP was not affected. Further studies will be required to delineate the ba-

solateral sorting signal in the ICL2 domain. For the α_{2A}-adrenergic receptor, it was proposed that the third cytoplasmic loop in its entirety (157 residues), rather than a small specific signal, is responsible for basolateral expression (Edwards and Limbird, 1999). Because of its relative shortness (24 residues), it seems unlikely that this applies to the ICL2 of the V_2 receptor.

Several arguments support the assumption that the apical transport of receptor fragment 71/CT.GFP is mediated by a transport signal within the C-terminal tail.

1. Most importantly, in the absence of an apical transport signal, one would expect the receptor fragment 71/CT.GFP to be delivered in a nonpolar fashion to the cell surface [i.e., that similar amounts would be expressed apically and basolaterally (as for the starting construct 71.GFP and the rescued construct 71/ICL3/R247-R252K.GFP)]. However, this was not the case.
2. It was recently shown that the C-terminal tail of rhodopsin contains an apical sorting signal (Chuang and Sung, 1998). Deletion of the C-terminal 32 residues abolished apical rhodopsin sorting in MDCKII cells and led to a randomized cell surface expression. Taking into account that the C-terminal tails of rhodopsin and the V_2 receptor are homologous in this region (44% identity; 78% similarity, based on the similarity matrix of Risler et al., 1988), it is reasonable to speculate that the C-terminal tail of the V_2 receptor contains a similar apical sorting signal. As stated above, in the case of the full-length V_2 receptor, the apical signal may be effectively suppressed by a dominant basolateral signal in the ICL2 domain. The nonpolarized transport of the starting construct 71.GFP seems to preclude the possibility that the *N*- and *O*-glycosylations in the extracellular N terminus of the V_2 receptor contribute significantly to an apical signal as shown for some other membrane proteins (Rodriguez-Boulan and Gonzalez, 1999).

Our data indicate that an RXR ER retention signal is contained within the sequence ^{247}RRRGRR^{252} of ICL3, which is unmasked in the case of receptor fragment 71/ICL3.GFP. It is possible that the same will happen to the full-length receptor if mutations lead to misfolding and thereby to exposure of the signal. The participation of such a signal in the ER quality control apparatus was recently described for the cystic fibrosis causing ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator protein (Chang et al., 1999). Here, mutation of all putative RXR signals led to a restoration of transport and also to functional rescue of the protein (Chang et al., 1999). The participation of a RXR retention signal was also described for γ-aminobutyric acid receptor subtype B receptor heterodimerization (Margeta-Mitrovic et al., 2000).

In the case of the NDI-causing V_2 receptor mutant ΔL62-R64, however, we failed to restore cell surface delivery by an additional R247-R252K mutation. Several explanations are possible.

1. It is not known whether the ΔL62-R64 mutation in particular leads to the exposure of the putative retention signal in ICL3. This may only be the case for a subset of mutants.
2. More than one arginine framed retention signal may be present in the full-length V_2 receptor, and mutation of the ^{247}RRRGRR^{252} sequence alone may not be sufficient to restore receptor transport. The sequence ^{65}RGR^{67} immediately C-terminal of the ΔL62-R64 mutation may constitute another retention signal, which may be exposed in this mutant receptor. The sequence may also be exposed in the case of the 71.GFP construct and cause the partial transport defect of this fusion. Interestingly, a receptor fragment containing only the N-terminal 71 amino acid residues of the V2 receptor without the GFP moiety is completely retained in the ER (Morello et al., 2001). The lack of the GFP moiety may in this case lead to a better exposure of this RXR sequence and consequently to a complete ER retention. Note, however, that an incorrect integration of the untagged construct into the ER membrane may also contribute to this phenomenon: the cytoplasmic portion without the GFP moiety is only nine amino acid residues long, and this may be too short for the translocation machinery to assess correctly the charge difference between the cytoplasmic portion and the N terminus. According to the statistical study of Hartmann et al. (1989), up to 15 amino acid residues may be necessary for this process. A receptor fragment containing the N-terminal 71 amino acids of the V2 receptor consistently seems to assure correct ER membrane integration only when fused at the C terminus to an arbitrary protein portion [e.g., a GFP moiety as demonstrated here, or an alkaline phosphatase moiety as described previously (Schülein et al., 1996b)]. The V2 receptor contains a third putative RXR sequence in the C terminus (^{344}RGR^{346}). However, it is unlikely that this sequence can function as a retention signal, because the construct 71/CT.GFP is transported successfully to the cell surface.
3. More than one system may be involved in the ER retention of misfolded V_2 receptors. In addition to the unknown proteins binding to exposed RXR retention signals, association of the misfolded receptors with chaperones may also contribute. A prolonged association with the calnexin/calreticulin system on the luminal side of the ER was indeed shown recently for misfolded V_2 receptors (Morello et al., 2001). Inhibition of only one retention system may thus be insufficient to restore cell surface transport.

The introduction of the R247-R252K mutation into a large number of transport defective NDI-causing mutant V_2 receptors together with inhibition of the chaperones involved in ER retention of misfolded forms of the V_2 receptor will help clarify these points in the future.

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Correction to "Sorting functions of the individual cytoplasmic domains of the G protein-coupled vasopressin V_2 receptor in Madin Darby canine kidney epithelial cells"

In the last sentence of the abstract of this article [Hermosilla R and Schulein R (2001) *Mol Pharmacol* **60:**1031–1039], the authors' original term "RXR" was changed during copy editing to "retinoid X receptor", and RXR was added to the abbreviation list with the incorrect definition. In this case, however, RXR was a sequence motif, not an abbreviation, and had absolutely nothing to do with a receptor.

We regret this error and apologize for any confusion or inconvenience it may have caused.