Rescue of a Nephrogenic Diabetes Insipidus-causing Vasopressin V₂ Receptor Mutant by Cell-penetrating Peptides*

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Mutant membrane proteins are frequently retained in the early secretory pathway by a quality control system, thereby causing disease. An example are mutants of the vasopressin V₂ receptor (V₂R) leading to nephrogenic diabetes insipidus. Transport-defective V₂Rs fall into two classes: those retained exclusively in the endoplasmic reticulum (ER) and those reaching post-ER compartments such as the ER/Golgi intermediate compartment. Although numerous chemical or pharmacological chaperones that rescue the transport of ER-retained membrane proteins are known, substances acting specifically in post-ER compartments have not been described as yet. Using the L62P (ER-retained) and Y205C (reaching post-ER compartments) mutants of the V₂R as a model, we show here that the cell-penetrating peptide penetratin and its synthetic analog KLAL rescue the transport of the Y205C mutant. In contrast, the location of the L62P mutant is not influenced by either peptide because the peptides are unable to enter the ER. We also show data indicating that the peptide-mediated transport rescue is associated with an increase in cytosolic Ca²⁺ concentrations. Thus, we describe a new class of substances influencing protein transport specifically in post-ER compartments.

The hormone 8-arginine vasopressin (AVP²; antidiuretic hormone) mediates water reabsorption in the kidney via the G protein-coupled human vasopressin V₂ receptor (V₂R) located at the cell surface (1, 2). Mutations in the V₂R gene cause X-linked nephrogenic diabetes insipidus (NDI), a disease characterized by the kidney’s inability to concentrate urine. The majority of the mutations result in transport-defective receptors (3), which are recognized and intracellularly retained by the quality control system (QCS). Besides NDI, transport-defective membrane proteins play a role in many other diseases (4, 5).

The QCS is located mainly in the endoplasmic reticulum (ER) and allows only correctly folded and/or assembled proteins to leave the early secretory pathway and to reach their final cellular destination (6, 7). Several components have been described to contribute to the QCS: (i) the lectin chaperones calnexin and calreticulin involved in the quality control of glycoproteins; (ii) “classical” chaperones such as the IgG heavy chain-binding protein, endoplasmic/grp90, and the ER-resident DnaJ-like proteins ERdj1–ERdj5; and (iii) enzymes such as protein-disulfide isomerase and the thiol oxidoreductase ERP57 (8). Misfolded or unassembled membrane proteins initially display a prolonged association with components of the QCS. They may then either accumulate in the ER, leading to the unfolded protein response (9), and/or be subjected to proteasomal degradation (10, 11). It has also been demonstrated that the QCS is not restricted to the ER but involves post-ER compartments such as the ER/Golgi intermediate compartment (ERGIC) (12, 13). For the V₂R, we have shown recently that transport-defective mutant receptors fall into two classes: mutants like L62P are retained exclusively in the ER, whereas others like Y205C escape to the ERGIC (14). In the ERGIC, they are recognized by an as yet incompletely understood mechanism.

Two strategies may lead to the successful treatment of diseases caused by transport-defective membrane proteins. The first comprises the use of substances promoting correct folding, thereby reducing the interaction with components of the QCS. In the case of receptors, membrane-permeable ligands may serve this purpose. Recently, such “pharmacological chaperones” were used to rescue the folding and plasma membrane transport of mutant G protein-coupled receptors such as, for example, the V₂R (15, 16) and rhodopsin (17, 18). The pharmacological chaperones have the advantage of acting protein-specific and at low concentrations. The known disadvantage is that tightly bound ligands, mostly receptor antagonists, cannot easily be removed from the protein (16).
A second, not intensively pursued strategy includes the development of inhibitors of the QCS. This approach should prove useful in cases in which mutant proteins still have some functional activity but are nevertheless retained by the QCS, i.e. if the QCS is overprotective. A well known example is the ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator protein found in 70% of patients with cystic fibrosis (19).

