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A Dileucine Sequence and an Upstream Glutamate Residue in the Intracellular Carboxyl Terminus of the Vasopressin V_2 Receptor Are Essential for Cell Surface Transport in COS.M6 Cells

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ABSTRACT

Little is known concerning the intracellular transport of the G protein-coupled receptors (GPCRs). Previous studies suggested a functional role for those residues immediately preceding the conserved palmitoylated cysteine residues in the intracellular carboxyl termini of some GPCRs in cell surface transport. For the human vasopressin V_2 receptor, we assessed the significance of a dileucine sequence with an upstream glutamate residue (ELRSLLCC) in mediating cell surface delivery. A series of deletion and point mutants in this region were constructed, and the mutant receptors were expressed in transiently transfected COS.M6 cells. By using [^{3}H]arginine vasopressin binding assays to intact cells and immunofluorescence studies with intact and permeabilized cells, we show that residues E335 (mutant E335Q) and L339 (mutant L339T) are obligatory for receptor transport to the plasma membrane. Residue L340 has a minor but significant influence. [^{3}H]Arginine vasopressin binding experiments on membranes of lysed cells failed to detect any intracellular binding sites for the transport-deficient mutant receptors, suggesting that residues E335 and L339 participate in receptor folding. Studies with green fluorescent protein-tagged receptors demonstrate that the bulk of the mutant receptors E335Q and L339T are trapped in the endoplasmic reticulum. Complex glycosylation was absent in these mutant receptors, supporting this conclusion. These data demonstrate that the glutamate/dileucine motif of the vasopressin V_2 receptor is critical for the escape of the receptor from the endoplasmic reticulum, most presumably by establishing a functional and transport-competent folding state. A databank analysis revealed that these residues are part of a conserved region in the GPCR family.

The heptahelical GPCRs represent one of the largest protein families in eukaryotic cells (Strader *et al*., 1994). The signal transduction pathways have been investigated for many different receptor types, and a large number of structure-function studies have defined receptor sequences that are essential for ligand binding, G protein coupling, and desensitization. In contrast, comparatively little is known concerning the sequence requirements for the transport of these proteins to the plasma membrane.

In several studies, carboxyl-terminally truncated receptor fragments have been used to determine the minimal sequence requirements for cell surface transport of a GPCR. For the rat m3 muscarinic receptor, it was shown by immunofluorescence studies that receptor fragments comprising only the amino-terminal two-, three-, four-, five-, or six-TMs were still transported to the plasma membrane (Schöneberg *et al*., 1995). In contrast, rat glucagon receptor fragments containing one, three, or five amino-terminal TMs were transport deficient (Unson *et al*., 1995). The fragments were localized by EndoH and PNGaseF deglycosylation studies in the ER, and it was proposed that seven TMs must be present for cell surface delivery. Equivalent results were obtained for bovine rhodopsin fragments containing one to five amino-terminal transmembrane segments that failed to escape from the ER (Heymann *et al*., 1997).

A crucial role of the intracellular carboxyl terminus for cell surface transport was shown in the case of the rat LH/CG receptor. Truncations carboxyl terminal to the conserved palmitoylated cysteine residues had no effect, whereas dele-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AVP, arginine vasopressin; ER, endoplasmic reticulum; EndoH, endoglycosidase H; GFP, green fluorescent protein; LH/CG, luteinizing hormone/choriogonadotrophin receptor; PhoA, *Escherichia coli* alkaline phosphatase; PNGaseF, peptide N-glycosidase F; TM, transmembrane domain.

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tion of the two cysteines and four additional residues located amino terminal to them abolished cell surface transport (Rodriguez *et al.*, 1992). Similar results were reported recently for the V_2 receptor: mutation of the palmitoylated cysteine residues did not abolish receptor transport but reduced it significantly (Schülein *et al.*, 1996a). Truncation at R337, deleting only four additional residues amino terminal of the palmitoylated cysteine residues, abolished receptor transport to the plasma membrane (Sadeghi *et al.*, 1997; Oksche *et al.*, 1998). In contrast, truncation at C341 resulted in transport-competent receptors (Sadeghi *et al.*, 1997). The results reported for the LH/CG and the V_2 receptors suggest that sequences amino terminal of the palmitoylated cysteine residues have a crucial role in the cell surface delivery of these proteins.

In the case of the V_2 receptor, the two preceding leucine residues L339 and L340 resemble a typical dileucine motif. For membrane proteins unrelated to GPCRs, these motifs have been shown to function as sorting signals for basolateral cell surface transport (Hunziker *et al.*, 1994; Sheikh *et al.*, 1996), lysosomal targeting (Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992), and endocytosis (Letourneur and Klausner, 1992). The dileucine motif is sometimes accompanied by an upstream glutamate residue (Pond *et al.*, 1995), as seen in the V_2 receptor (ELSRLLCC).

Here, we assessed in detail the functional significance of these residues for V_2 receptor transport by using point mutations. We show that residues E335 and L339 are critical for the escape of the V_2 receptor from the ER, most presumably by helping establish a correct and transport-competent folding state. Residue L340 has minor but significant influence on this process.

Experimental Procedures

Materials. [^{3}H]AVP (2.4 TB/mmol) was purchased from Du Pont (Bad Homburg, Germany). Lipofectin was purchased from GIBCO BRL (Eggenstein, Germany). Restriction enzymes EndoH and PNGaseF were from New England Biolabs (Schwalbach, Germany). Rhodamine 6G was from Molecular Probes (Leiden, The Netherlands). Trypan blue from Seromed (Berlin, Germany). Aprotinin, benzamidine, 1,4-diazabicyclo[2.2.2.]octane, phenylmethylsulfonyl fluoride, trypsin inhibitor, and nitro blue tetrazolium were from Sigma (München, Germany). 5-Bromo-4-chloro-3-indolylphosphate was from Biomol (Hamburg, Germany). The monoclonal anti-c-*myc* antibody, alkaline phosphatase-conjugated anti-mouse IgG, and the cyanine 3-conjugated anti-mouse IgG were from Dianova (Hamburg, Germany). The monoclonal anti-GFP antibody was from Clontech Laboratories (Heidelberg, Germany). All other reagents were from Merck (Darmstadt, Germany). Plasmid pRCDN2 (Schülein *et al.*, 1996a), encoding the V_2 receptor, and plasmid pEU367.PhoA, encoding a PhoA-tagged V_2 receptor, have been described previously (Schülein *et al.*, 1996b). Vectors pCDNA1.Neo and pEGFP-N1 were from InVitrogen (Leek, The Netherlands) and Clontech Laboratories (Heidelberg, Germany), respectively. African green monkey kidney (COS.M6) cells were a gift from F. Fahrenholz (Frankfurt, Germany).

