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Derlin-1 and p97/VCP Mediate the ER-Associated Degradation of Human V2 Vasopressin Receptors*

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Running Title

Degradation of V2 vasopressin receptors

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Abbreviations: AVP, 8-arginine vasopressin; BP, band pass; B_{MAX}, maximal binding; CP, core particle; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, concentration at which 50% of the maximal effect is obtained; ECL, enhanced chemiluminescence; *Endo*H, endoglycosydase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERGIC, ER/Golgi intermediate compartment; FBS, fetal bovine serum; GFP, green fluorescent protein; HEK, human embryonic kidney; IgG, immunoglobulin class G; K_D, dissociation constant; NDI, nephrogenic diabetes insipidus; p97/VCP, valosin-containing protein; *PNGase*F, peptide endoglycosydase F; RP, regulatory particle; Rpn1/S2, proteasome 26S non-ATPase subunit 2; Rpn10/S5a, 26S protease regulatory subunit 5a; Rpt1/S7, 26S protease regulatory subunit 7; UPR, unfolded protein response; V2R, human V2 vasopressin receptor.

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Abstract

The ER-associated degradation (ERAD), the main quality control pathway of the cell, is crucial for the elimination of unfolded or misfolded proteins. Several diseases are associated with the retention of misfolded proteins in the early secretory pathway. Among them is X-linked nephrogenic diabetes insipidus, caused by mutations in the gene encoding the V2 vasopressin receptor (V2R). We studied the degradation pathways of three intracellularly retained V2R mutants with different misfolded domains in HEK293 cells. At steady state, the wild type V2R and the complex-glycosylated mutant G201D were partially located in lysosomes, while core-glycosylated mutants L62P and V226E were excluded from this compartment. In pulse-chase experiments, proteasomal inhibition stabilized the non- and core-glycosylated forms of all studied receptors. In addition, all mutants and the wild type receptor were found to be polyubiquitinylated. Non- and core-glycosylated receptor forms were located in cytosolic and membrane fractions, respectively, confirming the deglycosylation and retrotranslocation of ERAD substrates to the cytosol. Recently, distinct Derlin-1-dependent and -independent ERAD pathways have been proposed for proteins with different misfolded domains (cytosolic, extracellular and membrane) in yeast. Here we show for the first time that V2R mutants with different misfolded domains are able to co-precipitate the ERAD components p97/valosin-containing protein, Derlin-1 and the 26S proteasome regulatory subunit 7. Our results demonstrate the presence of a Derlin-1 mediated ERAD pathway degrading wild type and disease-causing V2R mutants with different misfolded domains in a mammalian system.

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Introduction

Several human diseases are caused by the retention and/or accumulation of a protein within the cell. The quality control system of the secretory pathway screens for non-native protein conformations, e.g. unfolded or misfolded proteins, impedes their further transport and targets them to the degradation system. These proteins do not reach their site of action which leads to a loss of function and thereby to disease (Aridor and Hannan, 2000; Aridor and Hannan, 2002). Generally there are two fates for these retained proteins, either they are degraded or they accumulate. Protein accumulation in the endoplasmic reticulum (ER) may lead to an unfolded protein response (UPR) and/or ER overload response (Zhang and Kaufman, 2006). Other proteins may accumulate in the cytosol, which eventually leads to the formation of intracellular aggregates or aggresomes (Kopito, 2000), but ultimately in both cases, the cell is damaged and apoptosis is induced.

The intracellular retention of the V2 vasopressin receptor (V2R) causes X-linked nephrogenic diabetes insipidus (NDI). More than 170 different mutations of the *AVPR2* gene, each one of them causing NDI, have been described in the literature (http://www.medicine.mcgill.ca/nephros). The V2R is normally expressed at the plasma membrane of renal epithelial cells of the collecting duct and regulates vasopressin (AVP) mediated water reabsorption in the kidney. The majority of NDI-causing mutations produce receptors that are retained in different intracellular compartments, like the ER, ER/Golgi intermediate compartment (ERGIC) (Hermosilla et al., 2004) and Golgi apparatus. Few NDI-causing mutants are expressed at the cell surface, but fail to get activated by AVP (Robben et al., 2006). Patients with NDI suffer from polyuria, polydipsia, nocturia, and failure to thrive.