Here, we describe a new class of substances that rescue the transport of folding-defective membrane proteins. Using the L62P (ER-retained) and Y205C (reaching post-ER compartments) mutants of the V₂R as model proteins, we show that the cell-penetrating peptides (CPPs) penetratin and KLAL (20, 21), specifically rescue the transport of the Y205C mutant (but not the L62P mutant) at low micromolar concentrations. Thus, these peptides act specifically in post-ER compartments.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lipofectamine™ was purchased from Invitrogen (Eggenstein, Germany). FuGENE 6™, the Lumi-Light Western blotting substrate, and the Lumi-Imager F1™ workstation were from Roche Diagnostics (Mannheim, Germany). G418 and fetal calf serum were purchased from Calbiochem-Novabiochem (Bad Soden, Germany) and Biochrom (Berlin, Germany). Restriction enzymes were from New England Biolabs (Schwalbach, Germany). Trypan blue was obtained from Merck (Darmstadt, Germany). CaCl₂ was obtained from Roth (Karlsruhe, Germany). The Exper®S™-S™ protein labeling mixture was obtained from PerkinElmer Life Sciences (Rodgau, Germany). Aprotinin, benzamidine, 1,4-diazabicyclo[2.2.2]octane, Igepal CA-630 (Nonidet P-40), Aprotinin, benzamidine, 1,4-diazabicyclo[2.2.2]octane, Igepal CA-630 (Nonidet P-40), N-dodecyl β-D-maltoside, phenylmethylsulfonyl fluoride, and protein A-Sepharose were from Sigma (Munich, Germany). Sulfo-NHS-Biotin and Immunopure immobilized NeutrAvidin™ were obtained from Pierce. Horseradish peroxidase-conjugated anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG were purchased from Dianova (Hamburg, Germany). Horseradish peroxidase-conjugated anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The monoclonal anti-ERGIC-53 antibody was a gift from Hans-Peter Hauri (Biozentrum, University of Basel, Basel, Switzerland). The monoclonal anti-58-kDa Golgi protein antibody was obtained from Abcam (Cambridge, UK). The polyclonal anti-green fluorescent protein (GFP) serum for precipitation of the GFP-tagged receptors and detection of biotinylated GFP-tagged receptors was raised against a glutathione S-transferase/GFP fusion protein by us, and its specificity was verified.³ The polyclonal anti-GFP antiserum for immunogold electron microscopy (IEM), Fluoro-4/AM, and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR). Plasmids pRCDN2, encoding the V₂R (24), and pWT-GFP (25), encoding a C-terminally GFP-tagged V₂R, have been described. Vectors pCDNA1/Neo and pEGFP-N1 were from Invitrogen (Leek, The Netherlands) and Clontech (Heidelberg, Germany), respectively. Human embryonic kidney (HEK) 293 cells were a gift from Falk Fahrenholz (Mainz, Germany). All other reagents were obtained from Sigma.

**Plasmid Constructions**—Plasmids encoding the C-terminally GFP-tagged V₂R mutants L62P and Y205C (14) and wild-type V₂R (25) have been described. The corresponding cyan fluorescent protein (CFP) constructs of the wild-type receptor, L62P, and Y205C were generated by cloning the receptor moieties of the wild-type V₂R-GFP, L62P-GFP, and Y205C-GFP plasmids on a BamHI/SacI fragment into the pECFP-N1 vector. The nucleotide sequences of all DNA fragments were verified by sequencing.

**Cell Culture and Transfection**—Transiently or stably transfected HEK 293 cells were cultured at 37 °C and 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection of the cells with FuGENE 6™ was carried out according to the supplier’s recommendations. Stable transfection of HEK 293 cells was carried out using Lipofectamine™ according to the supplier’s recommendations. G418 (400 μg/ml) was used for selection and maintenance of the cell clones expressing wild-type V₂R-GFP, L62P-GFP, and Y205C-GFP.

**Visualization of GFP- and CFP-tagged Receptors in Transiently Transfected Living HEK 293 Cells**—HEK 293 cells (2.5 x 10⁵) grown for 24 h in a 35-nm diameter dish containing a poly-L-lysine-covered coverslip were transfected with 1 μg of plasmid DNA and FuGENE 6™ according to the supplier’s recommendations. Cells were incubated overnight, washed once with phosphate-buffered saline, and transferred immediately into a self-made chamber.³ Cells were covered with 1 ml of phosphate-buffered saline, and trypan blue was added to a final concentration of 0.05%. After 1 min of staining, GFP and trypan blue fluorescence signals were visualized at room temperature on a Zeiss LSM 510 META inverted confocal laser scanning microscope (objective lens, ×100/1.3 oil; optical section, <0.8 μm; multitrack mode; GFP, λex = 488 nm, argon laser; 500 – 530-nm band-pass filter; trypan blue, λex = 543 nm, helium-neon laser, 560-nm long-pass filter). The overlay of both signals was computed using Zeiss LSM 510 acquisition software (Release 3.2 SP2). Images were imported into Adobe Photoshop Version 5.5 software, and contrast was adjusted to approximate the original image. In the case of the cell-surface integrity assay, cells were stained for 15 min with trypan blue prior to recording. In the case of the receptor/peptide colocalization experiments, CFP-tagged receptors and 5(6)-carboxyfluorescein N-hydroxysuccinimide ester (FLUOS)-labeled peptides were analyzed at room temperature using the same microscope (objective lens, ×40/1.3 oil; optical section, <0.8 μm; multitrack mode; CFP, λex = 810 nm, Chameleon™ laser, 430 – 498-nm band-pass filter; FLUOS, λex = 488 nm, argon laser, 500 – 550-nm band-pass filter). The overlay of the signals was computed, and the images were processed as described above.