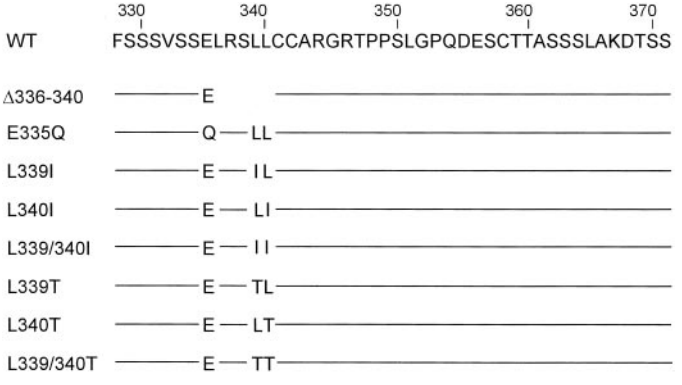

Fig. 1. Site-directed mutagenesis of the glutamate/dileucine sequence motif of the human V_2 receptor. *Top*, amino acid sequence of the entire intracellular carboxyl terminus of the wild-type V_2 receptor from F338 to S371. *Bottom*, mutants constructed by site-directed mutagenesis.

Isolation of crude membrane fractions of transiently transfected COS.M6 cells containing GFP-tagged receptors and EndoH/PNGaseF treatment. Crude membranes of transiently transfected COS.M6 cells were isolated from confluent cells grown on two 35-mm-diameter dishes as described previously (Schülein *et al.*, 1996b). Membranes (50 μg of protein) were incubated with or without EndoH or PNGaseF according to the supplier's recommendations.

Immunoblots. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide) and blotted onto nitrocellulose as described (Khyse-Andersen *et al.*, 1984). Filters were blocked for 1 hr with blotting buffer (20 mM Tris·HCl, 150 mM NaCl, 5% low-fat milk powder, 1% Triton X-100, pH 7.0) and incubated with monoclonal anti-GFP antibodies (dilution 1:1000 in blotting buffer) for 1 hr at room temperature. Filters were washed four times (15 min each) with blotting buffer and incubated with anti-rabbit alkaline phosphatase-conjugated IgG (dilution 1:5000 in blotting buffer) for 1 hr at room temperature. Filters were washed four times (10 min each) with blotting buffer, twice (10 min each) with the same buffer lacking milk powder, and once (5 min) with 10 mM Tris·HCl, pH 9.5. Filters were incubated in staining solution (0.56 mM 5-bromo-4-chloro-3-indolylphosphate, 0.48 mM nitro blue tetrazolium) until bands became visible.

Construction of V_2 receptor point mutations. For site-directed mutagenesis, the wild-type V_2 receptor cDNA was cloned from plasmid pRCDN2 (Schülein *et al.*, 1996a) into M13mp19 as a *Bam*HI/*Xba*I fragment. Site-directed mutagenesis was carried out with the Sculptor *In Vitro* Mutagenesis System (Braunschweig, Germany). The oligonucleotides were: 5′-CAGCGTGTCCTCAGAGCTCTGCTGTGCCCGG-3′ (Δ336–340), 5′-CAGCAGCGTGTCCTCACAGCTGCGAAGCTTGC-3′ (E335Q), 5′-CAGAGCTGCGAAGCATTCTCTGCTGTGCCCG-3′ (L339I), 5′-GCTGCGAAGCTTGATTTGCTGTGCCCGG-3′ (L340I), 5′-CAGAGCTGCGAAGCATTATTTGCTGTGCCCGGGG-3′ (L339/340I), 5′-CAGAGCTGCGAAGCACTCTCT-

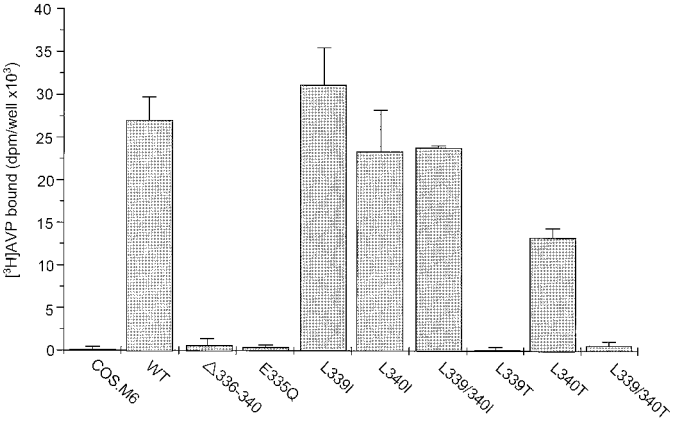

Fig. 2. Specific [^{3}H]AVP binding to intact, transiently transfected COS.M6 cells expressing wild-type and mutant V_2 receptors. Columns represent mean ± standard deviation values and are representative of three independent experiments. Vector-transfected COS.M6 cells were used as a control. The [^{3}H]AVP concentration (10 nM) was chosen close to saturation levels.

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GCTGTGCCCG 3′ (L339T), 5′-CAGAGCTGCGAAGCTTGACTT-GCTGTGCCCGGGGGAC-3′ (L340T), and 5′-C-AGAGCTGCGAA-GCACTACTTGCTGTGCCCGGGG-3′ (L339/340T). The mutant cDNAs were cloned as *Bam*HI/*Xba*I fragments into the eukaryotic expression vector pCDNAI.Neo, yielding plasmids pΔ336–340, pE335Q, pL339I, pL340I, pL339/340I, pL339T, pL340T, and pL339/340T, respectively.

Construction of c-*myc* epitope-tagged receptors. A c-*myc* epitope (EQKLISEEDL) was inserted by site-directed mutagenesis between Met3 and Ala4 in the amino termini of the V_2 receptors encoded by plasmids pRCDN2 (wild-type V_2 receptor), pE335Q, pL339T, pL340T, and pL339/340T, yielding plasmids pWT.myc, pE335Q.myc, pL339T.myc, pL340T.myc, and pL339/340T.myc (Anderson-Beckh B, Dehe M, Schülein R, Liebenhoff U, Wiesner B, Rosenthal W, and Oksche A, manuscript in preparation).

