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Molecular and Conformational Features of a Transport-Relevant Domain in the C-Terminal Tail of the Vasopressin V_2 Receptor

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ABSTRACT

We have previously shown a conserved glutamate/dileucine motif (^{335}ELRSLL^{340}) in the intracellular C terminus of the vasopressin V_2 receptor (V_2 receptor) to be essential for receptor transport from the endoplasmic reticulum (ER) to the Golgi apparatus. The motif may represent a transport signal that is recognized by a component of ER to Golgi vesicles. Alternatively, it may be necessary for transport-competent receptor folding to pass the quality-control system of the ER. To assess these two possibilities, we constructed a receptor fragment that allows transport studies independent of full-length receptor folding. Transmembrane domains II–VII were deleted, thereby fusing the intracellular C terminus to the first cytoplasmic loop. The mutations that impaired transport of the full-length receptor were introduced, and receptor fragments were localized in transiently transfected HEK 293 cells. All mutant receptor fragments were detectable at the plasma membrane, demonstrating that the glutamate/dileucine motif does not function as a small, linear vesicular transport signal. Instead, our data strongly suggest that this motif is required for transport-competent folding of the full-length receptor. To assess the underlying conformational features, a three-dimensional homology model of the V_2 receptor was computed. Our model predicts that the glutamate/dileucine motif contributes to a U-like loop within the intracellular C terminus. Residue Leu^{339} may be required for folding back the intracellular C terminus to residue Leu^{62} of the first cytoplasmic loop. We characterized the naturally occurring L62P and ΔL62-R64 mutations in the first cytoplasmic loop and show that they lead to transport-defective full-length V_2 receptors that are retained in the ER, consistent with the structure model.

The heptahelical G-protein-coupled receptors (GPCRs) play a key role in signal transduction and represent the largest protein family in eukaryotic cells. Numerous studies have been conducted to elucidate, e.g., ligand interactions (Ji et al., 1998), the mechanisms of agonist activation (Gether and Kobilka, 1998), and the mechanisms of desensitization and sequestration (Lefkowitz, 1998) of these receptors. Comparatively little is known concerning the transport of GPCRs via the intracellular membrane systems to the cell surface.

C-terminally truncated receptor fragments have been used to determine the sequence requirements for plasma membrane transport of GPCRs. Rat glucagon receptor fragments containing one, three, or five N-terminal transmembrane domains (TMs) were transport deficient and were localized in the endoplasmic reticulum (ER). It was proposed that all seven TMs must be present for cell surface delivery of this receptor (Unson et al., 1995). Equivalent results were obtained for bovine rhodopsin fragments containing one to five N-terminal transmembrane segments that failed to escape from the ER (Heymann and Subramaniam, 1997).

A crucial role of the intracellular C terminus for cell surface transport was previously demonstrated for the V_2 receptor. Mutation of the palmitoylated cysteine residues reduced receptor transport significantly (Schülein et al., 1996a). Truncation at residue Arg^{337}, deleting only four additional residues N-terminal of the palmitoylated cysteine residues, abolished receptor transport to the plasma membrane (Sadeghi et al., 1997; Oksche et al., 1998). These results suggested that sequences N-terminal of the palmitoylation site play a crucial role in the cell surface delivery of this receptor. In fact, it was shown recently that a glutamate/dileucine motif in this region is essential for ER to Golgi

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AVP, arginine vasopressin; ER, endoplasmic reticulum; EndoH, endoglycosidase H; GFP, green fluorescent protein; ICL, intracellular loop; PAGE, polyacrylamide gel electrophoresis; PhoA, *Escherichia coli* alkaline phosphatase; PNGaseF, peptide *N*-glycosidase F; TM, transmembrane domain.

transfer (Schülein et al., 1998). Two interpretations for the impaired ER to Golgi transport of V_2 receptors with mutations in this motif are possible: 1) The glutamate/dileucine motif may represent a transport signal that is recognized on the cytoplasmic side of ER to Golgi vesicles, by a component such as a vesicular coat protein (e.g., coatomer). Although transport signals of membrane proteins for ER to Golgi vesicles are not well defined, it was shown recently that a diacidic D*X*E motif in the cytoplasmic tail of the vesicular stomatitis virus glycoprotein facilitates ER to Golgi transport (Nishimura and Balch, 1997). The same was true for a dihydrophobic phenylalanine pair in the cytosolic tail of the p24 putative cargo receptor, which contributed to a motif specifying ER to Golgi transport by binding to coatomer β, γ, and ζ subunits (Fiedler et al., 1996). All signals described so far were small, linear sequence motifs in the cytoplasmic domains, and it is thus conceivable that the glutamate/dileucine motif of the V_2 receptor may function in a similar manner. 2) On the other hand, it is known that the ER contains a quality-control system that allows export only of correctly folded and assembled proteins (Hammond and Helenius, 1995). The glutamate/dileucine motif may thus be necessary for folding of the intracellular C terminus and, as a consequence, perhaps for folding of the entire receptor molecule. Establishment of a transport-competent folding state would thereby result from intramolecular interactions of the motif within the receptor molecule rather than of intermolecular interactions between the motif and a vesicular coat protein.

Here, we have assessed whether the glutamate/dileucine motif of the V_2 receptor is transport relevant in an experimental system that allows transport studies independent of full-length receptor folding. To gain insight into the conformational features of the receptor region containing the motif, we have also computed a three-dimensional (3D) structure model of the V_2 receptor with special emphasis on the intracellular C terminus.

Experimental Procedures

Materials. Lipofectamine was purchased from Life Technologies (Eggenstein, Germany). DNA-modifying enzymes, endoglycosidase H (EndoH) and peptide *N*-glycosidase F (PNGaseF) were from New England Biolabs (Schwalbach, Germany). Trypan blue was purchased from Seromed (Berlin, Germany). [^{3}H]Arginine vasopressin (AVP) for the binding assay (64.8 Ci/mmol) and [α-^{32}P]ATP for the adenylyl cyclase assay (30 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Oligonucleotides were from Biotez (Berlin, Germany). All other reagents were from Sigma Chemical Co. (Munich, Germany). Plasmid pE green fluorescent protein (GFP)-N1, encoding the red-shifted variant of GFP, was from Clontech Laboratories (Heidelberg, Germany). Plasmid pRCDN2, encoding the wild-type V_2 receptor cDNA, was described (Schülein et al., 1996a). Plasmids pEU71.alkaline phosphatase (PhoA) and pEU367.PhoA, encoding *Escherichia coli* PhoA fusions to residues Trp^{71} and Lys^{367} of the V_2 receptor, respectively, were described (Schülein et al., 1996b). Plasmid pWT.GFP, encoding a GFP fusion to residue K^{367} of the V_2 receptor, and plasmid pL339/340T.GFP, encoding the corresponding mutant GFP-tagged receptor, were previously described (Schülein et al., 1998). Anti-rabbit ^{125}I-labeled IgG (28–111 TBq/mmol) was purchased from Amersham Corp. (Braunschweig, Germany).

Antibodies. A polyclonal antiserum against the GFP moiety was raised against a synthetic peptide with an additional N-terminal tyrosine residue (Y-QSALSKDPNEKRDHMVL; comprising residues 205–221 of GFP). The peptide was coupled to keyhole limpet hemocyanin for immunization. Specificity of the antiserum was verified by an immunoblot with membranes from transiently transfected HEK 293 cells containing a C-terminally GFP-tagged V_2 receptor. The same immunoreactive core- and complex-glycosylated forms were detected as when a monoclonal anti-GFP antibody was used (see Fig. 3 of *Results* and Schülein et al., 1998). Labeling of these bands was abolished in the presence of 35 μg/ml of peptide immunogen (data not shown).

DNA Manipulations. Standard DNA manipulations were carried out according to the handbooks of Sambrook et al. (1989). The nucleotide sequences of DNA fragments were verified with the FS dye terminator kit from Perkin Elmer (Weiterstadt, Germany). Site-directed mutagenesis was carried out with the Quick Change site-directed mutagenesis kit from Stratagene (Heidelberg, Germany).