**Visualization of GFP-tagged Receptors in Stably Transfected HEK 293 Cell Clones by IEM**—Cell clones expressing similar amounts of the L62P and Y205C mutants were fixed in 2%

³ M. Oueslati, R. Hermosilla, and R. Schülein, unpublished data.

4 Details are available upon request.
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freshly prepared formaldehyde plus 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature. After three washes with phosphate buffer, the cells were scraped in 1% gelatin, spun down, and resuspended in 12% gelatin at 37 °C. Subsequently, the cells were centrifuged, and the gelatin was solidified on ice. The pellet was cut into small blocks, and the samples were infiltrated with 2.3 M sucrose in 0.1 M phosphate buffer (pH 7.2) overnight at 4 °C. The blocks were mounted on pins and frozen in liquid nitrogen until processing into 50–60-nm cryosections. Sections were double-labeled with primary antibodies and detected by protein A-gold as described previously (26).

Quantification of Plasma Membrane GFP Fluorescence Intensities—For quantification of the peptide-mediated transport rescue, the ratio of cell membrane to intracellular fluorescence signal intensities was calculated and statistically analyzed as described previously (16).

Calcium Measurements—HEK 293 cells were cultivated for 48 h in a 35-mm diameter dish containing a poly-L-lysine-coated coverslip. Cells were preloaded with the Ca²⁺ indicator Fluo-4/AM (2 μM in solution of 140 mM NaCl, 4.6 mM KCl, 2 mM CaCl₂, 10 mM glucose, and 0.01% Pluronic F-127 (pH 7.4)) for 30 min at 37 °C. The cells were washed three times with the same solution without Fluo-4/AM and incubated at 37 °C for chase periods of 1, 2, and 0.01% bromphenol blue (pH 6.8)) and separated by SDS-PAGE (10% acrylamide). The gel was incubated for 25 min in a fixing solution of 50% methanol and 10% acetic acid, dried, and exposed for 4 days to radiographic film.

Immunofluorescence—HEK 293 cells (1 x 10⁶) were spread on a poly-L-lysine-coated glass coverslip (12 mm in diameter) and incubated for 24 h. Cells were transiently transfected with 125 ng of plasmid DNA and FuGENE 6 according to the supplier’s recommendations. 18 h after transfection, the cell culture medium was supplemented with bafilomycin A₁ (1 μM) or vehicle and incubated for another 4 h. The immunofluorescence procedure using anti-ERGIC-53 or anti-58-kDa Golgi protein antibody was carried out as described (14).

Treatment of Cells with the Peptides, Bafilomycin A₁, and Brefeldin A (BFA)—Bafilomycin A₁ treatment of cells was carried out for 4 h at a final concentration of 1 μM. Treatment of cells with penetratin or KLAL was performed for 12 h using the peptide concentrations indicated under “Results.” In receptor/peptide colocalization studies, peptide treatment was performed for only 6 h due to the reduced stability of the FLUC-labeled peptides (peptide pretreatment = 2 h plus an additional 4 h together with bafilomycin A₁). Combined treatment with penetratin (1 μM) and BFA (0.5 μg/ml) was carried out for 18 h.

Peptide Synthesis and Labeling—Peptides were synthesized automatically (ABI 433A peptide synthesizer) by the solid-phase method using standard Fmoc chemistry in a batch-wise mode as described previously for the synthesis of corticotropin-releasing factor analogs (27). Carboxyfluorescein (FLUOS) (FLUOS) was N-terminally incorporated after the last Fmoc cleavage on peptide resin via the FLUOS-N-hydroxysuccinimide ester (2 eq in N,N-dimethylformamide overnight). After final cleavage/deprotection using trifluoroacetic acid/H₂O (9:1), crude peptides were purified by preparative HPLC to give final products of 95% purity according to HPLC analysis. The peptides were characterized by mass spectrometry, which yielded the expected masses.

Cell-surface Biotinylation Assay—Stably transfected HEK 293 cells (9 x 10⁵) were grown for 48 h in a 60-mm diameter dish to 80% confluence. The cell-surface biotinylation assay was carried out in the cold as described previously for transiently transfected HEK 293 cells (28). Biotinylated proteins were detected by immunoblotting using polyclonal anti-GFP serum and horseradish peroxidase-conjugated anti-rabbit IgG.