Disease-causing V2R mutants that are retained in different compartments of the secretory pathway are a good model to elucidate the interactions between the quality control system and degradation pathways. Intracellular degradation of membrane proteins is carried out by the lysosomes and/or the ubiquitin/proteasome system, depending on the intracellular localization of the protein. Proteins localized at the plasma membrane are usually degraded in lysosomes, such as the wild type V2R (Bouley et al., 2005). ER-retained proteins are targeted to the ER-associated degradation (ERAD) pathway. ER-retained misfolded membrane proteins are polyubiquitinylated by ubiquitin ligases (gp78, Hrd1) and pulled out of the ER membrane by AAA-ATPases (p97/valosin-containing protein

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(p97/VCP)) and ATPases of the 19S proteasome (Meusser et al., 2005; Wahlman et al., 2007) through channel proteins, like Sec61α and/or Derlin-1, -2 or -3. They are de-glycosylated, de-ubiquitinylated and unfolded before being degraded by the 26S proteasome (Hampton, 2002; Werner et al., 1996). G protein-coupled receptors (GPCR), as the δ opioid receptor, luteinizing hormone receptor and some disease-causing V2R mutants, have been associated with proteasomal degradation in mammalian cells (Petäjä-Repo et al., 2001; Pietila et al., 2005; Robben et al., 2005), however their degradation mechanisms are not fully understood. Furthermore, it is unknown how receptor proteins are degraded that are neither retained in the ER, nor localized at the cell membrane, like V2R mutants that are retained in the ERGIC (V226E) and Golgi apparatus (G201D).

In yeast, three distinct ERAD pathways (ERAD-L for misfolded ER-luminal domains; -M, for misfolded intramembrane domains; -C, for misfolded cytosolic domains of substrate proteins) have been proposed (Carvalho et al., 2006). ERAD-L and -M require the ubiquitin ligase Hrd1 and its cofactor Hrd3, but not Doa10 as the ERAD-C pathway. Der1 (Derlin-1, -2, -3 in mammals) is required in the ERAD-L pathway. All ERAD pathways require the ATPase complex of Ubx2, Npl4, Ufd1, and Cdc48 (p97/VCP in mammals). So far, it is unknown whether these distinct pathways also operate in mammalian cells. In the present work, we analyzed the degradation pathways of the wild type V2R and of three NDI-causing V2R mutants with misfolded cytosolic (L62P), luminal (G201D) and intramembrane (V226E) domains that are retained in different intracellular compartments, namely the ER, ERGIC and Golgi apparatus, respectively. The wild type receptor and all intracellular retained mutants are polyubiquitinylated and interact with the ERAD components Derlin-1, the AAA-ATPase p97/VCP, and the 26S proteasome regulatory subunit 7 (Rpt1/S7), regardless of the location of their misfolded domains. Our results show that substrates with different misfolded domains are degraded by a Derlin-1, p97/VCP-mediated ERAD pathway in HEK923 cells.

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Materials and Methods

Materials. [^{3}H]cAMP (0.925 TBq/mmol), [α-^{32}P] ATP (29.6 TBq/mmol) and [^{35}S] Easy tag protein labeling mix (43.48 TBq/mmol) were obtained from Perkin Elmer (Boston, USA). LipofectAMINE™ 2000 was purchased from Invitrogen (Karlsruhe, Germany) and FuGENE 6 from ROCHE Diagnostics-Applied Science (Mannheim, Germany). Restriction enzymes were from New England Biolabs (Schwalbach, Germany). Trypsin (sequencing grade) was obtained from Promega (Madison, USA). PMSF, deoxycholic acid sodium salt, Roti-Load 1 and SDS were from Roth (Karlsruhe, Germany). Dulbecco's modified Eagle's medium (without methionine and cysteine), benzamidine, chloroquine diphosphate salt, protein A-Sepharose, protein G-Agarose, the rabbit polyclonal α-Derlin-1 antibody, mouse monoclonal α-actin antibody, rabbit polyclonal α-FLAG antibody, mouse monoclonal α-FLAG M2 antibody and α-FLAG M2 Affinity Gel were from Sigma (Munich, Germany). Aprotinin (Trasylol), Tween-20, Trypsin inhibitor (STI), and the Chemiluminescence detection kit for horseradish peroxidase (POD) were from Applichem (Darmstadt, Germany). The POD-conjugated goat α-rabbit and goat α-mouse antibodies were purchased from Dianova (Hamburg, Germany). The rabbit α-p97/VCP antibody and the monoclonal rabbit α-GAPDH antibody were from Cell Signaling (Danvers, Ma, USA). The rabbit α-19S proteasome subunit Rpt1 antibody was from Biomol (Hamburg, Germany). The rabbit α-calnexin antibody and the monoclonal mouse anti-multi ubiquitin antibody were from Stressgen Bioreagents (Victoria, Canada). The polyclonal goat α-Derlin-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, USA). LysoTracker® Red was from Molecular Probes (Eugene, Oregon, USA). MG 132 was purchased from Calbiochem (Darmstadt, Germany). Plasmid pV2R.DNA3.1, coding for the untagged V2R, and plasmid pEU367.GFP, encoding a C-terminally GFP-tagged V2R, have been described before (Oksche et al., 1998; Schülein et al., 1998). The pEGFP-N1 vector was from Clontech Laboratories (Heidelberg, Germany). The QuikChange® site-directed mutagenesis kit was purchased from Stratagene (La Jolla, USA) and the Qproteome cell compartment kit was from Qiagen (Hilden, Germany). HEK293 cells were purchased from DSMZ (Hannover, Germany).