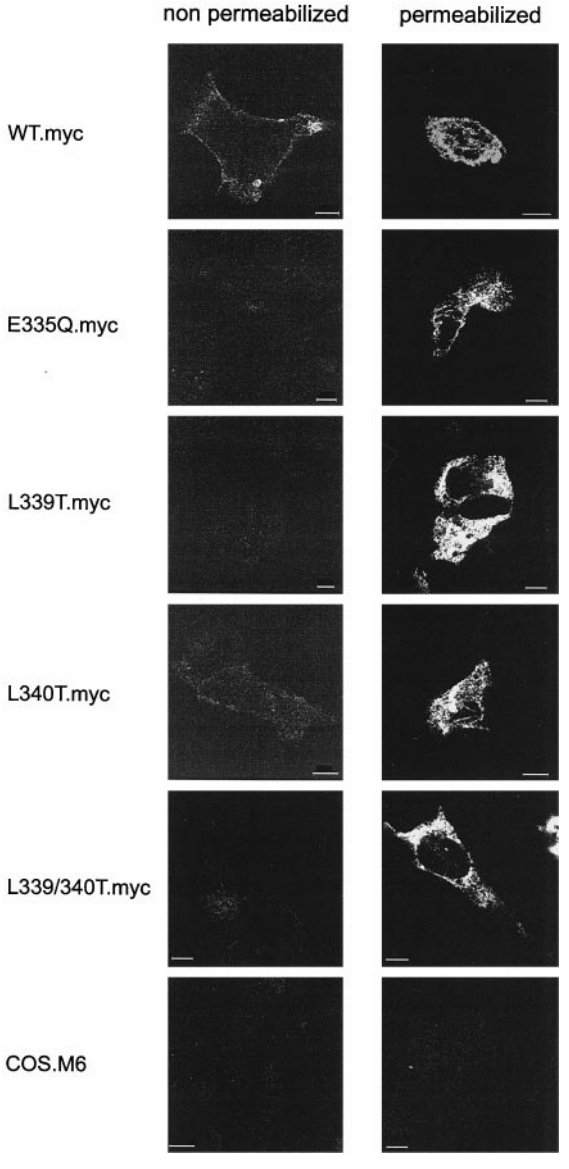

Fig. 3. Immunofluorescence studies with nonpermeabilized and permeabilized transiently transfected COS.M6 cells expressing amino-terminal c-*myc*-tagged wild-type and mutant V_2 receptors. Cells were grown on cover slips and stained with monoclonal anti-c-*myc* antibodies and cyanine 3-conjugated anti-mouse IgG. Immunofluorescence signals were analyzed by confocal microscopy. For the wild-type (WT.myc) and each mutant receptor (E335Q.myc, L339T.myc, L340T.myc, L339/340T.myc), nonpermeabilized cells (*left*) are compared with Triton X-100-permeabilized cells (*right*). Vector-transfected COS.M6 cells were used as a control. Each photograph shows a representative xy scan. *Scale bar*, 10 μm.

Construction of GFP-tagged receptors. We have recently shown that fusion of a 48-kDa PhoA enzyme portion to residue K367 of the V_2 receptor (i.e., to the entire receptor, lacking only the four carboxyl-terminal residues) yielded receptors with pharmacological properties similar to those of the untagged receptor (Schülein *et al*., 1996b). Therefore, the red shifted GFP variant (EGFP) was fused to the same residue of the wild-type V_2 receptor and mutants E335Q, L339T, L340T, and L339/340T. Polymerase chain reaction fragments were amplified from the corresponding plasmids using a 5′ primer (5′-CTGGGCCTGCTTTGCG-3′) complementary to nucleotides 647–662 of the V_2 receptor cDNA and a 3′ primer (5′- CCTCACGAT-GAAGGATCCTTGGCCAGGGAGG-3′) introducing a novel *Bam*HI site at nucleotide 1172 of the V_2 receptor cDNA. The PCR fragments were cut with *PmlI* and *Bam*HI and cloned first (for reasons not relevant to this report) into plasmid pEU367.PhoA (Schülein *et al*., 1996b), thereby replacing the wild-type *PmlI*/*Bam*HI fragment. From the resulting constructs, *Sac*I/*Bam*HI fragments were cloned into the GFP expression vector pEGFP-N1. The resulting plasmids were pWT.GFP, pE335Q.GFP, pL339T.GFP, pL340T.GFP, and pL339/340T.GFP.

Visualization of GFP-tagged receptors, cell surface, and ER staining in living transiently transfected COS.M6 cells. COS.M6 cells (4×10^4) in a 35-mm-diameter dish containing a cover slip were transfected with 500 ng of plasmid DNA and lipofectin according to the suppliers recommendations. Cells were incubated for 3 days (which resulted in optimal cell surface expression of transported receptors, data not shown). The cover slips with the adherent cells were washed twice with phosphate-buffered saline and transferred immediately into a self-made chamber (details provided on request). Cells were covered with 1 ml of phosphate-buffered saline, and GFP fluorescence was visualized on a Zeiss 410 invert laser scanning microscope (λ_{exc} = 488 nm, λ_{em} = >515 nm). Subsequently, either the cell surface or the ER of the same cells was stained with trypan blue (Wiesner B, Lorenz D, Krause E, Baeger I, Beyermann M, and Bienert M, manuscript in preparation) (0.05%, 1 min) or rhodamine 6G (5 μM, 20 min; Terasaki and Rees, 1992). Trypan blue (λ_{exc} = 543 nm, λ_{em} = >690 nm) and rhodamine 6G fluorescences (λ_{exc} = 543 nm, λ_{em} = >570 nm) were recorded on a second channel, and overlap with the GFP signals was computed.

Computer-based prediction of acidic/dihydrophobic motifs in the carboxyl-terminal tails of GPCRs. The data set was constructed from the SWISS-PROT database of the European Molecular Biology Laboratory (Heidelberg, Germany). GPCR sequences were analyzed with the Wisconsin Package (Genetics Computer Group, Madison, WI).

Miscellaneous. Standard DNA preparations and manipulations were carried out according to the handbooks of Sambrook *et al*. (1989). The nucleotide sequences of DNA fragments were verified using the FS Dye Terminator kit from Perkin Elmer (Weiterstadt, Germany). Cell culture, transient transfection of COS.M6 cells and [^{3}H]AVP binding assay to intact COS.M6 cells were carried out as described previously (Schülein *et al*., 1996a). Immunofluorescence studies with c-*myc* epitope-tagged V_2 receptors and [^{3}H]AVP binding assay to crude membranes of COS.M6 cells were as described previously (Oksche *et al*., 1998)

Results

Construction of V_2 receptors with mutations in the glutamate/dileucine sequence motif. To assess the significance of residues E335 and L339/340 in the carboxyl-terminal tail of the V_2 receptor for cell surface transport, we have constructed a series of deletion and point mutations in this region (Fig. 1). In mutant Δ336–340, both leucine residues and three upstream residues were eliminated. In mutant E335Q, the glutamate residue was replaced by glutamine. To determine whether the hydrophobicity or the two