Immunoprecipitation of Total Receptor Proteins—Immunoprecipitations were carried out in the cold. To immobilize polyclonal anti-GFP antibodies, protein A-Sepharose beads were incubated for 15 min in buffer A (0.3% N-dodecyl β-D-maltoside, 25 mM Tris-HCl, 10 mM CaCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM aprotinin, 0.5 mM benzamidine, 0.5 mM 1,4-diazabicyclo[2.2.2.]octane, and 0.5 mM Igepal CA-630 (pH 8.0)) and then washed three times with the same buffer. The beads (10 mg for the lystate of cells from one 100-mm diameter dish) were then incubated overnight with polyclonal anti-GFP serum (1:200) in a final volume of 1 ml of buffer A. Buffer A was removed by centrifugation at 500 x g for 5 min.

For immunoprecipitation, stably transfected HEK 293 cells were grown on 100-mm diameter dishes for 48 h to 80% confluence. Cells were washed twice with phosphate-buffered saline and lysed in buffer A for 1 h. Insoluble debris was
removed by centrifugation at 12,000 × g for 30 min. The lysates were incubated for 3 h with the antibody-coupled protein A-Sepharose and washed twice with buffer A, twice with buffer B (same as buffer A but without detergent), twice with buffer C (same as buffer B but with 2 mM EDTA instead of CaCl₂), and finally once with 12.5 mM Tris-Cl (pH 6.8). Precipitated proteins were eluted for 15 min at room temperature in Laemmli buffer (final concentrations of 60 mM Tris-Cl, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromphenol blue (pH 6.8)), boiled for 2 min at 95 °C, and finally subjected to SDS-PAGE/immunoblot analysis using monoclonal anti-GFP antibodies and horseradish peroxidase-conjugated anti-mouse IgG.

Miscellaneous—Standard DNA preparations and manipulations were carried out. [3H]AVP binding to intact HEK 293 cells was carried out as described previously for transiently transfected COS.M6 cells (29).

RESULTS

Intracellular Localization of the NDI-causing V₂R Mutants L62P and Y205C by Immunofluorescence and IEM—We have shown previously by immunofluorescence (14) that the intracellularly retained NDI-causing V₂R mutants fall into two classes: those retained in the ER (e.g. L62P) (Fig. 1A) and those reaching post-ER compartments such as the ERGIC and the Golgi apparatus (e.g. Y205C). This prompted us to search for post-ER-specific inhibitors of the QCS. To this end, we first confirmed the different intracellular locations of the receptor mutants L62P and Y205C by immunofluorescence (confocal laser scanning microscopy (LSM)) and refined these data by IEM. For immunofluorescence (Fig. 1B), HEK 293 cells were transiently transfected with plasmids encoding the C-terminally GFP-tagged V₂R mutants L62P and Y205C. We have shown previously that the GFP tag influences neither the pharmacological nor trafficking properties of the V₂R (30). The detection of a protein in the ERGIC or Golgi apparatus by immunofluorescence is difficult under steady-state conditions. The retrograde transport to the ER was therefore blocked by treating cells for 4 h with bafilomycin A₁. Under these conditions, cycling proteins accumulate in the post-ER compartments and become easily detectable (31, 32). The ERGIC was visualized using antibodies against the marker protein ERGIC-53, and the Golgi apparatus using antibodies against the 58-kDa Golgi protein. In the case of the L62P mutant, we failed to detect a colocalization of the receptor’s GFP fluorescence signals with either the ERGIC or Golgi marker (Fig. 1B), confirming the previously described retention of this mutant in the ER (see also Ref. 14). In contrast, the Y205C mutant accumulated in a perinuclear region. A substantial colocalization with the ERGIC marker and a weaker colocalization with the Golgi marker were observed, again in agreement with previous results (14).

Localization of the mutant receptors by IEM was performed using stably transfected cell clones expressing similar amounts of L62P and Y205C (Fig. 2). (Receptor expression was monitored by quantifying the receptors’ GFP fluorescence signals fluorometrically (data not shown).) Cells were double-labeled for ERGIC-53 (15-nm gold particles) and GFP (10-nm gold particles). Mutant L62P was detectable in the ER cisternae, whereas no label was found in the ERGIC and in other post-ER compartments (Fig. 2A; ERGIC-53-positive membranes are marked with an asterisk). An escape of minor amounts of L62P from the ER was detectable only when cell clones expressing very high levels of this mutant were used (data not shown). By contrast, Y205C-GFP was readily seen in the ERGIC (Fig. 2B; ERGIC-53-positive membranes are marked with an asterisk) and throughout the Golgi stack (Fig. 2C; the cis-side of the stack is indicated by the presence of ERGIC-53). Expression of the Y205C mutant in the ER was low in the stably transfected cell clone under steady-state conditions, in contrast to the situation in the transiently transfected cells, where bafilomycin A₁ treat-
Rescue of \( V_2R \) Mutants