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Plasmid constructions. Plasmid pFLAG.V2R encoding the V2R with a FLAG tag fused to the N terminus was made *via* PCR with a *BamH*I /FLAG insertion primer (CGGGATCCCGGCCACCATGGA-CTATAAGGACGATGACGATAAGATGCTCATGGCGTCCACTTCCGC) and the commercially available pcDNA3.1/BGH reverse sequencing primer from Invitrogen. For this reaction, pV2R.DNA3.1 was used as a template. The PCR product, containing the FLAG.V2R cDNA and a N-terminally inserted *BamH*I site, was cloned into the pcDNA3.1 vector using the introduced *BamH*I site and an *Xba*I site. Plasmids pFLAG.L62P, pFLAG.V226E and pFLAG.G201D were constructed with the help of the QuikChange® site-directed mutagenesis kit (Stratagene; primer sequences available on request); plasmid pFLAG.V2R was used as template. Plasmids pL62P.GFP, pV226E.GFP and pG201D.GFP encoding the receptor mutants L62P, V226E and G201D tagged with GFP at their C terminus (residue K367) have been described elsewhere (Krause et al., 2000). Standard DNA preparations and cloning techniques were carried out according to the handbooks of Sambrook et al. (Sambrook and Russell, 2001). The nucleotide sequences of the DNA fragments were verified by sequencing, using the BigDye® Terminator kit (Applied Biosystems).

Cell culture. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) from Sigma, supplemented with 10% (v/v) heat-inactivated FBS, 20 mM glutamine, 100 IU of penicillin and 100 µg/ml streptomycin in a 5% CO_2 atmosphere at 37 °C. Stably expressing cell lines were generated through transfection with FuGENE 6 (Roche) or LipofectAMINE™ 2000 (Invitrogen) according to the manufacturer's recommendations. Clones were selected with 400 µg/ml G418. The culture medium for the stably expressing cell clones was additionally supplemented with 400 µg/ml G418.

Adenylyl cyclase assay. The adenylyl cyclase assays were carried out with nuclei-free crude membranes of stably transfected HEK293 cells as described previously for stably transfected mouse Ltk^- cells (Schülein et al., 1996).

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Total cell lysis. HEK293 cells stably expressing V2R constructs were grown to confluence on a 60 mm culture dish. Cells were washed two times with ice-cold PBS and lysed in 500 µl Ripa buffer, the method has been described elsewhere (Gazit et al., 1999). After determination of protein concentration, 30 µg of the total amount of proteins were used for SDS-PAGE.

Immunoprecipitation of FLAG-tagged V2Rs and co-immunoprecipitation. HEK293 cells stably expressing V2R constructs were grown to confluence on a 60 mm culture dish. Cells were washed two times with ice-cold PBS and lysed in 1 ml of ice-cold lysis buffer (1% Triton® X-100, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM Na-EDTA, 0.5 mM PMSF, 10 µg/ml STI, 1 µg/ml Trasylol and 0.5 mM benzamidine, pH 8.0) and transferred to microfuge tubes. The insoluble fraction of one 60 mm culture dish was removed by centrifugation (20 min, 4 °C, 18,620 x *g*) and FLAG-tagged proteins were isolated from the supernatant with an immune complex of 5 mg protein A-Sepharose beads and 5 µg α-FLAG Sigma M2 antibodies at 4 °C overnight. Beads were centrifuged (1 min, 4 °C, 13,680 x *g*), washed three times with buffer A (0.5% Triton® X-100, 0.1% SDS, 50 mM Tris-HCl, 500 mM NaCl, 1 mM Na-EDTA, pH 8.0) and once with buffer B (0.5% Triton® X-100, 0.1% SDS, 50 mM Tris-HCl, 1 mM Na-EDTA, pH 7.4). Bound proteins were eluted by 5 min boiling (95 °C) in 40 µl of 2x Roti-Load 1 sample buffer and separated by SDS-PAGE. For co-immunoprecipitation experiments, FLAG-tagged proteins were isolated with 40 µl of α-FLAG M2 Affinity Gel (Sigma) (same preparation as described above) or with an immune complex of 5 mg protein G-Agarose beads and 2 µg of polyclonal α-Derlin-1 antibodies (Santa Cruz) at 4 °C overnight.