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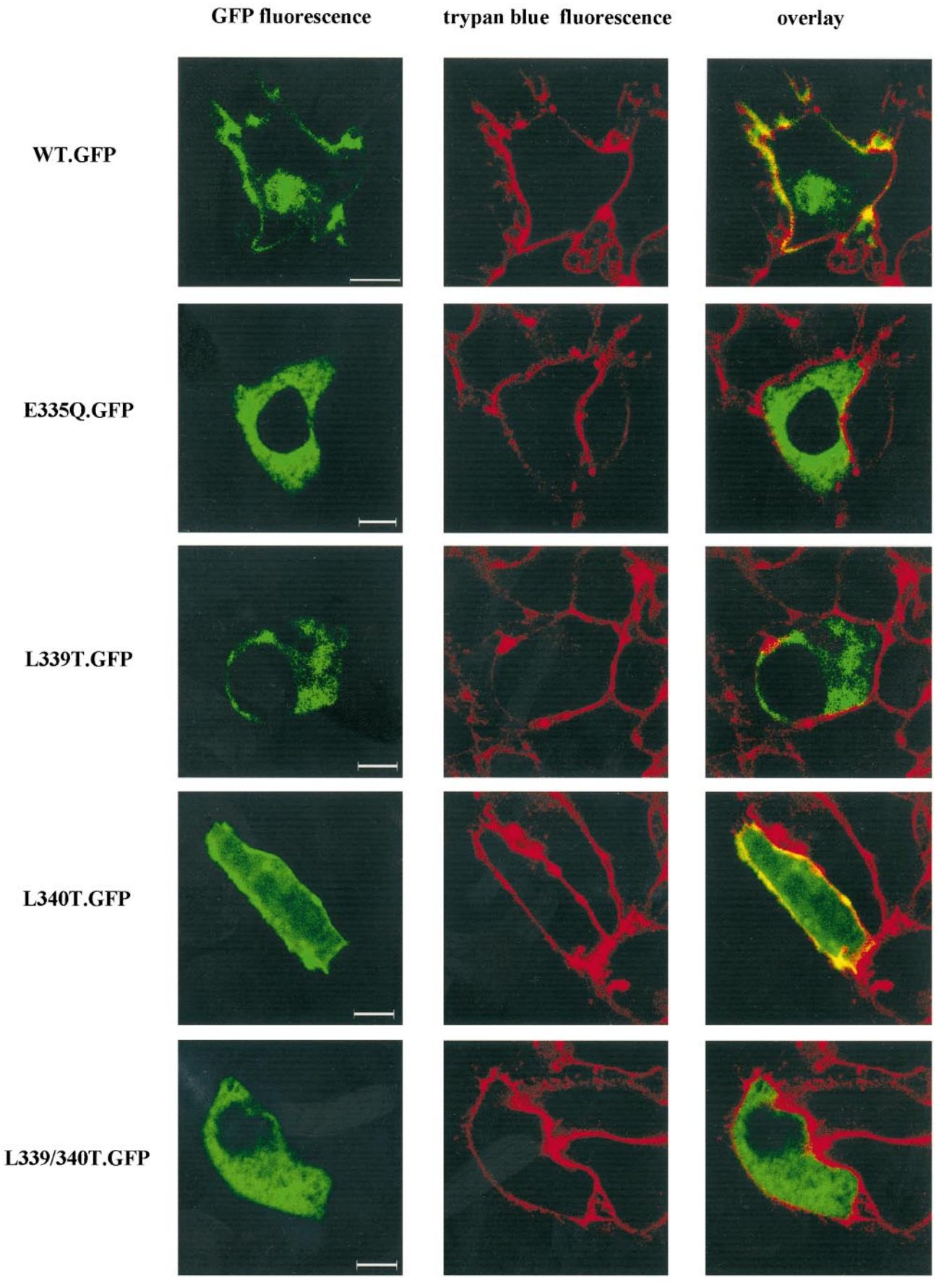

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leucine residues themselves are significant, they were exchanged both for hydrophobic isoleucine (single mutants L339I, L340I; double mutant L339/340I) and polar threonine (single mutants L339T, L340T; double mutant L339/340T).

Residues E335 and L339 are essential for cell surface transport of the V_2 receptor in transiently transfected COS.M6 cells. To assess the roles of E335, L339, and L340 for cell surface delivery, the mutant plasmids were transfected into COS.M6 cells, and [^{3}H]AVP binding assays to intact cells were performed (Fig. 2). We assumed that mutation of the carboxyl-terminal cytoplasmic loop would not interfere with the hormone binding site and that [^{3}H]AVP binding to intact cells would correlate with receptor transport to the plasma membrane. Deletion of residues L339 and L340 (mutant Δ336–340) eliminated [^{3}H]AVP-binding, indicating that cell surface expression of the receptor had been abolished. The same was true when exchanging residues L339 and L339/340 for threonine (mutants L339T, L339/340T) and E335 for glutamine (mutant E335Q). These results show the significance of residues E335 and L339 of the glutamate/dileucine sequence motif for cell surface transport of the V_2 receptor. Residue L340 seems not to be absolutely essential for receptor transport to the plasma membrane but has a significant influence on this process because its replacement by threonine reduced [^{3}H]AVP binding to 50% of the wild-type level (mutant L340T). Replacement of residues L339 and L340 by isoleucine (single mutants L339I, L340I; double mutant L339/340I) did not influence receptor transport, indicating that the hydrophobicity of the leucine residues, rather than their other properties, is critical for mediating cell surface delivery.

To confirm the results of the [^{3}H]AVP binding assays and to demonstrate that transport-deficient receptors are located inside the cells, immunofluorescence studies with COS.M6 cells transiently expressing amino-terminal c-*myc*-tagged receptors (WT.myc, E335Q.myc, L339T.myc, L340T.myc, L339/340T.myc) were performed (Fig. 3). To detect cell surface bound and intracellular receptors, monoclonal anti-c-*myc* antibodies were used with nonpermeabilized and permeabilized cells, respectively. For cells expressing WT.myc, a fluorescence signal was detected at the plasma membrane of intact cells and in the intracellular membrane compartments of permeabilized cells, indicating that WT.myc is transported to the cell surface. The intracellular signal of WT.myc was relatively strong, which may result from saturation of the transport system caused by overexpression. However, paraformaldehyde fixation may also explain this result because it causes aggregation of the ER and, consequently, an overproportionally strong intracellular signal. For mutant receptor L340T.myc, a fluorescence signal was detectable on intact cells and in permeabilized cells. However, the cell surface signal was much weaker than that of WT.myc, which is in agreement with the data from [^{3}H]AVP binding. In cells expressing E335Q.myc, L339T.myc, and L339/340T.myc, immunofluorescence was observed exclusively inside permeabilized cells, demonstrating that these mutant receptors were expressed but were not transported to the cell surface. In summary, the immunofluorescence data correlate with those from [^{3}H]AVP binding studies and confirm that residues E335 and L339 of the glutamate/dileucine sequence motif are essential for cell surface transport of the V_2 receptor and that residue L340 has an significant, although not absolute influence on this process.

To assess whether the transport defective mutant receptors were functional in the internal membrane system, binding of [^{3}H]AVP (10 nM) to crude sonicated membranes of disrupted cells (plasma membranes and internal membranes) was determined. With this methodology, essentially the same binding patterns were observed for wild-type and mutant receptors as for intact cells (data not shown, see Fig. 2). For mutants E335Q and L339T, the absence of internal binding sites suggests that the mutant receptors are not functional and may be misfolded. The residues concerned therefore may be necessary to establish a functional and transport-competent folding state.

Residues E335 and L339 are essential for receptor exit from the ER in transiently transfected COS.M6 cells. The data above raise the question as to the identity of the intracellular membrane compartment in which the mutant receptors are trapped. To localize the transport defective receptors in the intracellular membrane compartments, we constructed GFP fusion proteins. The autofluorescence of the GFP moiety, together with membrane compartment-specific fluorescence dyes, should allow investigation of this question in living cells. GFP was fused to the wild-type and relevant mutant receptor fragments consisting of 367 residues (the entire receptors lacking the four carboxyl-terminal residues), and fusion proteins (E335Q.GFP, L339T.GFP, L340T.GFP, and L339/340T.GFP) were expressed in COS.M6 cells. To first determine whether the GFP moiety affects the intracellular transport of the V_2 receptor, [^{3}H]AVP binding assays to intact cells expressing the wild-type GFP-tagged receptor were performed. The [^{3}H]AVP binding characteristics of the receptor-GFP fusion were similar to those of the receptor alone (K_D, 2.04 and 1.53 nM, respectively; sites/cell, 120,142 and 128,356, respectively), demonstrating that the GFP moiety affects neither ligand-binding properties nor receptor transport to the plasma membrane.