![Diagram: Rescue of \( V_2R \) Mutants](image)

FIGURE 2. IEM of stably transfected HEK 293 cell clones expressing the GFP-tagged \( V_2R \) mutants L62P and Y205C. Cell clones expressing similar amounts of each mutant (quantified by analyzing their total GFP signals fluorometrically) were used. Mutants L62P (A) and Y205C (B and C) were double-labeled for GFP (10-nm gold particles) and the ERGIC marker protein ERGIC-53 (15-nm gold particles). A, L62P-GFP was clearly visible in the ER cisternae, whereas no label was found in the ERGIC. The asterisk indicates ERGIC-positive membranes. M, mitochondrion. B, by contrast, Y205C-GFP levels in the ER were low or undetectable, whereas label was readily seen in the ERGIC. The asterisk indicates ERGIC-53-positive membranes. C, L62P-GFP was clearly visible in the ER cisternae, whereas no label was found in the ERGIC. The asterisk indicates ERGIC-positive membranes. M, mitochondrion. Scale bars = 200 nm.

Penetratin (16 aa) RQIKIWFQNRRMKWKK
KLAL (18 aa) KLALKLALKALKAL/KAALKA

KALK (18 aa) KALKLKLALALLALKLA
TAT (12 aa) GCGRKRRQR

FIGURE 3. Sequences and helical wheel projections of the peptides penetratin, KLAL, KALK, and TAT. Hydrophobic residues are indicated by open circles; charged and hydrophilic residues by shaded circles. Penetratin and KLAL are CPPs with amphipathic properties. The hydrophilic and hydrophobic residues of penetratin are not as strictly distributed on one side of the helix as those of the synthetic analog KLAL. The TAT peptide is a non-amphipathic CPP. The KALK peptide represents a scrambled version of KLAL. aa, amino acids.

ment was needed to facilitate detection of the Y205C mutant in post-ER compartments (see Fig. 1B and Ref. 14). This is explicable if one assumes that transient transfections lead to stronger overexpression and to more ER aggregates of the Y205C mutant. In summary, these data confirm that the transport-defective NDI-causing \( V_2R \) mutants fall into two classes: those retained in the ER and those reaching post-ER compartments.

Cell-surface Delivery of the Y205C Mutant of the \( V_2R \) Is Rescued by Treatment with Penetratin or KLAL—Experiments using CPPs as carriers indicated that they may improve trafficking of some misfolded \( V_2Rs\). Two properties of these peptides allowed us to consider it possible that these peptides inhibit the QCS specifically in post-ER compartments. (i) The amphipathic nature of some of these peptides (penetratin and KLAL) (Fig. 3) may allow multiple hydrophobic and non-hydrophobic interactions with target proteins and may consequently enable the displacement of quality control components. (ii) It has been described that CPPs enter the cell predominantly via the endocytotic pathway and that they are transported retrogradely but without reaching the ER (22, 23).

To determine whether the peptides rescue the transport of proteins retained by the QCS in post-ER compartments, transiently transfected HEK 293 cells expressing the ER-retained L62P mutant and the ER-escaping Y205C mutant were treated with the CPPs penetratin and TAT and the synthetic analog KLAL at 1 \( \mu \)M for 12 h (Figs. 3 and 4). We used a scrambled version (KALK) of the KLAL peptide as a negative control. Cell-surface expression of the receptors was analyzed by confocal LSM using the fluorescent dye trypan blue as a plasma membrane marker (Fig. 4, upper panels). In untreated cells, a colocalization of GFP and trypan blue signals was observed in the case of the wild-type receptor. The intracellular fluorescence signals represent transport intermediates en route to the cell surface and/or receptors that are retained as a consequence of overexpression (25, 33). Neither peptide influenced the transport of the wild-type receptor. In the case of mutant Y205C, the GFP signals were detectable intracellularly in untreated cells. The cell-surface delivery of mutant Y205C was restored by treatment with both penetratin and KLAL; TAT and the scrambled peptide were not effective. In the case of mutant L62P, neither peptide worked. These results show that the cell-penetrating peptides penetratin and KLAL specifically rescue the transport of mutant Y205C. Taking the different intracellular locations of Y205C and L62P into account (see Figs. 1 and 2), it is conceivable that the peptides act specifically in post-ER compartments. The results also show that the ability to rescue receptor trafficking is not attributable to the whole CPP family because TAT was ineffective in the case of the Y205C mutant.