Construction of Wild-Type and Mutant GFP-Tagged V_2 Receptor Fragments. For reasons not relevant to this work, receptor fragments were initially tagged with the PhoA protein of *E. coli*. The resulting plasmids were used for the construction of equivalent GFP-tagged receptor fragments. Plasmid pEU367.PhoA, encoding a PhoA fusion to residue Lys^{367} of the V_2 receptor (i.e., to the entire receptor, except for the four C-terminal residues) was described (Schülein et al., 1996b). Plasmid pEU71.PhoA encodes a PhoA fusion to residue Trp^{71}, i.e., to a fragment consisting of the N terminus, first transmembrane helix, and first cytoplasmic loop of the V_2 receptor (Schülein et al., 1996b). To insert the wild-type C terminus of the V_2 receptor between the first cytoplasmic loop and the PhoA portion of this construct, plasmid pEU367.PhoA was used as a template for polymerase chain reaction. A sequence encoding the C terminus of the V_2 receptor with the fused PhoA tag was amplified (5′ primer: 5′-CACCAACCCCTGGATAGATCTATCTTTCAGCAGCAG-3′; 3′ primer: 5′-GATTTAGGTGACACTATAG-3′). The 5′ primer introduced a *Bgl*II site at nucleotide 1043 of the V_2 receptor cDNA (at the end of the seventh transmembrane helix). The sequence for the PhoA-tagged C terminus of the V_2 receptor was cloned as a *Bgl*II/*Xba*I fragment into *Bam*HI/*Xba*I cut pEU71.PhoA, thereby replacing the PhoA portion of the latter plasmid. In the resulting construct (p71C/WT.PhoA), the first 71 amino acids of the V_2 receptor are followed by 41 wild-type C-terminal amino acids (residues S^{327}–K^{367} containing the glutamate/dileucine motif) and the PhoA moiety. To replace the PhoA tag of this fragment with GFP, the *Sac*I/*Bam*HI fragment of this plasmid was cloned into *Sac*I/*Bam*HI cut vector pEGFPN-1, yielding the plasmid p71C/WT.GFP. The cloning procedures above are for wild-type sequences. The construction of GFP-tagged receptor fragments with mutations in the glutamate/dileucine motif (E335Q, L339T, L340T, L339/340T; Schülein et al., 1998) followed the same scheme as above but proceeded from the corresponding pEU367.PhoA mutants. The resulting plasmids encoding mutant GFP-tagged receptor fragments were designated p71C/E335Q.GFP, p71C/L339T.GFP, p71C/L340T.GFP, and p71C/L339/340T.GFP, respectively.

Construction of Mutant Receptors L62P and ΔL62-R64. The L62P and the ΔL62-R64 mutations were introduced directly by site-directed mutagenesis into plasmids pRCDN2, encoding the wild-type untagged V_2 receptor (Schülein et al., 1996a), and plasmid pWT.GFP, encoding a GFP fusion to residue Lys^{367} of the wild-type V_2 receptor (i.e., to the entire V_2 receptor, lacking only the four C-terminal residues; Schülein et al., 1998). The primer sequences were 5′-GGTGCTGGCG-GCCCCAGCTCG-3′ (and its complementary equivalent) for the L62P mutation and 5′-CCTGGTGCTGGCGGCCCGGGGCCGGCGGGGCC-3′ (and its complementary equivalent) for the ΔL62-R64 mutation.

Cell Culture and Transfection Methods. HEK 293 cells were cultured at 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown on poly-L-lysine-coated plastic material to improve adherence. Cells were transfected with Lipofectamin according to the supplier's recommendations. For

a 15-mm-diameter well of a 24-well plate, 5×10^4 HEK 293 cells were transfected with 250 ng of plasmid DNA and 2 μl of Lipofectamin (for laser scanning microscopy, lower cell densities were used; see below). After removal of the transfection reagent, cells were incubated for 48 h.

[^{3}H]AVP Binding Assay and Adenylyl Cyclase Assay. The [^{3}H]AVP binding assay was carried out with intact, transiently transfected HEK 293 cells essentially as described previously for African green monkey kidney (COS.M6) cells (Schülein et al., 1996a). However, HEK 293 cells were grown in 24-well plates (15 mm diameter/well) instead of the 35-mm-diameter dishes described for COS.M6 cells. The adenylyl cyclase assay was carried out with nuclei-free crude membranes of transiently transfected HEK 293 cells as described previously for stably transfected L^{tk-} cells (Schülein et al., 1996a).

Visualization of GFP-Tagged Receptors: Cell Surface Staining in Living, Transiently Transfected HEK 293 Cells. HEK 293 cells, 4×10^4, in a 35-mm-diameter dish containing a poly-L-lysine (M_r 300,000)-coated cover glass were transfected with 500 ng of plasmid DNA and 7.5 μl of Lipofectamin according to the supplier's recommendations. Cells were incubated for 16 h, and cover glasses with cells were washed twice with PBS and transferred immediately into a self-made chamber (details on request). Cells were covered with 1 ml of PBS, and GFP fluorescence was visualized on a Zeiss 410 invert laser scanning microscope (λ_{exc} = 488 nm, $\lambda_{em} > 515$ nm).

Isolation of Crude Membrane Fractions of Transiently Transfected HEK 293 Cells Containing GFP-Tagged V_2 Receptor Fragments: EndoH/PNGaseF Treatment and Immunoblots. Crude membranes of transiently transfected HEK 293 cells were isolated from confluent cells grown in two 35-mm-diameter dishes as described previously for COS.M6 cells (Schülein et al., 1996b). Membranes were incubated with or without EndoH or PNGase F according to the supplier's recommendations. For the detection of the GFP-tagged receptor fragments, proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% acrylamide) and blotted onto nitrocellulose filters as described (Khyse-Andersen, 1984). Filters were blocked for 1 h with blocking buffer (10 mM Tris-HCl, 0.9% NaCl, 1% casein, 1% gelatin, pH 7.2), supplemented with polyclonal anti-GFP antiserum (dilution, 1:15,000) and incubated for 2 h at room temperature. Filters were washed four times (10 min each) with wash buffer (10 mM Tris-HCl, 0.9% NaCl, 0.01% NaN_3, pH 7.2). Anti-rabbit ^{125}I-labeled IgG was added to a final concentration of 1 μg/ml (1 μCi/ml), and the filters were incubated for 2 h at room temperature. Filters were washed two times with wash buffer (10 min each), dried, and exposed to X-ray film (2 days).

V_2 Receptor Model Building. Previously published modeling data for the V_2 receptor considered mainly the ligand binding site or the TMs (Chini et al., 1995; Czaplewski et al., 1998; Ala et al., 1998). Our structure model was computed with special emphasis on the intracellular C terminus. The procedure for the construction of a 3D structure model of the transmembrane regions and the connecting loops of the human V_2 receptor was analogous to that described by Biebermann et al. (1998). Packing of the transmembrane helices was based on electron-density maps of frog rhodopsin (Unger et al., 1997). The starting conformations of the intracellular loops (ICLs) 1, 2, 3, and the first portion of the C-terminal tail comprising the putative ICL4 of the V_2 receptor (confined by the palmitoylation sites Cys^{341} and Cys^{342}) were adopted from the NMR structure of the rhodopsin cytosolic loop peptide complex (Yeagle et al., 1997). For the remaining sections of the ICLs and for the extracellular domains, fragments of four to six residues were selected and tested against the Brookhaven 3D protein databank (Brookhaven National Laboratory, Brookhaven, CT). Only those loop fragments occurring more than once with a similar backbone conformation in the database were used. The homology model was finally computed on the transmembrane template of rhodopsin by addition of the conformations of the ICL peptides and the homologous fragments from the 3D database.