We next determined whether the results for cell surface transport obtained by [^{3}H]AVP binding and immunofluorescence studies (see Figs. 2 and 3) could be confirmed by the GFP methodology: localization of GFP fluorescence of mutants E335Q.GFP, L339T.GFP, L340T.GFP, and L339/340T.GFP was monitored by laser scanning microscopy in transiently transfected living COS.M6 cells (Fig. 4, *left*, *green*). The cell surface of the same cells was identified by the use of trypan blue (Fig. 4, *middle*, *red*; trypan blue does not penetrate living cells, and its autofluorescence is suitable to visualize the cell surface; Wiesner B, Lorenz D, Krause E, Baeger I, Beyermann M, and Bienert M, manuscript in preparation). Computer overlay of green GFP fluorescence and red trypan blue fluorescence allows identification of receptors

← **Fig. 4.** Cell surface localization of wild-type and mutant V_2 receptor-GFP fusion proteins in living transiently transfected COS.M6 cells. *Left*, GFP fluorescence of cells expressing fusions to the wild-type (WT.GFP) and mutant V_2 receptors (E335Q.GFP, L339T.GFP, L340T.GFP, L339/340T.GFP) is shown (*green*). *Middle*, after recording the GFP fluorescence, the cell surface of the same cells was stained with trypan blue whose fluorescence was recorded on a second channel (*red*). GFP fluorescence is detectable only in the case of cells that were successfully transfected, whereas cell surface trypan blue fluorescence is detectable for every cell present in the field of view. *Right*, GFP and trypan blue fluorescence were computer overlaid. *Yellow*, overlap. Each photograph shows a representative horizontal (xy) scan. Scale bar, 10 μm.

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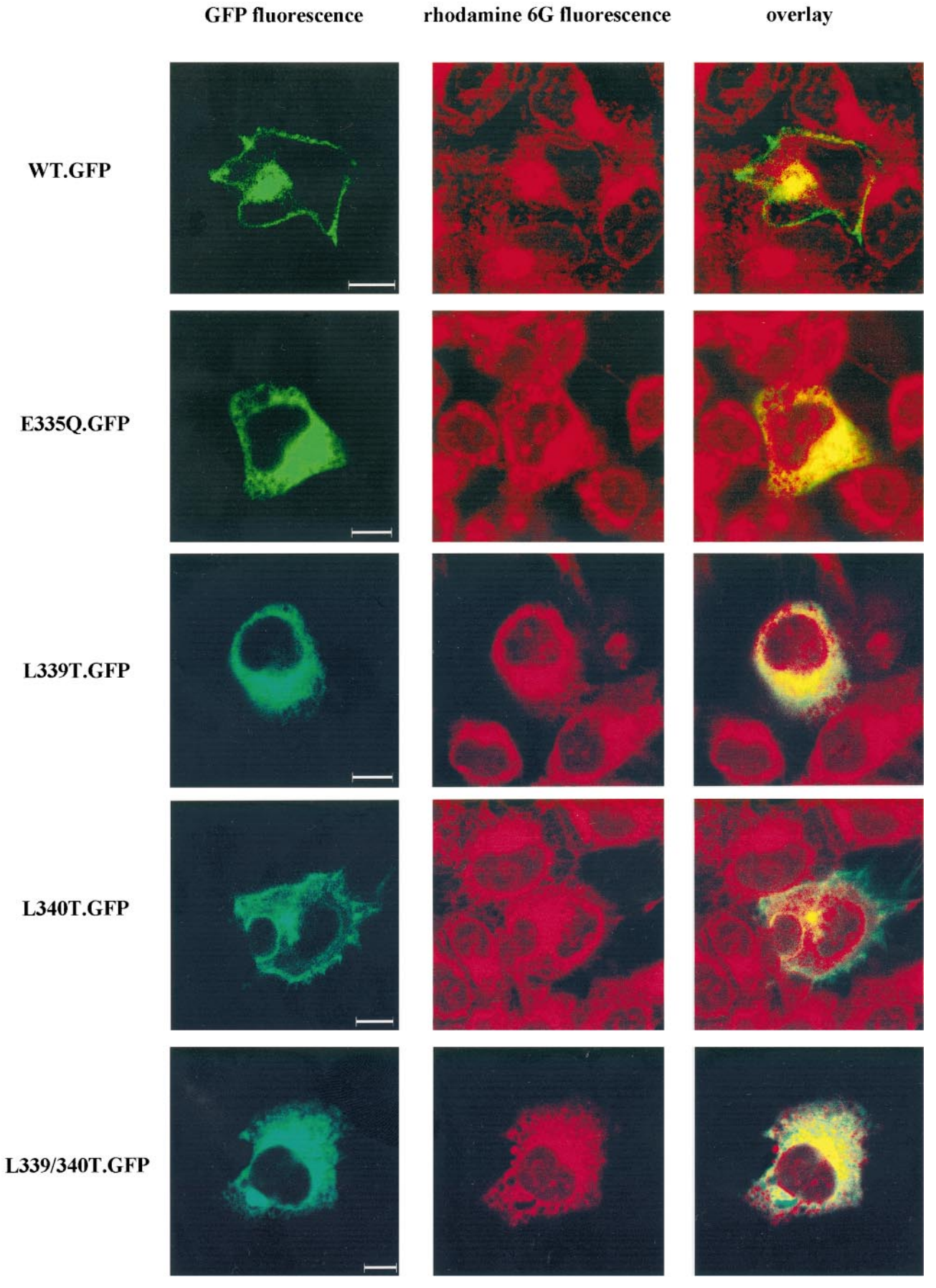

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that are transported to the cell surface (Fig. 4, *right*, colocalization is indicated by *yellow*).

For the wild-type receptor, a GFP signal was detected at the cell surface (Fig. 4, *WT.GFP*), indicated by its overlap with the trypan blue signal. Thus, WT.GFP is transported to the cell surface. Additional GFP signals were located inside the cells, presumably representing transport intermediates en route to the cell surface. However, the cell surface and internal GFP signals seem to be roughly equivalent, suggesting that a fraction of WT.GFP may also become trapped as a consequence of overexpression and saturation of the transport system. For mutant L340T.GFP, results similar to those with WT.GFP were obtained. However, a greater proportion of the GFP fluorescence was distributed diffusely throughout the cell, which may reflect the partial transport defect of this mutant. In contrast to WT.GFP and L340T.GFP, no overlap of GFP and trypan blue fluorescence was observed for mutants E335Q.GFP, L339T.GFP, and L339/340T.GFP. Instead, diffuse GFP fluorescence filled the interior of the cells with the exception of the nucleus, supporting the postulate that residues E335 and L339 are essential for cell surface transport.