To quantify the effects of the peptides, the ratios of cell membrane to intracellular fluorescence signal intensities of the receptors were determined in transiently transfected HEK 293 cells \( (n = 30 \) cells) (Fig. 4, lower panels) (see also Ref. 20). In the case of vehicle-treated cells expressing the Y205C mutant, a ratio of <1 was obtained, indicating a predominant intracellular...
lar localization of the mutant. Peptide treatment with either penetratin or KLAL significantly increased this ratio, consistent with cell-surface delivery. TAT and the scrambled KALK peptide were ineffective in the case of the Y205C mutant. The ratios of the L62P mutant and the wild-type receptor were not significantly influenced by any peptide treatment.

To confirm the result that penetratin and KLAL treatment leads to specific rescue of the transport of the Y205C mutant, we performed a cell-surface biotinylation assay with stably transfected HEK 293 cells expressing the GFP-tagged wild-type receptor and both mutants. Cells were treated with penetratin (Pe), KLAL, TAT, or KALK (1 μM) or with vehicle (−) and analyzed by confocal LSM. The GFP fluorescence signals of the receptors were recorded (left panels; green); plasma membranes were stained with trypan blue (middle panels; red), and overlay of the signals was computed (right panels; colocalization is indicated in yellow). All scans were recorded using the same setup parameters and are representative of four independent experiments. WT, wild-type V2R. Scale bars = 5 μm. The bar graphs show the results from quantitative analysis of the peptide effects. The ratio of cell membrane to intracellular fluorescence signals was determined. Ratios >1 indicate a predominantly intracellular localization of the receptors, and ratios >1 a predominant localization at the cell membrane. Error bars indicate the mean ± S.D. (n = 30 cells). The results are representative of two independent experiments.

To assess whether the peptide-mediated rescue requires the secretory pathway, we induced disassembly of the Golgi

FIGURE 4. Subcellular distribution of the GFP-tagged mutants L62P (A) and Y205C (B) and wild-type receptor (C) in transiently transfected HEK 293 cells: influence of peptide treatment. Cells were treated with penetratin (Pe), KLAL, TAT, or KALK (1 μM) or with vehicle (−) and analyzed by confocal LSM. The GFP fluorescence signals of the receptors were recorded (left panels; green); plasma membranes were stained with trypan blue (middle panels; red), and overlay of the signals was computed (right panels; colocalization is indicated in yellow). All scans were recorded using the same setup parameters and are representative of four independent experiments. WT, wild-type V2R. Scale bars = 5 μm. The bar graphs show the results from quantitative analysis of the peptide effects. The ratio of cell membrane to intracellular fluorescence signals was determined. Ratios >1 indicate a predominantly intracellular localization of the receptors, and ratios >1 a predominant localization at the cell membrane. Error bars indicate the mean ± S.D. (n = 30 cells). The results are representative of two independent experiments.
apparatus by concomitant BFA treatment. Transiently transfected HEK 293 cells expressing mutants L62P and Y205C were incubated with penetratin alone or with a penetratin/BFA combination, and the GFP fluorescence signals of the receptors were analyzed by confocal LSM (Fig. 5C). BFA treatment abolished the penetratin-mediated rescue of the Y205C mutant, demonstrating that restoration of the cell-surface transport takes place only when the secretory pathway is functional. Similar results were obtained for KLAL-treated cells (data not shown).

Control Experiments—CPPs may disturb plasma membrane integrity (20, 21) and probably also intracellular membrane compartments. Thus, we needed to exclude that the peptides do not affect overall cell structure and function in the concentrations used in this study (1 μM). We first examined whether the integrity of the plasma membrane was maintained after the addition of the peptides. Untransfected HEK 293 cells were incubated for 12 h with 1, 5, 10, 50, and 100 μM penetratin or KLAL. Subsequently, cells were incubated for 15 min with the plasma membrane marker trypan blue, and membrane integrity was tested by analyzing the trypan blue fluorescence signals using confocal LSM. Only an intact plasma membrane prevents trypan blue from entering into the cells. Fig. 6A shows that the trypan blue fluorescence signals remained surface-bound at peptide concentrations as high as 50 μM for each peptide. We next examined the function of the Golgi apparatus. Stably transfected HEK 293 cell clones expressing the GFP-tagged wild-type V2R were pretreated with penetratin or KLAL (1 μM) or left untreated for 12 h. Maturation of the V2R was then assessed by pulse-chase experiments. The receptor was metabolically labeled with [35S]Met, collected by immunoprecipitation following chase periods of up to 4 h, and detected by autoradiography. Fig. 6B shows that receptor maturation from the high mannos form (asterisk, 60–65 kDa) (see Ref. 33 for the identity of the protein bands) to the mature form (arrowhead, 75–80 kDa) proceeded in the presence and absence of penetratin or KLAL, demonstrating that normal Golgi function is unaffected by the CPPs at the concentrations used in this study. These experiments also demonstrate that the early secretory pathway (ER and ERGIC) remains intact upon peptide treatment.