Immunoblots. Proteins were separated by SDS-PAGE (10% or 15% acrylamide) and blotted onto nitrocellulose membranes as described elsewhere (Kyhse-Andersen, 1984). Membranes were blocked for 1 h with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO4, pH 7.4) supplemented with 0.1% Tween-20 and 5% low fat milk powder and, in case of detection of the V2R, incubated with a mouse monoclonal α-FLAG M2 antibody (1:1500 in PBS with 0.1% Tween-20 and 2% low fat milk powder) for 1 h at RT. Membranes were washed three times (15 min each) with wash buffer (PBS supplemented with 0.1% Tween-20). Blots were incubated with a POD-conjugated α-mouse antibody

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(1:10,000) in PBS supplemented with 0.1% Tween-20 and 2% low fat milk powder for 45 min at RT. Membranes were washed again three times with wash buffer (15 min each). Finally, membranes were incubated in ECL for three minutes and developed with a luminescence X-Omat film (Kodak). For co-immunoprecipitation experiments a polyclonal α-p97/VCP antibody (Cell Signaling), a polyclonal rabbit α-Derlin-1 (Sigma), a polyclonal α-19S Regulator, ATPase subunit Rpt1 antibody (Biomol) (all at 1:1000 dilution) or a mouse monoclonal α-FLAG M2 antibody (Sigma) (1:1500 dilution) were used.

Ubiquitinylation assay. Stably transfected HEK293 cells were treated with 20 µM MG 132 in serum-free medium for 16 h. Lysis of cells expressing FLAG-tagged receptors was carried out, the insoluble fraction was removed by centrifugation (20 min, 4 °C, 18,620 x *g*) and polyubiquitinylated proteins were isolated, as described in immunoprecipitation of FLAG-tagged V2Rs with 40 µl of α-FLAG M2 Affinity Gel (Sigma). After the washing steps, the proteins were boiled in 40 µl 2x Roti-Load 1 sample buffer, separated by SDS-PAGE (10% acrylamide) and blotted onto nitrocellulose membranes. For detection, a mouse monoclonal anti-multi ubiquitin antibody (Stressgen) (1:400) and a POD-conjugated α-mouse IgG (1:10,000) were used. The receptor expression was analyzed with a mouse monoclonal α-FLAG M2 antibody (Sigma) (1:1500) and a POD-conjugated α-mouse IgG (1:10,000). Immunoreactive bands were developed with ECL.

Visualization of GFP-tagged receptors by confocal laser-scanning microscopy in living HEK293 cells. Stably transfected HEK293 expressing the wild type and mutant V2Rs tagged with GFP were grown for 24 - 48 h in a 35 mm culture dish containing a poly-L-lysine-coated cover slip. Cells were incubated with 150 nM LysoTracker® for 1 h and for inhibition of degradation additionally incubated with 100 µM chloroquine for 3 h, washed once with PBS and transferred immediately into a heating chamber (details on request). Cells were covered with 1 ml PBS and GFP fluorescence was visualized on a Zeiss 510 invert laser-scanning microscope (optical section: <0.9 µm; λ_{exc}: 488 nm, LP filter: 505 nm). GFP and LysoTracker® fluorescence signals were recorded (optical section: <0.9 µm; multitrack

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mode; GFP, λ_{exc}: 488 nm, BP filter: 494–548 nm; LysoTracker® Red, λ_{exc}: 543 nm, LP filter: 560 nm) and an overlay of both signals was computed.

Pulse-chase assay. Stably transfected HEK293 cells were grown in 100 mm culture dishes until confluence. Cells were starved in 5 ml serum-free DMEM without methionine and cysteine (Sigma) for 16 h. The cells were washed twice with PBS, treated with trypsin/EDTA, and collected in a 15 ml Falcon® tube. Cells were washed with PBS two more times (1,000 x *g*, RT, 5 min). Metabolic labeling with 220 µCi of [^{35}S] Easy tag protein labeling mix in 500 µl medium lacking methionine and cysteine was performed for 45 min. The labeling process was stopped with 4 ml DMEM supplemented with unlabeled methionine (2.3 mM) and cysteine (0.75 mM). To indicated times cells were resuspended and 1 ml suspension was harvested for each sample, mixed with 750 µl ice-cold DMEM supplemented with protease inhibitors (0.5 mM PMSF, 10 µg/ml STI (trypsin inhibitor), 1 µg/ml Trasylol and 0.5 mM benzamidine), collected by a centrifugation step (1,000 x *g*, 4 °C, 5 min) and lysed with 500 µl ice-cold lysis buffer. The FLAG-tagged receptors were immunoprecipitated as described above. After separation by SDS-PAGE (10% acrylamide), the gels were dried and exposed to X-ray film. Lysosomal and proteasomal activity was inhibited with 100 µM chloroquine and 40 µM MG 132, respectively, supplemented in all buffers from the labeling step until the end of the chase.