To determine the membrane compartment in which the mutant receptors are trapped, we assessed colocalization of GFP fluorescence with the ER fluorescence marker rhodamine 6G. Localization of GFP fluorescence was monitored by laser-scanning microscopy in transiently transfected living COS.M6 cells (Fig. 5, *left*, *green*). The ER of the same cells was stained with rhodamine 6G (Fig. 5, *middle*, *red*), and signal overlap was computed (Fig. 5, *right*, colocalization is indicated by *yellow*). For the wild-type receptor (WT.GFP), the cell surface GFP signal surrounded the ER rhodamine 6G signal. Additional GFP fluorescence was detected inside the cell. These signals overlapped with the rhodamine 6G signals, indicative of transport intermediates. For mutant L340T.GFP, similar results were obtained (i.e., cell surface bound GFP signals were present, which did not overlap with the rhodamine 6G signals, and internal GFP signals were present, which did overlap). In contrast, the GFP fluorescence of mutants E335Q.GFP, L339T.GFP, and L339/340T.GFP was observed exclusively in the cell interior and overlapped almost exclusively with rhodamine 6G fluorescence. The slight GFP fluorescence of these mutants that did not overlap may be caused by the methodology: the GFP pictures had to be taken before the 20-min rhodamine 6G stain, and small changes in cell shape during this period may cause an overlay that is slightly out of focus. These data suggest that residues E335 and L339 are essential for normal transfer of the receptor from the ER to the Golgi.

If the transport-deficient fusion proteins indeed fail to escape from the ER, only core-glycosylated fusion proteins should be present in membrane fractions. In contrast, for WT.GFP, one would predict relatively large amounts of complex-glycosylated fusion proteins, and for mutant L340T.GFP, rather lower ones would be predicted, representing receptors at the cell surface. To address this question, membranes were isolated from transiently transfected COS.M6 cells expressing wild-type and mutant GFP fusion proteins and treated with EndoH to remove core glycosylations and with PNGaseF to remove both core and complex glycosylations. Fusion proteins were detected on Western blots with monoclonal anti-GFP antibodies (Fig. 6).

In all samples, an immunoreactive protein band with an apparent molecular mass of 57 kDa was detected. This protein is not related to the fusion proteins because it was also detected in the membranes of vector transfected COS.M6 cells. For WT.GFP, two specifically stained bands were detected: one broad band with an apparent molecular mass of 75–88 kDa and another of 60–65 kDa. On EndoH treatment, the 60–65-kDa band was shifted to a narrow 60-kDa band. The broad 60–65-kDa band therefore represents core-glycosylated forms of the wild-type fusion protein, and the narrow 60-kDa band represents the unmodified form. As expected, on PNGaseF treatment, the 60–65-kDa band also shifted to 60 kDa. The broad 75–88-kDa band was EndoH resistant, demonstrating that this band represents complex-glycosylated forms. On PNGaseF treatment, the complex-glycosylated 75–88-kDa band was shifted to 70 kDa rather than to

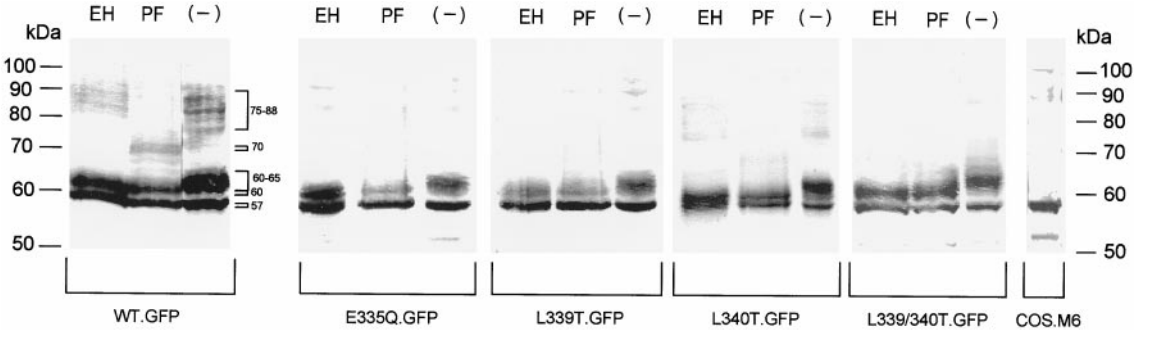

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblot analysis of transiently transfected COS.M6 cells expressing wild-type and mutant V_2 receptor-GFP fusion proteins. Crude membranes were isolated from COS.M6 cells expressing GFP-fusions to the wild-type (WT.GFP) and mutant V_2 receptors (E335Q.GFP, L339T.GFP, L340T.GFP, L339/340T.GFP). Membranes (50 μg of protein) were either untreated (−) or treated with EndoH (*EH*; to remove core glycosylations) or PNGaseF (*PF*; to remove core and complex glycosylations). Immunoreactive proteins were detected with monoclonal anti-GFP antibodies and alkaline phosphatase-conjugated anti-mouse IgG. Protein bands described in the text are indicated. Vector-transfected COS.M6 cells were used as a control. The immunoblot is representative of three independent experiments.

← **Fig. 5.** ER localization of wild-type and mutant V_2 receptor-GFP fusion proteins in living transiently transfected COS.M6 cells. *Left*, GFP fluorescence of cells expressing fusions to the wild-type (WT.GFP) and mutant V_2 receptors (E335Q.GFP, L339T.GFP, L340T.GFP, L339/340T.GFP) is shown (*green*). *Middle*, after recording the GFP fluorescence, the ER of the same cells was stained with rhodamine 6G whose fluorescence was recorded on a second channel (*red*). GFP fluorescence is detectable only in the case of cells which were successfully transfected, whereas ER rhodamine 6G fluorescence is detectable for every cell present in the field of view. *Right*, GFP and rhodamine 6G fluorescence were computer overlaid. *Yellow*, overlap. Each photograph shows a representative horizontal (xy) scan. *Scale bar*, 10 μm.