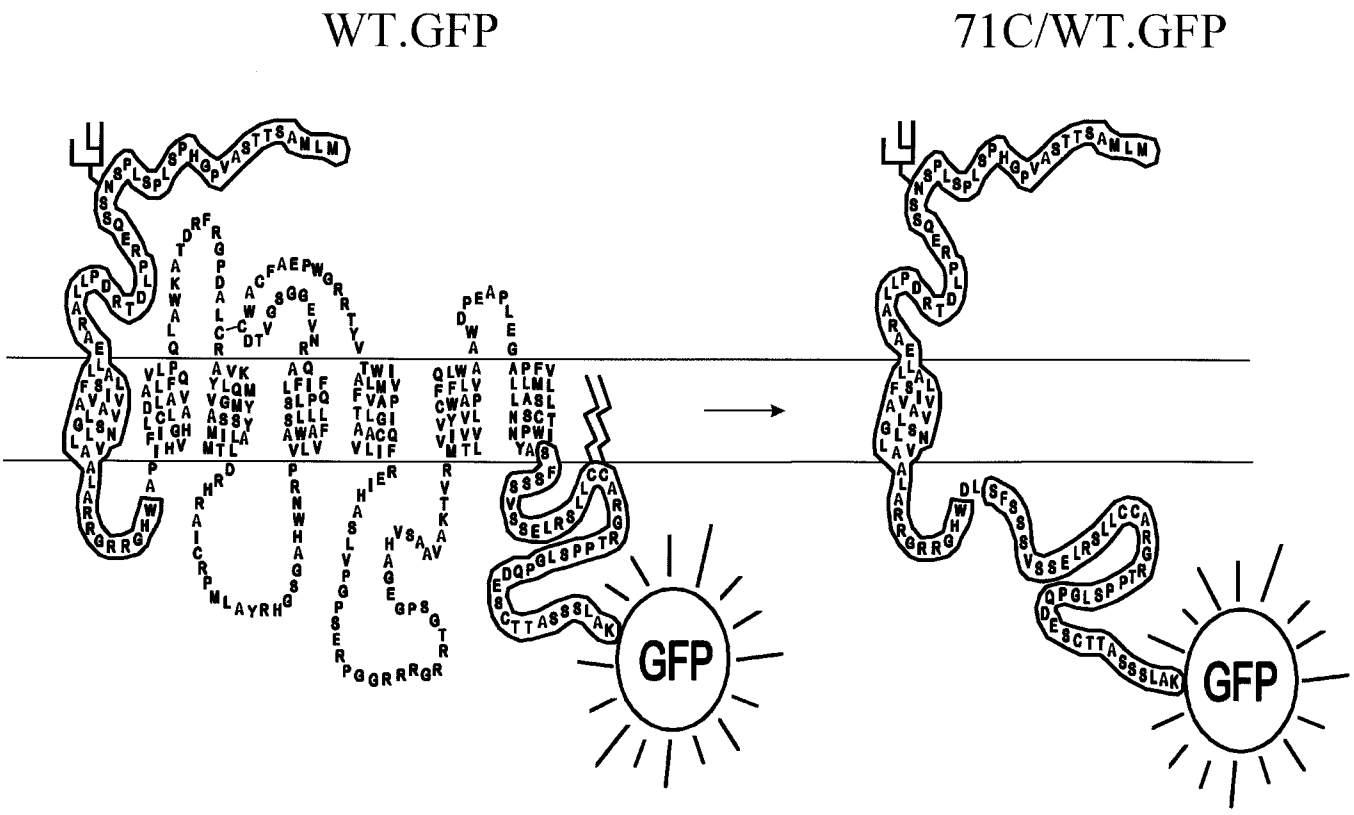

Fig. 1. Amino acid sequences and topology models of WT.GFP and receptor fragment 71C/WT.GFP. Fragment 71C/WT.GFP was constructed by fusing the intracellular C terminus to a fragment comprising the N terminus, first TM and first cytoplasmic loop. Fragment 71C/WT.GFP allowed the study of transport functions of the intracellular C terminus independent of full-length receptor folding.

Model components were assembled with the biopolymer and loop search modules of the Sybyl program package (Tripos Inc., St. Louis, MO) and minimized by an assisted model building with energy refinement 4.1 force field. The stability of the resulting receptor model was assessed as previously described (ter Laak et al., 1999). Molecular dynamics simulations maintaining helix stability were performed at 300 K for 200 ps using assisted model building with energy refinement force-field conditions in vacuo.

Results

The Glutamate/Dileucine Motif of the V_2 Receptor Does Not Function as a Linear Transport Signal for ER to Golgi Vesicles. To assess whether the glutamate/dileucine motif represents a sorting signal for ER to Golgi vesicles or is essential for transport-competent receptor folding, we have established an experimental system that allows transport studies of the intracellular C terminus independent of full-length receptor folding. To this end, we have deleted a major portion of the GFP-tagged receptor (comprising TM2 through 7) by fusing the intracellular C terminus to the first cytoplasmic loop (construct 71C/WT.GFP; Fig. 1). If the glutamate/dileucine motif functions as a sorting signal, mutants should be transport deficient in this system. If it is essential for transport-competent folding, however, transport should be retained. The mutations of the glutamate/dileucine motif that impaired ER to Golgi transport of the full-length receptor (E335Q, L339T, L340T, L339/340T; Schülein et al., 1998) were introduced into the C terminus of 71C/WT.GFP, and the resulting mutant receptor fragments (71C/E335Q.GFP, 71C/L339T.GFP, 71C/L340T.GFP, and 71C/L339/340T.GFP) were expressed in transiently transfected HEK 293 cells. The GFP fluorescence signals were recorded by laser scanning microscopy (Fig. 2). The wild-type GFP-tagged V_2 receptor consisting of 367 residues (WT.GFP), representing the full-length receptor lacking only the four C-terminal residues and the corresponding transport-deficient L339/340T receptor mutant (L339/340T.GFP) (Schülein et al., 1998) were used as respective positive and negative controls for cell surface transport. For the receptor fragment with the wild-type C terminus and for all receptor fragments with mutations in the glutamate/dileucine motif, GFP-fluorescence signals were clearly detectable at the cell surface in horizontal xy scans and vertical z scans (Fig. 2B). The same was true for WT.GFP, whereas the signals of the mutant receptor L339/340T.GFP diffusely filled the cell's interior with the exception of the nucleus (Fig. 2A). Plasma membrane localization of the GFP signals of all receptor fragments was verified by demonstrating their overlap with cell surface-specific fluorescence signals obtained after trypan blue staining (data not shown).

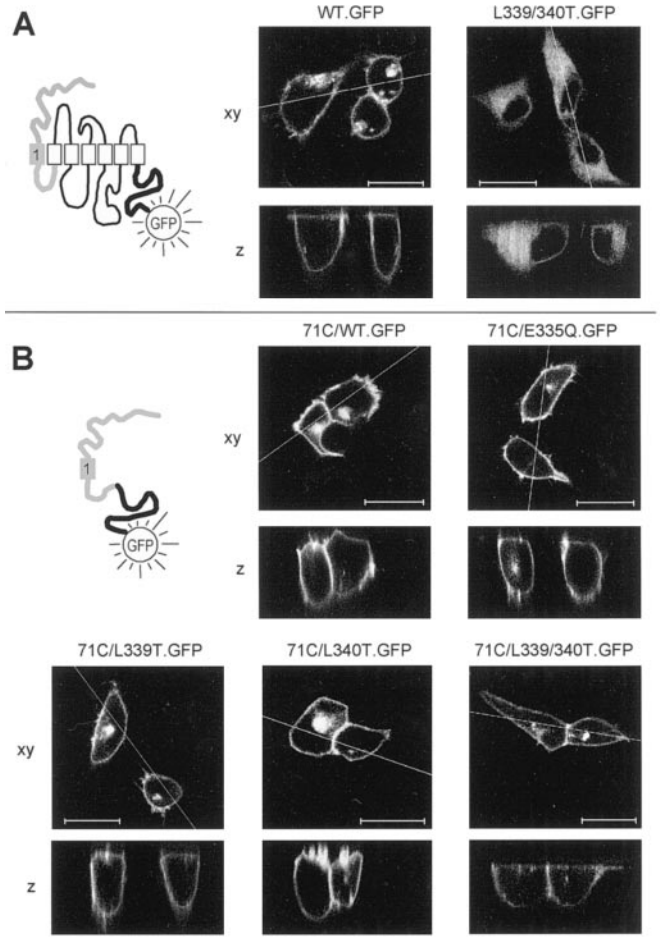

Fig. 2. Cell surface localization of GFP-tagged V_2 receptor fragments in living transiently transfected HEK 293 cells. The GFP fluorescence signals were analyzed by confocal laser scanning microscopy with horizontal xy-scans and with vertical z-scans at the indicated lines. The scans show representative cells. Scale bar = 25 μm. A, GFP fluorescence signals of cells expressing the transport-competent wild-type receptor (WT.GFP) and the corresponding transport-deficient mutant receptor L339/340T (L339/340T.GFP) as respective positive and negative controls for cell surface delivery. B, GFP fluorescence signals of cells expressing receptor fragments with wild-type (71C/WT.GFP) and mutant (71C/E335Q.GFP, 71C/L339T.GFP, 71C/L340T.GFP, 71C/L339/340T.GFP) C termini.