Protein identification by mass spectrometry. Proteins of HEK293 cells treated with and without MG 132 were immunoprecipitated, prepared as described above, and subjected to SDS-PAGE. Sample preparation, in-gel digestion, peptide extraction, and spectra acquisition were performed as described elsewhere (Czupalla et al., 2006). In brief, proteins were stained with Coomassie blue, excised from the gel and subjected to in-gel digestion by trypsin. NanoLC-MS/MS experiments were performed on a quadruple orthogonal acceleration time-of-flight mass spectrometer Q-Tof Ultima™ (Micromass, Manchester, UK). A Micromass CapLC liquid-chromatography system with a capillary column (PepMap C_{18}, 3 µm, 150 mm x 75 µm i.d., Dionex, Idstein, Germany) was used to deliver the peptide solution to the nanoelectrospray source (PicoTip spray capillaries, New Objective, Woburn, MA, USA). For protein identification, data were acquired in a data-dependent mode (survey scanning)

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using one MS scan followed by MS/MS scans of the most abundant peak. The processed MS/MS spectra and MASCOT server version 1.9 (Matrix Science Ltd, London, UK) were used to search in-house against the SwissProt protein database (210906, 234112 sequences).

Subcellular fractionation. Proteins of different cellular compartments were obtained with the Qproteome cell compartment kit (Qiagen, Germany). Cytosolic and membrane fractions were isolated according to the manufacturer's protocol. Equal amounts of proteins were used for SDS-PAGE. The purity of the fractions was controlled by detection of calnexin and GAPDH, concurrently the proteins served as a loading control. Detection of receptor proteins was carried out with a monoclonal α-FLAG M2 antibody (Sigma) and a POD-conjugated α-mouse IgG. Immunoreactive bands were developed with the ECL system.

Results

Lysosomal degradation of V2 vasopressin receptors

The degradation of intracellularly retained GPCRs has not been characterized in detail. The subject of the present study was the degradation pathway of three intracellularly retained V2R mutants, with misfolded domains located in different parts of the receptor. The L62P mutation is localized in the first intracellular domain at the interface to the first transmembrane domain; G201D lays in the second extracellular loop and V226E in the fifth transmembrane domain (Fig. 1 *A*). Mutants L62P and V226E are deficient in their ability to stimulate the adenylyl cyclase; mutant G201D is partially deficient, thereby confirming their disease-causing nature (Fig. 1 *B*). All mutants also show a different subcellular localization. The L62P mutant is found exclusively in the ER, the V226E mutant in the ER and ERGIC and the G201D mutant localizes in the ER, ERGIC, Golgi apparatus and partially at the plasma membrane (Hermosilla et al., 2004) (see Fig. 2 and supp. Fig. 2). We first determined whether lysosomes are involved in the degradation of all mutants. To this end, the presence of the mutants in lysosomes was assessed in living HEK293 cells by confocal laser scanning microscopy. Cell clones stably expressing the V2R mutants tagged with the green fluorescent protein (GFP) were stained with

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the fluorescent lysosome marker LysoTracker®. GFP fluorescence signals were recorded on the green and the LysoTracker® signals on the red channel; overlays were computed. L62P and V226E were diffusely localized within the cell, excluding the nucleus (Fig. 2, *left panels*). LysoTracker® signals were detected as intracellular vesicles, frequently nearby the nucleus (Fig. 2, *middle panels*) and no co-localization with GFP signals could be identified in overlays of mutants L62P and V226E (Fig. 2, *right panels*). Mutant G201D was detected in vesicular structures and at the plasma membrane. Colocalization with LysoTracker® signals was observed (Fig. 2, *right panel*). GFP fluorescence signals from the wild type V2R were localized predominantly at the plasma membrane and in vesicular structures, which also colocalized with LysoTracker® signals.

To confirm these results, we inhibited lysosomal enzymes, which should lead to an increase in the co-localization between GFP fluorescence and LysoTracker® signals. Stably transfected HEK293 cells were treated with 100 µM chloroquine for 3 h. Only in the case of mutant G201D and the wild type receptor GFP fluorescence signals were increased (Fig. 3, *left and right panels*). No change in the GFP fluorescence signals and colocalization status with lysosomes of mutant L62P and V226E could be observed. These results demonstrate that G201D and the wild type V2R, which reach the Golgi apparatus and plasma membrane, are transported to the lysosomal compartment for degradation at steady state. In contrast, mutants L62P and V226E retained by the quality control system in the ER or ER/ERGIC are excluded from lysosomal degradation.