The Peptides Penetratin, KLAL, and TAT Colocalize with the Y205C Mutant in the ERGIC—It was shown previously that penetratin reaches the Golgi apparatus, but not the ER (22, 23). These data may explain why the L62P mutant, which is retained in the ER, is not rescued by the peptides. It is not known whether penetratin is also transported beyond the Golgi apparatus to the ERGIC. To clarify whether the peptides reach the Y205C mutant in the ERGIC, but not the L62P mutant in the ER, we assessed colocalization of the peptides with the receptors after bafilomycin A1 treatment (see also Fig. 1B). To this end, we synthesized FLUOS-labeled penetratin, KLAL, and TAT. Mutant receptors were C-terminally tagged with CFP instead of GFP to allow the distinction of receptor and peptide fluorescence signals. Transiently transfected HEK 293 cells expressing the mutant receptors were pretreated with modified peptides (1 μM each) for 2 h. Bafilomycin A1 was added, and cells were incubated for an additional 4 h to accumulate receptors in the ERGIC (see also Fig. 1B). Analysis of cells by confocal LSM revealed that the Y205C mutant colocalized in the perinuclear ERGIC with either peptide (Fig. 7). (We also showed that the peptides colocalized with the marker protein ERGIC-53 in this case (data not shown)). In contrast, the L62P mutant did not colocalize with the peptides. Rescue of the Y205C mutant by the FLUOS-labeled peptides penetratin and KLAL was not detectable, in contrast to the experiments shown in Fig. 4. This is due to the fact that treatment with FLUOS-labeled peptides was performed for only 6 h (2-h peptide pretreatment + an additional 4 h together with bafilomycin A1) instead of 12 h in the rescue experiments shown above. Incubation of cells with FLUOS-labeled penetratin and KLAL peptides for 12 h also led to rescue of the Y205C mutant (data not shown). However, the fluorophore was not stable under these conditions, and peptide fluorescence disappeared, thus necessitating a decreased incubation time in the receptor/peptide colocalization experiment.

Taken together, the results of the receptor/peptide colocalization study suggest that the peptides penetratin and KLAL reach the ERGIC. Taking into account that they fail to reach the ER (22, 23), this finding provides an explanation for the selective rescue of mutant Y205C. Our data show that the TAT peptide reaches the ERGIC, too. This result demonstrates that the
Peptide Treatment with Penetratin or KLAL Leads to an Increase in Intracellular Ca\(^{2+}\) Concentrations—It has been shown previously that Ca\(^{2+}\) release into the cytosol mediated by inhibitors of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs), such as thapsigargin and curcumin, leads to rescue of the transport of some misfolded proteins (34). It is believed that decreased Ca\(^{2+}\) levels in the ER caused by these compounds influence the function of molecular chaperones and/or other quality control components (35). Recently, a thapsigargin-mediated transport rescue has also been reported for a V\(_2\)R mutant (36).

High luminal Ca\(^{2+}\) concentrations are found not only in the ER, but also throughout the whole secretory pathway (37). We thus addressed the question of whether CPPs may cause Ca\(^{2+}\) release. To this end, HEK 293 cells were preloaded with the Ca\(^{2+}\)-sensitive fluorophore Fluo-4/AM and incubated with thapsigargin and the peptides penetratin, KLAL, TAT, and KALK. Changes in cytosolic Ca\(^{2+}\) were monitored by measuring Fluo-4/AM fluorescence using confocal LSM (Fig. 9, A and B). In agreement with previous results (e.g. Ref. 36), thapsigargin treatment led to a quick increase in cytosolic Ca\(^{2+}\) with a peak after 30 s. Ca\(^{2+}\) levels then decreased to a steady-state level, which was higher than the level under the starting conditions.

**DISCUSSION**

We have demonstrated that the CPPs penetratin and KLAL promote cell-surface delivery of the ER-escaping V\(_2\)R mutant Y205C. In contrast, the transport of the ER-retained mutant L62P is not influenced. It was shown previously that CPPs are transported to the Golgi apparatus via the retrograde transport pathway but fail to reach the ER (22, 23). We have shown here that the peptides reach the ERGIC, i.e. the compartment between the ER and the Golgi apparatus. Taken together, these data indicate that the penetratin- and KLAL-mediated transport rescue is associated with an increase in cytosolic Ca\(^{2+}\) levels.