If all mutant receptor fragments do indeed leave the ER and reach the cell surface, complex-glycosylated fusion proteins should be present in membrane fractions. To address this question, membranes were isolated from transiently transfected HEK 293 cells expressing the receptor fragments and treated with EndoH to remove high-mannose glycosylations or with PNGaseF to remove both high-mannose and complex glycosylations. Fusion proteins were detected on Western blots with polyclonal anti-GFP antibodies (Fig. 3B). Membranes from cells expressing the transport-competent wild-type GFP-tagged V_2 receptor (WT.GFP) and from cells expressing the corresponding transport-defective mutant receptor L339/340T (L339/340T.GFP) were used as respective positive and negative controls for complex glycosylations (Fig. 3A). For each receptor fragment, two sharp bands with apparent molecular masses of 42 and 45 kDa and one broader band with an apparent molecular mass ranging from 55 to 60 kDa were detected in untreated membranes. The 42-kDa bands represent the unmodified form of the receptor fragments, verified by expression in *E. coli*, which yielded a comigrating band (data not shown). The 45-kDa but not the 55- to 60-kDa bands were sensitive to EndoH, whereas both bands were sensitive to PNGaseF. The 45-kDa bands thus represent the high-mannose-glycosylated and the 55- to 60-kDa bands represent the complex-glycosylated forms of the receptor fragments. The 55- to 60-kDa bands were shifted to 50 kDa rather than to the expected 42 kDa on PNGase treatment. As stated previously (Schülein et al., 1996b, 1998), an additional post-translational modification must occur within the N terminus of the V_2 receptor in a post-ER compartment, increasing the apparent molecular mass. In fact, it was shown recently that *O*-glycosylation of the N

terminus is responsible for this increase (Sadeghi and Birnbaumer, 1999). The calculated molecular mass of the unmodified receptor fragment is 38.5 kDa (11.5-kDa V_2 receptor + 27-kDa GFP moiety). The observed molecular mass of 42 kDa may be the result of incomplete unfolding of the receptor fragments in the presence of SDS. The control proteins WT.GFP and L339/340T.GFP displayed a glycosylation pattern that was similar to that previously described for transiently transfected COS.M6 cells (Schülein et al., 1998). For the transport-competent receptor WT.GFP, complex-glycosylated (75- to 80-kDa) and high-mannose-glycosylated (60- to 65-kDa) forms were detectable. In HEK 293 cells, however, the complex-glycosylated 75- to 80-kDa band was narrower than in COS.M6 cells (75–88 kDa; Schülein et al., 1998), which might reflect minor differences between the glycosylation machineries of the two cell types. For the transport-deficient mutant receptor L339/340T.GFP, only the high-mannose-glycosylated forms were present.

The presence of complex-glycosylated forms for all receptor fragments is consistent with their transport to the plasma membrane, confirming the laser scanning microscopy localization study. Taken together, our results strongly suggest that the glutamate/dileucine motif is required for transport-competent folding of the full-length receptor. The experiments are not consistent with the possibility that the motif represents a small linear transport signal that is recognized by a component of ER to Golgi vesicles.

Homology Model of the V_2 Receptor. If the glutamate/dileucine motif is indeed required for transport-competent receptor folding, this should be reflected in the conformational features of the motif-bearing receptor region. To address this question, a 3D homology model of the vasopressin V_2 receptor was computed, based on the electron-density map structure of the TMs of frog rhodopsin (Unger et al., 1997) and the NMR structure of the complexed, intracellular portions of bovine rhodopsin (Yeagle et al., 1997; see *Experimental Procedures* for details of the modeling procedure). There were several arguments for the use of rhodopsin as a struc-

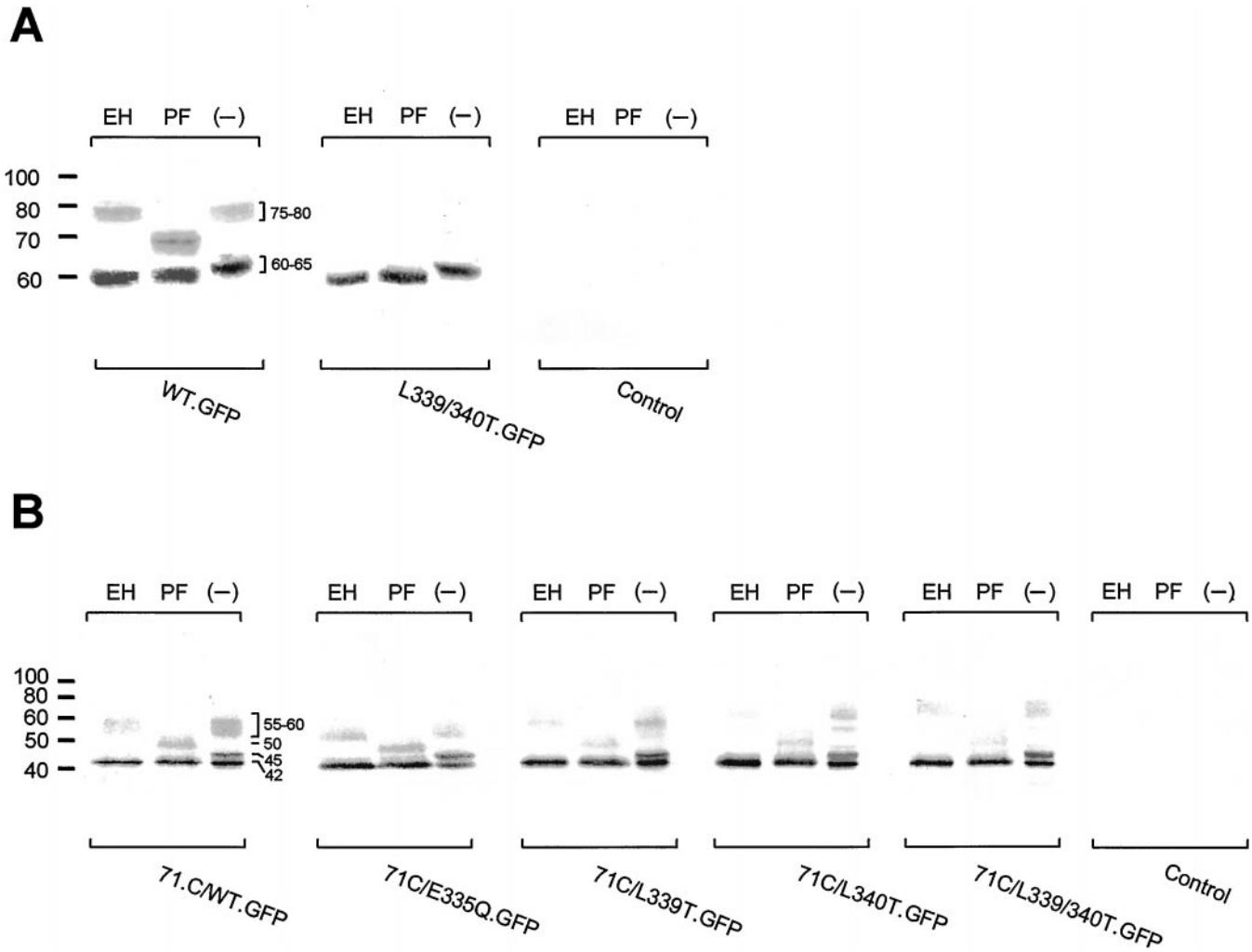

Fig. 3. SDS-PAGE/immunoblot analysis of transiently transfected HEK 293 cells expressing GFP-tagged V_2 receptor fragments. Crude membranes were isolated and treated with EndoH (EH), to remove high mannose glycosylations, or PNGaseF (PF), to remove both high mannose and complex glycosylations; untreated controls (−). Immunoreactive proteins were detected with a polyclonal anti-GFP antiserum and anti-rabbit ^{125}I-IgG. Untransfected HEK 293 cells were used as a control for antibody specificity. Protein bands described in the text are indicated. The immunoblots are representative of three independent experiments. A, membranes (50 μg of protein) from cells expressing the transport-competent wild-type GFP-tagged V_2 receptor (WT.GFP) and from cells expressing the corresponding transport-defective mutant receptor L339/340T (L339/340T.GFP) as respective positive and negative controls for complex glycosylations. B, membranes (30 μg of protein) from cells expressing GFP-tagged receptor fragments with wild-type (71C/WT.GFP) and mutant (71C/E335Q.GFP, 71C/L339T.GFP, 71C/L340T.GFP, 71C/L339/340T.GFP) C termini.

tural template. Most important, there is remarkable sequence homology (82% similarity, based on the similarity matrix of Risler et al., 1988) between the C-terminal tails of the two receptors (Fig. 4, bottom). In addition, the largest number of intermolecular interactions (nuclear Overhauser effect data) were reported to occur between the C-terminal tail and the ICL1 peptides of rhodopsin (Yeagle et al. 1997). Thus, the best-described regions in the rhodopsin peptide complex corresponded to those of the V_2 receptor in which we were interested. Additional structural information of the intracellular portions of other GPCRs also supports the view that rhodopsin is a suitable template for GPCR modeling studies. The NMR structure of ICL3 of the parathyroid hormone receptor (Pellegrini et al. 1996), for example, was similar to that described for rhodopsin.