MG 132 inhibits the degradation of immature forms of all mutants and the wild type V2R

Mutants L62P and V226E were excluded from lysosomal degradation by their retention in the ER and ERGIC. Hence, their degradation should be carried out by the proteasome. Therefore, we studied the proteasomal and lysosomal degradation of all mutants and wild type V2R in HEK293 cells stably expressing N-terminally FLAG-tagged V2R constructs (see Supplemental Data and supp. Fig. 1 for the pharmacological characterization). Pulse-chase metabolic labeling experiments were done in the presence of the lysosomal enzyme inhibitor chloroquine, the proteasomal inhibitor MG 132 and under control conditions (untreated). Immunoprecipitated proteins were identified by autoradiography. At 0 h the wild type V2R showed one prominent band at 38 and another at 35 kDa, the first corresponding

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to the core-glycosylated (*EndoH* and *PNGaseF* sensitive) and the second to the non-glycosylated receptor form (*EndoH* and *PNGaseF* resistant; see supp. Fig. 3) (Fig. 4, *left panel*). Both bands were present at 2.5 h of chase, additionally a third broader band running between 55 and 45 kDa appeared. This band corresponds to the mature, complex-glycosylated form of the receptor (see supp. Fig. 3). After 5 h and 10 h of chase the 35-kDa band became undetectable and only bands corresponding to the glycosylated receptor forms were present.

A similar band pattern was detected for mutant G201D. However, bands representing the complex-glycosylated receptor forms were less prominent. In contrast to the wild type V2R and mutant G201D, mutants V226E and L62P showed two bands only, corresponding to immature forms of the receptor (non- and core-glycosylated); complex-glycosylated forms were not detectable (Fig. 4, *left panel* and supp. Fig. 3). Under control conditions, the immature forms of mutants L62P and V226E persisted longer then that of mutant G201D and the wild type receptor, indicating that the forward transport of the latter is also responsible for a signal reduction (Fig. 4, *left panel*).

Treatment with 100 µM chloroquine had no apparent effect on the band pattern of the wild type V2R, indicating that its lysosomal degradation half-life is longer then 10 h. For mutant G201D a change was only observed in the case of the complex-glycosylated band, confirming that the mutant is degraded in lysosomes. Mutants V226E and L62P showed no change in their band pattern, demonstrating that they do not reach the lysosomal compartment.

Additionally, cell clones were treated with the proteasomal inhibitor MG 132 and bands representing the non-glycosylated and core-glycosylated receptors (immature receptor forms) could be stabilized in all samples (Fig. 4, *right panel*). This result shows that proteasomal activity is involved in receptor degradation of the immature receptor forms. Taken together, these results demonstrate that the complex-glycosylated receptors are subjected to lysosomal degradation whereas non- and core-glycosylated receptor proteins are degraded by the proteasome. Thus, the ERGIC seems to be the last compartment in the secretory pathway that excludes membrane proteins from lysosomal degradation.

Wild type V2R and NDI-causing mutants are polyubiquitinylated

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Mature forms of the mutant G201D and wild type V2R were degraded in lysosomes. In contrast, the immature forms of the wild type and mutant V2Rs were stabilized when proteasomal activity was blocked, demonstrating a participation of the proteasome in their degradation. ERAD substrates are usually polyubiquitinylated and retrotranslocated from the ER to the cytosol for proteasomal degradation. We investigated if the NDI-causing mutants and the wild type V2R are polyubiquitinylated and degraded *via* the ERAD. FLAG-tagged receptors were precipitated with a α-FLAG M2 Affinity Gel and ubiquitinylated receptor proteins were identified with a monoclonal α-polyubiquitin antibody. An immunoreactive smear starting at 250 kDa was only detected in samples that contained receptor constructs (Fig. 5 *A*). MG 132 treatment increased the intensity of this smear that ranged from 250 to 60 kDa due to ubiquitin chains of different length. The wild type V2R and intracellular retained mutants were polyubiquitinylated, indicating that they are suitable substrates for ERAD.

Furthermore, the precipitation efficiency and expression levels of the V2R constructs used for the immunoprecipitations were analyzed by western blot. No major difference in the expression of FLAG-tagged constructs could be detected between cell clones subjected to immunoprecipitation (Fig. 5 *B*). As expected, for the V2R wild type and mutant G201D all three receptor forms, the non-glycosylated, core-glycosylated (immature), and complex-glycosylated form could be detected after MG 132 treatment (compare supp. Fig. 3 & Fig. 4, *right panel*). Mutants V226E and L62P showed only immature receptor forms . Bands with an apparent molecular mass of ~160 and 80 kDa are presumably multimers of immature receptor proteins (Fig. 5 *B*, (*)). The apparent smear detected between 200 and 80 kDa represents polyubiquitinylated forms of the V2R.