The CPPs penetratin and KLAL also caused a significant (albeit delayed) Ca\(^{2+}\) mobilization. Steady-state concentrations were higher than those following thapsigargin treatment. No response was seen for the TAT and KALK control peptides. Taken together, these data indicate that the penetratin- and KLAL-mediated transport rescue is associated with an increase in cytosolic Ca\(^{2+}\) levels.
stores in post-ER compartments of the secretory pathway such as the ERGIC. This may affect Ca\textsuperscript{2+}/H\textsubscript{11001}-dependent chaperones involved in post-ER quality control in the ERGIC such as the IgG heavy chain-binding protein (13). Because of their delayed mode of action, it is unlikely that the peptides affect plasma membrane Ca\textsuperscript{2+}/H\textsubscript{11001} permeability in an ionomycin-like mechanism, although this possibility has not been excluded rigorously as yet.

The exact mechanism by which the peptides increase cytosolic Ca\textsuperscript{2+} concentrations after endocytic uptake remains elusive. SERCAs are also present in post-ER compartments of the secretory pathway, and the peptides may inhibit these pumps directly, as has been shown for thapsigargin (38). Alternatively, the peptides may assemble into pore-like structures that are permeable for Ca\textsuperscript{2+} in these post-ER compartments. Pore formation was observed for many other cationic and amphipathic peptides (20, 21).

We have shown that all three CPPs, penetratin, KLAL, and TAT, reach the ERGIC. In the case of the TAT peptide, however, rescue of the transport of the Y205C mutant was not observed. These data raise the question as to the structural

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**FIGURE 7.** Receptor/peptide colocalization experiment. Transiently transfected HEK293 cells expressing the C-terminally CFP-tagged mutants L62P and Y205C were treated with FLUOS-labeled penetratin (Pe), KLAL, or TAT. All cells were treated with bafilomycin A\textsubscript{1} for 4 h to accumulate proteins in the ERGIC. The CFP fluorescence (left panels; red) and the peptides’ FLUOS fluorescence (middle panels; green) were visualized by confocal LSM. Overlays were computed (right panels; colocalization is indicated in yellow). The scans were recorded using the same setup parameters and are representative of three independent experiments. Scale bars = 5 μm.

**FIGURE 8.** Specific [H]AVP binding to stably transfected intact HEK 293 cell clones expressing the GFP-tagged wild-type receptor and mutants L62P and Y205C. The [H]AVP concentration was 50 nM, i.e. saturating. Cells were treated with penetratin (1 μM) prior to the binding assay (+) or with vehicle (−). Untransfected cells were used as negative control (Ctrl). Error bars represent the means ± S.D. of triplicates that differed by <10%. Unspecific binding contributed up to 25% of the total binding. The results are representative of two independent experiments. WT, wild-type V\textsubscript{2}R.

**FIGURE 9.** Calcium measurements. A, fluorescence intensity (means ± S.E.) of the Ca\textsuperscript{2+} indicator Fluo-4/AM in HEK 293 cells following treatment with thapsigargin (Tpg; magenta; n = 10 cells), penetratin (Pe; green; n = 30 cells), KLAL (orange; n = 20 cells), TAT (blue; n = 18 cells), and KALK (black; n = 18 cells). The fluorescence intensities of the cells were recorded following the addition of the substance in a time series (2 s, 300 images) and were normalized to the initial values (100%). The black arrowhead indicates the addition of the substances (1 μM each), and the red arrowheads indicate the time points for recording the pictures shown in B. The data are representative of two independent experiments. B, images of HEK 293 cells showing Fluo-4/AM fluorescence intensity. The scans were recorded at the time points indicated in A (0, 50, and 550 s).
properties of the peptides responsible for this rescue. TAT is a cationic CPP; KLAL and penetratin are more or less amphipathic. Thus, amphipathicity may be relevant. However, TAT is also significantly shorter than penetratin and KLAL, and peptide length must also be considered. Further studies are needed to address these questions in more detail.

CPPs are used to target compounds into cells. Our data indicate that care should be taken when using penetratin and KLAL for this purpose because it is conceivable that they also affect Ca\(^{2+}\)-dependent signal transduction. For use as a carrier, the TAT peptide seems to be the best choice.

We have shown that the endocytosed CPPs penetratin and KLAL represent a new class of substances leading to a transport rescue of misfolded membrane proteins specifically from post-ER compartments. Penetratin and KLAL may thus represent powerful tools to study the mechanisms of post-ER quality control.

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