The V_2 receptor model was assembled by fusing the intracellular and TM domains on the rhodopsin template. A reasonable match of the distances between the attachment sites of the transmembrane helices and those of the ICLs was observed. Especially TM1, TM2, and TM7 matched perfectly with ICL1 and the C-terminal tail structures of the rhodopsin loop complex. The model of the C-terminal tail of the V_2 receptor and the structure of the C-terminal tail of rhodopsin are shown in Fig. 4. For the V_2 receptor, the region between the seventh TM and the palmitoylated cysteine residues C^{341} and C^{342} (constituting the putative ICL4) formed a U-like loop that turned up back toward the membrane and exposed the palmitoylated cysteine residues to the membrane surface. This U-like loop remarkably resembled the corresponding rhodopsin structure. For rhodopsin, fragments of other

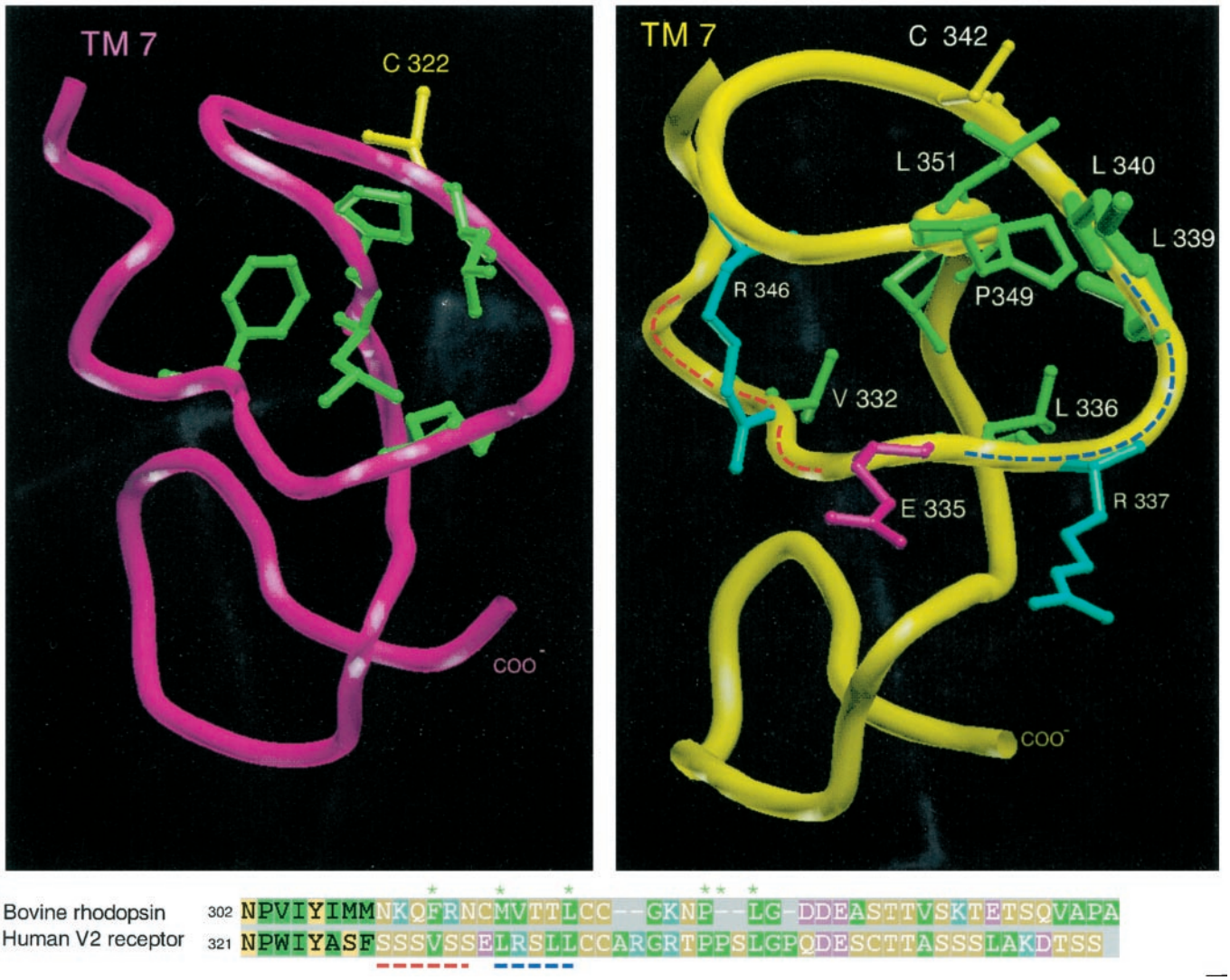

Fig. 4. Putative conformation of the intracellular C terminus of the V_2 receptor based on the corresponding rhodopsin structure. The conformation of the C-terminal tail of rhodopsin (left) was adopted from the NMR structural data of the cytosolic loop peptide complex (Yeagle et al. 1997). The ICL4 of rhodopsin, comprising the sequence from TM7 to the palmitoylated cysteine residues (yellow, only residue Cys^{322} is visible), forms a U-like loop that is stabilized by a hydrophobic core (green residues). The homology model of the intracellular C terminus of the V_2 receptor (right) was computed based on the remarkable sequence similarity between the two C-terminal tails (bottom; residues forming the hydrophobic core are indicated by an asterisk). The model was refined by considering the conformations of identical sequence fragments of other proteins retrieved from a 3D database. Fusion of Ig (red dashed line) and lysozyme (blue dashed line) fragments resulted in a U-like loop in the V_2 receptor similar to that of rhodopsin. A comparable interior hydrophobic core (green residues), which is predicted to be the major force for the development of the loop, is also present. The hydrophobic core is formed by residues Val^{332}, Leu^{336}, and Leu^{340} of ICL4. From the distal portion of the intracellular C terminus, residues Pro^{343}, Leu^{351}, and Pro^{353} may also contribute. Residue Leu^{339} faces outward from the hydrophic core and is necessary for an interaction with ICL1 (see Fig. 5). Residue Glu^{335} (magenta) may form a salt bridge with either the adjacent residue Arg^{337} or with residue Arg^{346} (blue residues)

proteins with identical sequences retrieved from the Brookhaven 3D databank revealed backbone conformations similar to those in the rhodopsin structure (data not shown). This approach was also used to refine the V_2 receptor model. Fragments of four to six residues were selected and tested against the databank. For the sequence ^{329}SSSVSS^{334} of ICL4, an identical sequence was found in the database for two Ig structures (Ser^{26} to Ser^{31}; database identification numbers 1FIG, 2FBJ). The same was true for the adjacent sequence ^{336}LRSLL^{340}, for which a counterpart was found in the lysozyme structure (Leu^{103} to Leu^{107}, database identification number 1LBA). Again, the connected fragments adopted a U-shaped conformation similar to the corresponding rhodopsin structure (red and blue dashed lines in Fig. 4). Taken together, these data strongly suggest that the putative ICL4 of the V_2 receptor and rhodopsin have comparable conformations.

ICL4 of rhodopsin is stabilized mainly by internally oriented hydrophobic residues that constitute a hydrophobic core. A similar hydrophobic core is predicted to occur in the V_2 receptor. It is formed mainly by residues Val^{332}, Leu^{336}, and residue Leu^{340} of the glutamate/dileucine motif. Residues Pro^{349}, Leu^{351}, and Pro^{353} may also contribute to these hydrophobic interactions. Most likely, residue Glu^{335} of the glutamate/dileucine motif is also required to stabilize the U-like loop by forming a salt bridge to the adjacent residue Arg^{337}. Alternatively, a salt bridge to residue Arg^{346} may be present. Taking the complete receptor model into account, residue Leu^{339} of the glutamate/dileucine motif appears to not be involved in the stabilization of the U-like loop but seems to have a different function. In the rhodopsin structure, one side of ICL4 interacts with ICL1. In the V_2 receptor model, residue Leu^{339} faces outward from the hydrophobic core and constitutes a similar linkage by forming a close hydrophobic interaction with residue Leu^{62} of ICL1 and probably also with the adjacent residue Ala^{61} (Fig. 5). Our model suggests that residue Leu^{339} of the glutamate/dileucine motif contributes to a long-range interaction within the receptor molecule and that this residue in particular is required for transport-competent full-length receptor folding.