Wild type V2R and NDI-causing mutants interact with ERAD components

If the immature wild type V2R and its mutants are degraded *via* the ERAD, canonical ERAD components that interact with polyubiquitinylated proteins should be identified after precipitation of V2R from HEK293 cells. Stably transfected cells expressing the FLAG-tagged wild type V2R were treated with and without MG 132 overnight and receptors were immunoprecipitated and separated by SDS-PAGE. Corresponding slices from Coomassie-stained gels of MG 132-treated and untreated

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samples were excised (for details see supp. Fig. 4). Co-precipitated proteins were in-gel digested with trypsin and identified by NanoLC-tandem mass spectrometry. In samples of untreated cells only the V2R and Ig gamma-1 chain of precipitating antibodies were detected. In samples of MG 132-treated cells, several proteins related to cell stress and protein degradation were identified additionally: immunoglobulin heavy chain-binding protein (BiP/Grp78), the 26S proteasome regulatory subunit 7 (Rpt1/S7), the proteasome 26S non-ATPase subunit 2 (Rpn1/S2), elongation factor 1-α 1 (eEF1A-1), heat shock protein 90kDa α (HSP 90-α/HSP 86) and heat shock protein 90kDa β (HSP 90-β/HSP 84) (Table 1). Both identified proteasomal proteins are subunits of the 19S proteasome regulatory complex.

To verify the mass spectrometry results, we first studied the endogenous expression levels of Rpt1/S7 in whole cell lysates from stable cell clones. A specific immunoreactive band running at 48 kDa was identified in all lysates (Fig. 6, *upper panel*); no difference in the expression of Rpt1/S7 could be detected between cell clones. Next, blots of immunoprecipitated samples pretreated with or without MG 132 showed also a specific band with an apparent mass of 48 kDa, representing Rpt1/S7, and an unspecific band of 60 kDa. In untreated samples of mutant L62P, more Rpt1/S7 could be co-precipitated then in other samples (Fig. 6, *middle panel, Rpt1/S7*). As expected, a strong increase in the intensity of the specific immunoreactive band could be observed, when cells were treated with MG 132 (Fig. 6, *lower panel, Rpt1/S7*). As a control for V2R precipitation levels the same membranes were stripped and used for V2R detection (Fig. 6, *V2R*). In the presence of MG 132 immature V2R forms were increased, partially explaining the increased co-immunoprecipitation of Rpt1.

Another canonical ERAD component is p97/VCP, an AAA-ATPase involved in the extraction of misfolded proteins from the ER. It binds to polyubiquitinylated ERAD substrates. Expression levels of p97/VCP were analyzed in whole cell lysates by immunoblotting and again no differences could be detected between cell clones (Fig. 7, *upper panel, p97/VCP*). Co-immunoprecipitation studies showed an immunoreactive band with an apparent size of 89 kDa in samples of whole cell lysates detected against p97/VCP. No specific signals were found in co-immunoprecipitated samples from untreated cell clones. The only exception was mutant L62P, where a specific band running at 89 kDa could be identified, indicating an interaction of this mutant with p97/VCP at steady state (Fig. 7, *middle panel,*

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p97/VCP). As expected, treatment with MG 132 increased the amount of co-precipitated p97/VCP significantly (Fig. 7, *lower panel, p97/VCP*). V2R precipitation levels were assayed in the same membranes used for the co-immunoprecipitation study (Fig. 7, *V2R*).

Similar experiments were also performed with an antibody against Derlin-1, a membrane protein that acts in concert with the p97/VCP to remove dislocation substrate proteins from the ER membrane. A single band with an apparent size of 22 kDa could be identified in samples of whole cell lysates, with no differences in the expression levels between cell clones (Fig. 8 *A, upper panel, Derlin-1*). In immunoprecipitated samples of untreated cells, an immunoreactive band of 22 kDa could be detected with a α-Derlin-1 antibody (data not shown). As expected, the intensity of this band increased, when cells were treated overnight with MG 132 (Fig. 8 *A, middle panel, Derlin-1*). V2R precipitation levels were verified in the same membranes used for the detection of Derlin-1 (Fig. 8 *A, lower panel, V2R*). Additionally, we also precipitated Derlin-1 and detected against the V2R with a mouse monoclonal α-FLAG M2 antibody. As expected, only immature forms of all mutants and the wild type V2R were co-precipitated by Derlin-1 in a MG 132-dependent manner (Fig. 8 *B*). Unfortunately, reverse co-immunoprecipitation studies were not successful using α-Rpt1 and α-p97/VCP antibodies for precipitation.