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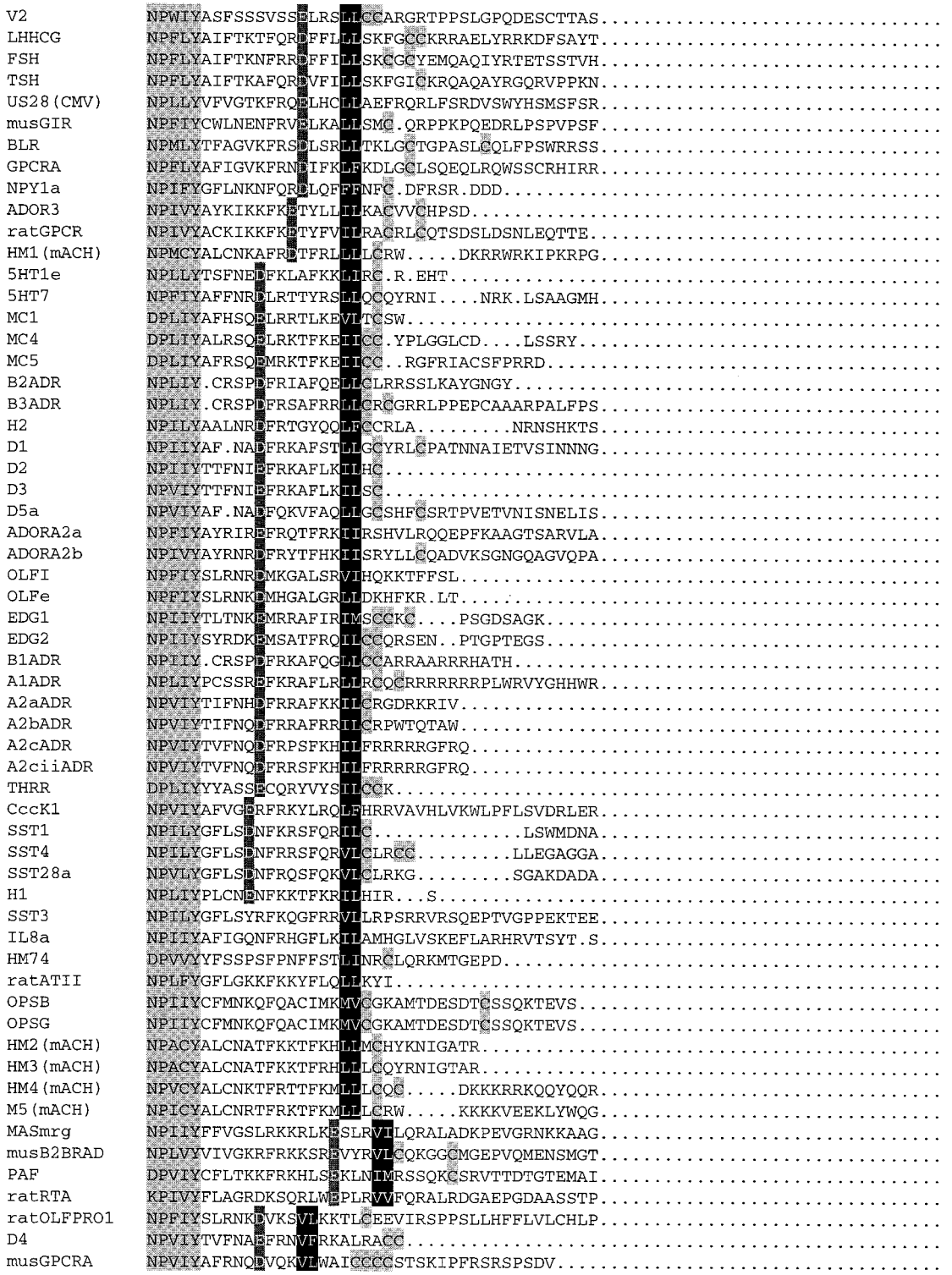

Fig. 7. Putative acidic/dihydrophobic sequence motifs in the carboxyl-terminal tails of GPCRs. The data set was constructed from the SWISS-PROT databank and sequences were aligned with the Wisconsin package. Human sequences are shown when available. The frames show (*left to right*) the conserved NPXXY motif indicating the end of the seventh TM, the conserved acidic residues (E or D), the dihydrophobic sequence (containing L, I, F, V, and M), and the putative palmitoylated cysteine residues (C). Abbreviations and descriptions were adopted from the original SWISS-PROT data files: V_2, vasopressin V_2 receptor; *LHHCG*, LH/CG; *FSH*, follicle-stimulating hormone receptor; *TSH*, thyrotropin receptor; *US28 (CMV)*, cytomegalovirus GPCR; *musGIR*, mouse probable GPCR (glucocorticoid-induced receptor, GIR); *BLR*, Burkitt's lymphoma receptor 1; *GPCRA*, human putative GPCR; *NPY1a*, neuropeptide Y receptor type 1a; *ADOR3*, A_3 adenosine receptor; *ratGPCR*, rat putative GPCR; *HM1 (mACH)*, muscarinic acetylcholine receptor HM1; *5HT1e*, 5-hydroxytryptamine receptor type 1e; *5HT7*, 5-hydroxytryptamine receptor type 7; *MC1*, melanocortin receptor type 1; *MC4*, melanocortin receptor type 4; *MC5*, melanocortin receptor type 5; *B2ADR*, β_2-adrenergic receptor; *B3ADR*, β_3-adrenergic receptor; *H2*, histamine receptor type 2; *D1*, dopamine receptor type 1; *D2*, dopamine receptor type 2; *D3*, dopamine receptor type 3; *D5a*, dopamine receptor type 5a; *ADORA2a*, adenosine receptor type 2a; *ADORA2b*, adenosine receptor type 2b; *OLFI*, olfactory receptor-like protein HGMP07I; *OLFe*, olfactory receptor-like protein HGMP07E (OR17–4); *EDG1*, epithelial cell GPCR type1; *EDG2*, epithelial cell GPCR type 2; *B1ADR*, β_1-adrenergic receptor;

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the expected unmodified 60-kDa form. These results correlate with our previous data with PhoA fusion proteins indicating that an additional post-translational modification must be present in the V_2 receptor that increases the apparent molecular mass (Schülein *et al*., 1996b). This modification seems to occur in a post-ER compartment because it is present only in the complex-glycosylated forms and not in the core-glycosylated forms, which can be shifted to the unmodified form on PNGaseF treatment. The calculated molecular mass (minus the 27-kDa GFP moiety) for the untagged complex-glycosylated receptor is 48–61 kDa, which is in good agreement with previous results (Tsukaguchi *et al*., 1995; Innamorati *et al*., 1996). For WT.GFP, the bulk of the immunoreactive receptor seems to exist in the core-glycosylated 60–65-kDa form because this band stained more deeply than that of the complex-glycosylated 75–88-kDa form. It must be noted, however, that the amount of the latter may be significantly underestimated because of the weaker signal density resulting from the broad molecular mass range of these forms. The observed amounts of complex- and core-glycosylated forms may, in this case, thus reflect the roughly equal distribution of intracellular and cell surface signals observed for WT.GFP in living cells (see Figs. 4 and 5). The core-glycosylated fraction of WT.GFP may represent transport intermediates en route to the cell surface and/or receptors that become trapped as a consequence of overexpression.

For mutant L340T.GFP, both core- and complex-glycosylated receptors were detected, although the complex-glycosylated forms were present only at low levels. The low levels of complex-glycosylated forms are consistent with a partial transport defect of this mutant. In contrast, for mutants E335Q.GFP, L339T.GFP, and L339/340T.GFP, only the core-glycosylated receptors were detected, and this is consistent with the trapping of these mutants in the ER. In summary, the observed glycosylation patterns of the mutant GFP-tagged receptors thus support the GFP and rhodamine 6G fluorescence data obtained by confocal microscopy in living cells (i.e., that residues E335 and L339 are necessary for escape from the ER and that residue L340 has a minor but significant influence on this process).

The total amount of immunoreactive protein was higher for WT.GFP than for all mutant GFP-tagged receptors, even in the case of the core-glycosylated forms. This may result either from lower synthesis levels of the mutant receptors, a possibility that was not supported by Northern blot analysis (data not shown) or, more likely, by an increased degree of degradation of the transport-incompetent mutant receptors in the ER. For mutants E335Q.GFP, L339T.GFP, and L339/340T.GFP, however, even lower synthesis levels would not explain the lack of complex-glycosylated forms: for mutant receptor L340T.GFP, the amount of core-glycosylated forms was equally low as for the other mutants, although complex-glycosylated forms were detectable.