The idea that a hydrophobic cluster stabilizes the U-like loop of ICL4 and that another hydrophobic residue within this loop may be required for an interaction with ICL1 is also supported by the results of bioinformatic analysis and basic local alignment search tool searches (Fig. 6). Alignment of the C-terminal tails identified about 70 GPCRs that have an equivalent hydrophobic sequence pattern (**hxxxhxxhh**) in the N-terminal part of their C termini, corresponding to

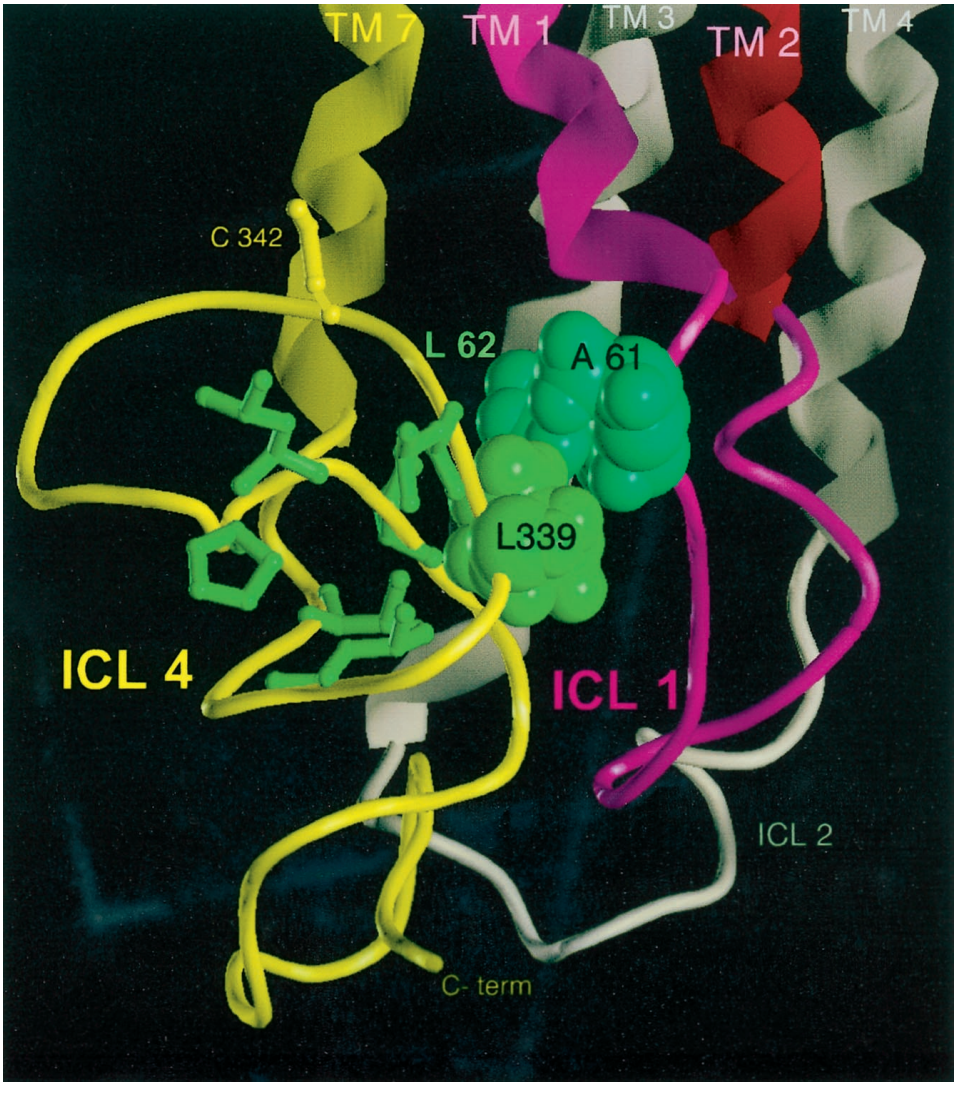

Fig. 5. Detail of a complete V_2 receptor model based on fused templates for transmembrane and intracellular portions. Only the helix attachments, the C-terminal tail (yellow), ICL1 (magenta), and ICL2 (blue) are shown. Within the C-terminal tail, the putative ICL4 forms a U-like loop that is stabilized by hydrophobic interactions (green residues). Residue Leu^{339} is directed out from the hydrophobic core and constitutes a close hydrophobic interaction with Leu^{62} and Ala^{61} (green-blue) of ICL1.

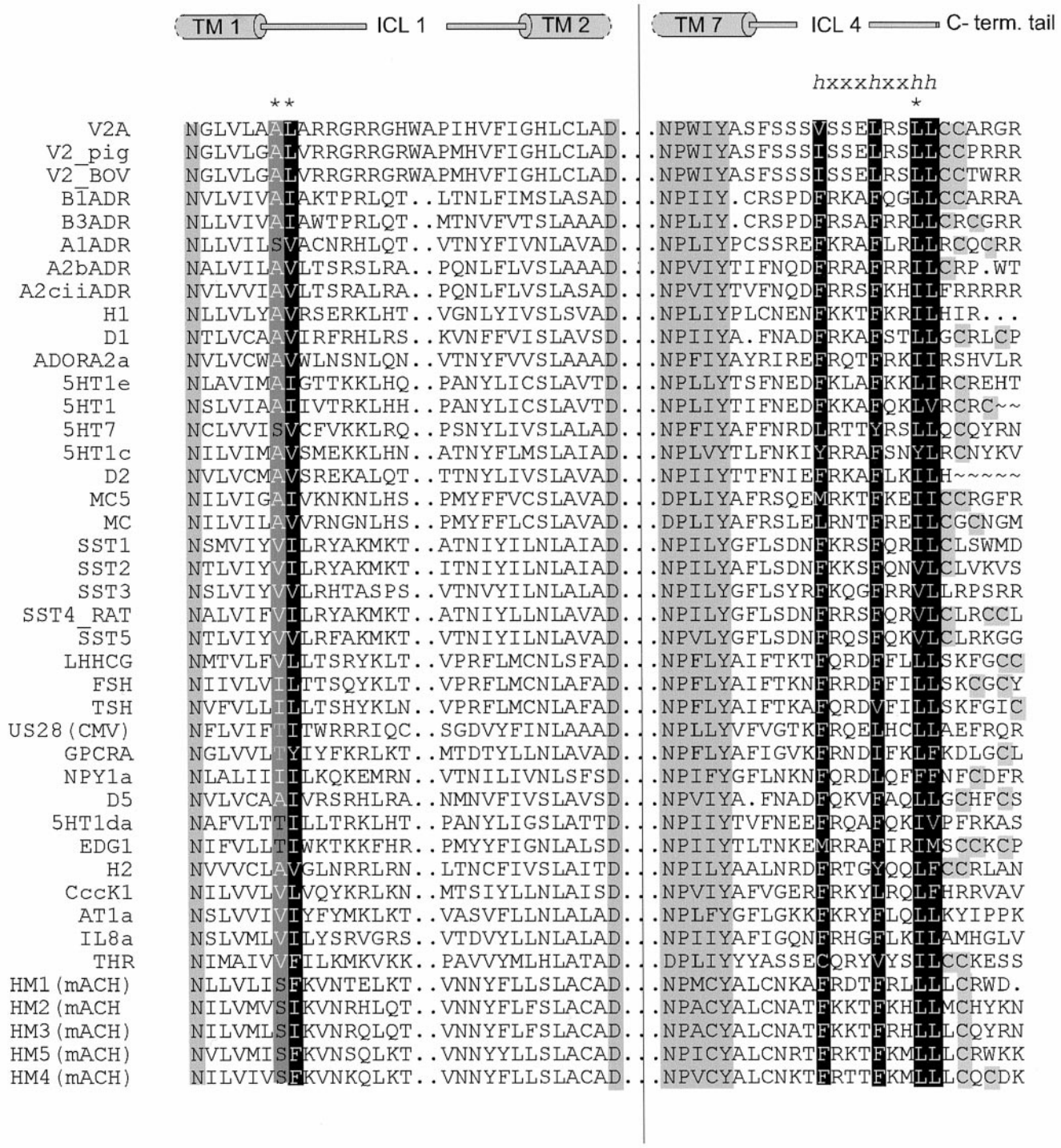

Fig. 6. Putative hydrophobic folding motifs in ICL1 and ICL4 of GPCRs. Only homologies of residues relevant to this work are shown. The data set was constructed from the SWISS-PROT and EMBL databanks. ICL1 and ICL4 sequences were aligned with the Wisconsin package. Human sequences are shown when available. The frames show (from left to right) 1) the highly conserved asparagine residue of TM1; 2) a pair of residues in ICL1 (hydrophobicity is conserved more weakly for the first and strongly for the second residue); 3) the highly conserved aspartate residue of TM2; 4) the highly conserved NPXXY motif indicating the end of TM7; 5) ; the hydrophobic sequence motif **h**xxx**h**x**h**h corresponding to residues Val^{332}, Leu^{336}, and the dileucine motif Leu^{339}/Leu^{340} of the V_2 receptor; and 6) the putative palmitoylated cysteine residues. Potentially interacting residues between ICL4 and ICL1 are indicated by an asterisk. Abbreviations were adopted from the original SWISS-PROT or EMBL data files: B1ADR, β_1-adrenergic receptor; B3ADR, β_3-adrenergic receptor; A1ADR, α_1-adrenergic receptor; A2bADR, α_{2b}-adrenergic receptor; A2ciiADR, α_{2cII}-adrenergic receptor; H1, histamine receptor type 1; D1, dopamine receptor type 1; ADORA2a, adenosine receptor type 2a; 5HT1e, 5-hydroxytryptamine receptor type 1e; 5HT7, 5-hydroxytryptamine receptor type 7; D2, dopamine receptor type 2; MC5, melanocortin receptor type 5; MC, melanocortin receptor; SST1, somatostatin 1 receptor; SST4, somatostatin 4 receptor; SST28a, somatostatin 5 receptor (SSTR5); SST3, somatostatin 3 receptor; LHHCG, luteinizing hormone human choriogonadotrophin receptor; FSH, follicle-stimulating hormone receptor; TSH, thyrotropin receptor; US28 (CMV), cytomegalovirus G-protein-coupled receptor; GPCRA, human putative G-protein-coupled receptor; NPY1a, neuropeptide Y receptor type 1a; D5a, dopamine receptor type 5a; EDG1, epithelial cell G-protein-coupled receptor type 1; CccK1, C-C chemokine receptor type 1; AT1a, vascular type-1 angiotensin II receptor; IL8a, interleukin 8 receptor; THR, thrombin receptor precursor; HM1 (mACH), muscarinic acetylcholine receptor HM1; HM2 (mACH), muscarinic acetylcholine receptor HM2; HM3 (mACH), muscarinic acetylcholine receptor HM3; HM4 (mACH), muscarinic acetylcholine receptor HM4.

residues Val[332], Leu[336], and the dileucine motif Leu[339]/Leu[340] of the V_2 receptor (Fig. 6). For the identified receptors, hydrophobic residues that could serve a similar function as residues Leu[62] and Ala[61] of the V_2 receptor are also conserved in the ICL1 domains.

Mutant Receptors L62P and ΔL62-R64 Are Retained in the ER. Mutations in the V_2 receptor gene cause X-linked nephrogenic diabetes insipidus (NDI; Oksche and Rosenthal, 1997). Interestingly, in two affected families, mutations were found in the ICL1 region, which is predicted to interact with residue Leu[339] of the intracellular C terminus. In one mutant receptor, residue Leu[62] is replaced by a proline residue, and hydrophobicity is decreased (L62P mutant; Knoers et al., 1994). In the other mutant receptor, the sequence [62]LAR[64] is deleted, and hydrophobicity is decreased even further (ΔL62-R64 mutant; Bichet et al., 1994). Although the NDI phenotype implicates that these mutant receptors are not functional; their precise defects are not yet characterized. Taking the V_2 receptor model into account, these mutant receptors should also be trapped in the ER. To prove this hypothesis, the L62P and the ΔL62-R64 mutation were introduced by site-directed mutagenesis into the cDNAs encoding the full-length untagged and GFP-tagged V_2 receptors. The pharmacological properties of the mutant receptors were determined with the untagged receptors; [^{3}H]AVP binding assays (Fig. 7A) with intact cells revealed a typical binding curve for cells expressing the wild-type V_2 receptor (K_D = 1.6 nM), whereas no specific binding sites were detected for cells expressing mutant receptors L62P or ΔL62-R64. Similarly, efficient adenylyl cyclase stimulation (EC_{50} = 1.3 nM). was only observed with crude membrane preparations expressing the wild-type V_2 receptor (Fig. 7B). For mutant receptor L62P, cAMP formation was barely detectable but only when very high AVP concentrations up to 10 μM were used. The adenylyl cyclase assay is more sensitive than the [^{3}H]AVP binding assay, and it can be concluded that ligand binding is not completely abolished for this mutant receptor. In contrast to mutant receptor L62P, no adenylyl cyclase stimulation was detected in membranes expressing mutant receptor ΔL62-R64, demonstrating that this receptor is nonfunctional.

The intracellular transport of the mutant receptors was assessed with GFP fusions in transiently transfected HEK 293 cells by laser scanning microscopy localization of GFP fluorescence signals (Fig. 8A). For both mutant receptors, the GFP fluorescence signals diffusely filled the cell's interior, with the exception of the nuclei, demonstrating a transport defect for mutant receptors L62P and ΔL62-R64. Consistently, no overlap with the fluorescence signals of the cell surface marker trypan blue was detected (data not shown). Analysis of the glycosylation state (Fig. 8B) revealed only the presence of the 60- to 65-kDa high-mannose forms, demonstrating that mutant receptors L62P and ΔL62-R64 are retained in the ER. The barely detectable adenylyl cyclase stimulation in crude membranes expressing mutant receptor L62P (see above) thus seems to derive from these intracellular rather than from cell surface receptors.

The ER retention of the NDI-causing mutant receptors L62P and ΔL62-R64 is consistent with a requirement of residue Leu[62] of the ICL1 region for full-length receptor folding and with the predictions of the structure model, i.e., with the proposed hydrophobic interaction of residue Leu[339] of ICL4 with residue Leu[62] of ICL1.

Discussion

By deleting the major part of the V_2 receptor, we have constructed a receptor fragment that allows study of the transport functions of the intracellular C terminus independent of full-length receptor folding. Mutation of the glutamate/dileucine motif within the C-terminal tail did not affect

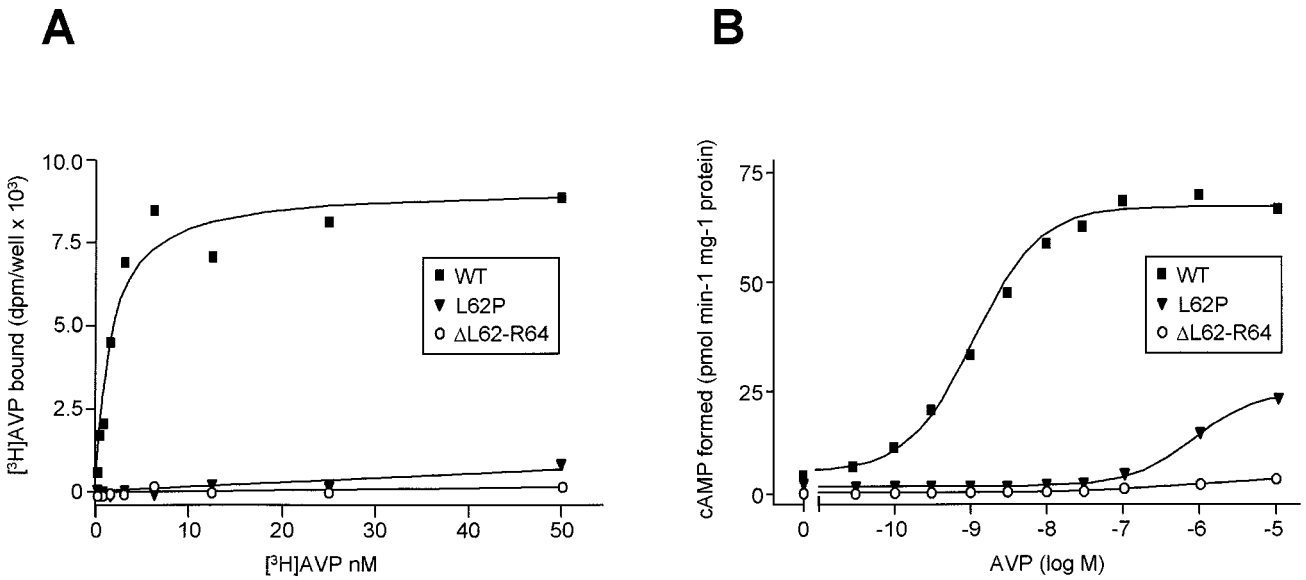

Fig. 7. Pharmacological properties of the NDI-causing full-length mutant V_2 receptors L62P and ΔL62-R64. A, specific [^{3}H]AVP binding to intact, transiently transfected HEK 293 cells expressing wild-type (WT) and mutant V_2 receptors. Data represent mean values of duplicates that differed by <5%. The calculated K_D value for the wild-type V_2 receptor is 1.6 nM. In all binding experiments, unspecific binding contributed up to 30% of total binding. The results are representative of two individual experiments. B, AVP dose-response curves of crude membranes derived from transiently transfected HEK 293 cells expressing wild-type and mutant V_2 receptors. Data represent mean values of duplicates that differed by <10%. The calculated EC_{50} for the wild-type V_2 receptor was 1.3 nM. Note that an EC_{50} value for the residual adenylyl cyclase stimulation by mutant receptor L62P cannot be calculated, because saturation was not observed even with 10 μM AVP.

cell surface transport in this system, strongly suggesting that the motif is required for transport-competent folding of the full-length receptor to pass the quality-control system of the ER rather than representing a small, linear transport signal that is recognized by a component of ER to Golgi transport vesicles. Our homology model consistently predicted that this motif is part of a folding relevant U-like loop within the C-terminal tail. The individual residues of the glutamate/dileucine motif, however, appear to have different functions.