Acidic/dihydrophobic motifs are conserved in the carboxyl-terminal tails of GPCRs. Because no glutamate/dileucine sequence motif had previously been reported for GPCRs, we conducted a sequence databank search for acidic/dihydrophobic structures within this protein family. Our results show that equivalent sequences are conserved in the carboxyl-terminal tails of GPCRs (Fig. 7). The majority are located between the seventh TM and the conserved putative palmitoylation sites at a distance of 10–15 residues from the seventh transmembrane segment. Several classes seem to exist regarding the distance of the acidic from the two hydrophobic residues (ranging from three to eight residues). Some receptors have a dihydrophobic sequence but lack an upstream acidic residue. The acidic residues aspartate and glutamate seem to have an almost equal frequency of distribution within the GPCR family (57% and 43%, respectively). In the dihydrophobic sequence, however, leucine residues are most frequent (57%), followed by isoleucine (22%), valine (13%), phenylalanine (5%), and methionine residues (3%). These findings may prove relevant for other GPCRs

Discussion

Our data demonstrate that residues E335 and L339 are necessary for the exit of the V_2 receptor from the ER. Residue L340 seems to have a minor but significant influence on this process.

One plausible interpretation of these results may be derived from the recent finding that a dihydrophobic phenylalanine pair of a membrane protein may contribute to a motif signaling ER-to-Golgi transport by binding to coatomer β-, γ-, and ζ subunits (Fiedler *et al*., 1996). Assuming that the dihydrophobic leucine pair of the V_2 receptor could substitute the phenylalanines, the corresponding region of the V_2 receptor might function in a similar manner as an ER-to-Golgi transport signal.

For mutants E335Q and L339T, the lack of intracellular [^{3}H]AVP binding suggests that the residues concerned are required for receptor folding because it is unlikely that they play a direct role in ligand binding. Therefore, residues E335Q and L339T may be necessary to establish a correct and transport-competent folding state in the ER rather than functioning as a transport signal. However, we do not know whether V_2 receptors are actually able to bind ligand at the ER level. We cannot exclude the possibility that a maturation process along the transport pathway is necessary to confer this ability. If the residues concerned are necessary to such a folding state in the ER, one would predict an interaction with another, as yet unidentified, region of the receptor. For rhodopsin, the solution structure of the carboxyl terminus and the three other intracellular loops demonstrated seven intermolecular constrained distances between the loops. Interestingly, four of them were formed between the amino-terminal

A1ADR, α_1-adrenergic receptor; *A2aADR*, α_{2A}-adrenergic receptor; *A2bADR*, α_{2B}-adrenergic receptor; *A2cADR*, α_{2C}-adrenergic receptor (C4); *A2ciiADR*, α_{2CII}-adrenergic receptor; *THRR*, thrombin receptor precursor; *CccK1*, C-C chemokine receptor type 1; *SST1*, somatostatin 1 receptor; *SST4*, somatostatin 4 receptor; *SST28a*, somatostatin 5 receptor (SSTR5); *H1*, histamine receptor type 1; *SST3*, somatostatin 3 receptor; *IL8a*, interleukin 8 receptor; *HM74*, probable GPCR; *ratATII*, rat vascular type 1 angiotensin II receptor; *OPSB*, blue opsin; *OPSG*, green opsin; *HM2 (mACH)*, muscarinic acetylcholine receptor HM2; *HM3 (mACH)*, muscarinic acetylcholine receptor HM3; *HM4 (mACH)*, muscarinic acetylcholine receptor HM4; *M5*, M5 muscarinic acetylcholine receptor; *MASmrg*, mas-related [human, genomic, 2416-nucleotide] mas product homolog; *musB2BRAD*, mouse B_2 bradykinin receptor; *PAF*, platelet-activating factor receptor; *ratRTA*, rat probable GPCR RTA; *ratOLFPRO1*, rat olfactory protein receptor-like protein; *D4*, dopamine receptor type 4; *musGPCRA*, mouse probable GPCR.

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part of the carboxyl terminus (between TMVII and the conserved palmitoylated cysteines) and the first intracellular loop (Yeagle *et al.*, 1997). Therefore, the amino-terminal part of the carboxyl terminus of a GPCR may contribute, by binding back to the first intracellular loop, to a compactly folded state that may be obligatory for ER exit. It must be noted, however, that rhodopsin contains no conserved acidic/dihydrophobic sequence. Clearly, further experiments are needed to determine whether the glutamate/dileucine sequence represents an ER-to-Golgi sorting signal or a motif necessary for correct and transport-competent folding.

Dileucine motifs were previously shown to function as sorting signals at the *trans*-Golgi network for basolateral cell surface transport (Hunziker *et al.*, 1994; Sheikh *et al.*, 1996) and at the plasma membrane for endocytosis (Letourneur and Klausner, 1992). Both transport pathways occur via clathrin-containing vesicles, and dileucine motifs mediate sorting by specifically binding to adaptin molecules, which themselves bind to clathrin (Heilker *et al.*, 1996). In the case of the V_2 receptor, we demonstrated that a glutamate/dileucine motif is essential for the escape from the ER, most presumably by establishing a transport-competent folding state. Our results, however, do not preclude the possibility that these residues may contribute to a transport signal at a later step of intracellular transport, such as at the *trans*-Golgi network for sorting to the basolateral membrane in the epithelial cells where the V_2 receptor naturally occurs or at the plasma membrane for internalization. Folding of this motif may change due to the reversible palmitoylation of the adjacent cysteines, thereby exposing novel signals. However, if mutation of the glutamate/dileucine sequence motif does prevent ER exit, these questions become difficult to address for the V_2 receptor.

By a databank analysis, we have identified acidic/dihydrophobic motifs in the carboxyl-terminal tails of other GPCRs, suggesting that our results have general implications for the intracellular transport of other receptors. Limited experimental data are available for mutations immediately amino terminal of the palmitoylated cysteines of other GPCRs: truncation of the rat LH/CG four residues amino terminal of the two palmitoylated cysteines abolished receptor transport to the plasma membrane (Rodriguez *et al.*, 1992). The consequence of this mutation is that the second leucine residue of an LL pair (see Fig. 7) loses its hydrophobicity because it becomes the carboxyl-terminal residue of the truncated receptor. Therefore, the LL pair of the LH/CG receptor may be essential for ER exit in a similar manner to that of the V_2 receptor. The same may be true for the α_{2A}-adrenergic receptor, in which alanine substitution of the five residues proximal of the palmitoylated cysteine residue (containing an IL pair, see Fig. 7) caused a strong reduction in specific ligand binding sites (Kennedy and Limbird, 1994). However, intracellular transport of this mutant was not investigated in detail in this study. While this work was in revision, Gabilondo *et al.* (1997) demonstrated that alanine substitution of the dileucine motif of the β_2-adrenergic receptor (see Fig. 7) yielded receptors that are transported to the cell surface. However, it is not certain whether the hydrophobicity of this motif is actually abolished by alanine substitutions. It would be interesting to see whether, for example, polar threonine residues would impair ER-to-Golgi transfer in a similar manner as that described here for the V_2 receptor. At least one GPCR, the rat m3 muscarinic receptor, seems to have no special sequence requirements in the intracellular carboxyl terminus for cell surface expression because even very short receptor fragments are transported to the plasma membrane (Schöneberg *et al.*, 1995). Therefore, cell surface transport mechanisms may vary within the GPCR family. Our results also support previous data (Barak *et al.*, 1997; Tarasova *et al.*, 1997) demonstrating that GFP fusion proteins offer a powerful tool with which to address these questions in future studies.

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