Leu^{339} may contribute to a long-range hydrophobic interaction within the receptor molecule by binding to residue Leu^{62} and probably also to residue Ala^{61} of ICL1. We thus postulate that Leu^{339} is a key residue for the establishment of a transport-competent folding state in the intracellular C terminus. The NDI-causing mutations L62P and ΔL62-R64 led to receptors that are retained in the ER. Although not a direct proof, these results are consistent with the view that residue Leu^{62} of ICL1 is required for full-length receptor folding, as is the case for residue Leu^{339} of ICL4. It is also compatible with the postulated hydrophobic ICL1-ICL4 interaction that replacement of the adjacent residue Ala^{61} by a similarly hydrophobic valine residue leads to receptors with wild-type properties (Pan et al., 1994).

In contrast to residue Leu^{339}, residue Leu^{340} is predicted to be involved in local hydrophobic interactions. It stabilizes the U-like loop by strengthening its hydrophobic core and should thus contribute indirectly to the ICL4-ICL1 interaction, because the formation of the U-like loop appears to be a prerequisite for the correct exposure of Leu^{339}.

The model-derived functions of residues Leu^{339} and Leu^{340} are also supported by our previous data (Schülein et al., 1998). Transport of the full-length receptor was impaired only when residues Leu^{339} and Leu^{340} were replaced by threonine residues. In contrast, isoleucine residues were tolerated. The significance of the hydrophobicity rather than the absolute identity of the leucine residues is in agreement with the prediction that both are involved in (albeit different) hydrophobic interactions. Moreover, the observation that threonine substitution of residue Leu^{339} abolished receptor transport, whereas the substitution of residue Leu^{340} only reduced it to 40% of wild-type level (Schülein et al., 1998), is also explicable by the model. It is conceivable that mutation of residue Leu^{339} would largely impair receptor folding by disrupting the linkage to ICL1, whereas mutation of residue Leu^{340} may be compensated, at least in part, by the hydrophobic residues Val^{332} or Leu^{336}, which should also stabilize the core of the U-like structure.

Residue Glu^{335} most likely forms a salt bridge with the adjacent residue Arg^{337} and may thus also enable the correct exposure of residue Leu^{339} by stabilizing the U-like loop. The strong influence of residue Arg^{337} on receptor delivery to the plasma membrane is consistent with this proposal (Oksche et al., 1998). However, because of the location of residue Glu^{335} in the central part of the U-like loop, the formation of an alternative salt bridge with residue Arg^{346} cannot be excluded, and further experiments are needed to clarify this point. In any case, residue Glu^{335} appears to be folding relevant, explaining the transport defect of the corresponding full-length mutant receptor.

The V_2 receptor model also allows predictions concerning the transport relevance of the other residues of ICL4. As mentioned above, the hydrophobicity of residues Leu^{336} and Val^{332} should be required for the stabilization of the hydrophobic core of the U-like turn similar to residue Leu^{340}. Decreasing hydrophobicity at these positions should lead to a decreased cell surface delivery of the full-length receptors, as observed previously for an L340T mutant (Schülein et al., 1998). The same may be true for the hydrophobic residues Pro^{349}, Leu^{351}, and Pro^{353} in the distal portion of the intracellular C terminus, which may also stabilize the hydrophobic core. The observation that truncation of the intracellular C terminus at residue P^{349} led to decreased cell surface transport (40% of the wild-type level; Oksche et al., 1998) is consistent with this view.

Our results do not support the concept that the glutamate/dileucine motif of ICL4 contains a small linear transport signal that is recognized on the cytoplasmic side by a component of ER to Golgi transport vesicles, such as a coat protein. Instead, they strongly suggest that an interaction between

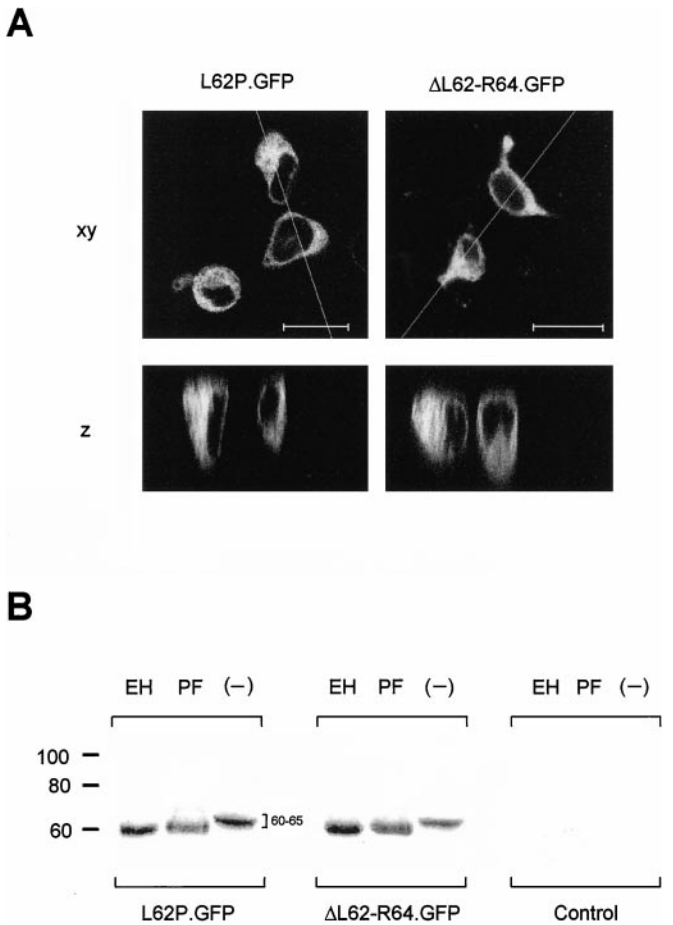

Fig. 8. Intracellular transport of the full-length mutant V_2 receptors L62P and ΔL62-R64. A, localization of GFP-tagged wild-type (WT.GFP) and mutant V_2 receptors in living transiently transfected HEK 293 cells. The GFP fluorescence signals were analyzed by confocal laser scanning microscopy with horizontal xy-scans and with vertical z-scans at the indicated lines. The scans show representative cells. Scale bar = 25 μm. B, SDS-PAGE/immunoblot analysis of transiently transfected HEK 293 cells expressing wild-type and mutant V_2 receptors. Crude membranes (50 μg of protein) 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 with a polyclonal anti-GFP antiserum and anti-rabbit ^{125}I-IgG. Untransfected HEK 293 cells (control) were used as a control for antibody specificity. See Fig. 3A for positive (WT.GFP) and negative (L339/340T.GFP) controls for complex glycosylations.

ICL4 and ICL1 is required for transport competent folding of the V_2 receptor in the ER. The reasons why prevention of this interaction might disfavor passage of the protein through the ER are completely unknown. The chaperones of the ER proofreading system that are involved in the retention of inappropriately folded membrane proteins, e.g., calnexin-calreticulin and/or Ig heavy-chain binding protein, are located in the ER lumen, whereas misfolding because of a lack of any ICL4-ICL1 interaction would occur on the cytoplasmic side of the receptor. An abolished ICL4-ICL1 interaction may, however, lead not only to a folding defect on the cytoplasmic side of the receptor but also, as a consequence, to misfolding of the entire receptor molecule; a prolonged association with the ER chaperones Ig heavy-chain binding protein and/or calnexin-calreticulin on the luminal side might then be promoted. Such a relatively strong influence of the intracellular portions of a GPCR on full-length receptor folding is in agreement with the work of Yeagle et al. (1997), whose data indicate that the complexed intracellular portions of rhodopsin fold correctly even in the absence of the extracellular and TM domains. Recently, it was shown for the cystic fibrosis transmembrane conductance regulator protein that chaperones on the cytoplasmic side of the ER (human DnaJ 2/heat-shock cognate 70) may also interact with the cytoplamic domains of membrane proteins (Meacham et al., 1999). If a prolonged association of these chaperones with misfolded cytoplasmic domains were to contribute to a proofreading mechanism, as is the case for luminal ER chaperones, our results would be explainable by misfolding of the cytoplasmic domains alone.

The hypothesis that the cytoplasmic domains of GPCRs may be in tight contact may also have implications for receptor signaling and desensitization. The rigid body motion theory for GPCRs, initially developed for rhodopsin, states that movements of TMs relative to one another occur during receptor activation and that these movements may cause the conformational changes of the cytoplasmic receptor domains that are needed, e.g., for G-protein coupling (Farrens et al., 1996). If the cytoplasmic domains are in tight contact, movement of a TM may not only induce a conformational change to the cytoplasmic domain to which it is directly connected but also to those interacting with it. In particular, binding of ICL1 to the intracellular C terminus may constitute a structural basis for transducing long-range conformational changes within the cytoplasmic face of a GPCR that may be necessary for receptor signaling and desensitization.

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