Proteasomal inhibition leads to an accumulation of immature, polyubiquitinylated and non-polyubiquitinylated proteins, thereby increasing the amount of immunoprecipitated receptors and co-precipitated proteins, in this case Rpt1/S7, p97/VCP and Derlin-1, confirming that these proteins bind polyubiquitinylated ERAD substrates (Wang et al., 2004) (see Fig. 5 *A & B*, Fig. 6 – 8, *V2R*). Our results show that immature and/or polyubiquitinylated V2Rs interact with ERAD components, namely Rpt1/S7, p97/VCP and Derlin-1. However, these results do not preclude the possibility that other ERAD proteins participate in this complex.

Immature receptor proteins are retrotranslocated into the cytosol

V2R mutants are polyubiquitinylated and interact with membrane and cytosolic components of the ERAD complex. For determination of the site of turnover, cytosolic and membrane fractions of HEK293 cells stably expressing FLAG-tagged L62P mutants and wild type receptors were isolated.

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Immunoblot analysis revealed specific bands in the cytosolic and membrane fractions of MG 132-treated cells (Fig. 9). In the cytosolic fraction of L62P a specific band of approximately 35 kDa could be detected that corresponds to the un-glycosylated receptor form. In the membrane fraction a specific band of 38 kDa could be identified, representing the core-glycosylated receptor form. For the wild type receptor a similar band pattern could be recognized, however in the membrane fraction of MG 132-treated cells two additional immunoreactive bands could be detected, one between 53 - 45 kDa and a second of 35 kDa. These bands represent the complex- and un-glycosylated receptor forms, respectively. As expected, an increase in the intensity or emergence of bands representing the immature receptor forms was observed in cells treated with MG 132, confirming our results obtained by pulse-chase analysis. These results indicate that ERAD substrates are de-glycosylated and retrotranslocated completely into the cytosol. Furthermore, glycosylated receptor forms are restricted to the membrane fraction.

Taken together, our results demonstrate that core-glycosylated disease-causing mutants of the V2R are retrotranslocated, de-glycosylated and degraded *via* a Derlin-1 and p97/VCP mediated ERAD pathway. This degradation pathway is independent of the location of the misfolded domain within the V2R.

Discussion

Two major pathways may operate in the cell to degrade intracellularly retained GPCRs: the ubiquitin-dependent proteasomal and the lysosomal pathways (Ciechanover, 2006). It is accepted that integral membrane proteins expressed at the cell surface are internalized and degraded by the lysosome. On the other hand, membrane proteins retained in the ER by the quality control system are retrotranslocated into the cytosol and degraded by the ubiquitin/proteasome system. This particular degradation pathway is known as ERAD (Brodsky and McCracken, 1999; Hampton, 2002). In mammalian cells the ERAD pathway is still not well characterized. In addition, the degradation pathways utilized by the cell when proteins are retained outside the ER are also not well defined.

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In the present study, we analyzed the degradation pathways of the wild type V2R and three intracellularly retained disease-causing mutants. These particular mutants are good tools to analyze the degradation pathways, because they are retained in different compartments of the secretory pathway (Hermosilla et al., 2004). Furthermore, the defects resulting from mutations are localized in different domains of the protein; L62P is located in a cytosolic domain (beginning of the first intracellular loop), G201D in a luminal (second extracellular loop) and V226E in a transmembrane domain (fifth helix) (Fig. 1 *A*). The co-localization studies in living cells showed that the wild type V2R and mutant G201D are localized in lysosomes. Furthermore, pulse-chase experiments demonstrated that the complex glycosylated (mature) forms of mutant G201D are degraded by lysosomes. Similar results have been reported for the wild type V2R and mature mutants at steady state and after activation by AVP (Robben et al., 2004). Our results demonstrate that membrane proteins, which reach the Golgi apparatus or are expressed at the plasma membrane, are transported to the lysosomal compartment for degradation at steady state. However, it is not known whether receptors that reach the Golgi apparatus are first transported to the plasma membrane and then to lysosomes, or if a direct route is followed or if both routes are operating.

All immature V2R forms (mutant and wild type) were degraded *via* the ERAD, regardless of their intracellular localization (Fig. 6 – 8; supp. Fig. 2). As expected, all mutants were found out to be polyubiquitinylated (Fig. 5 *A & B*). We could demonstrate that in the presence of a proteasome inhibitor all non- and core-glycosylated V2R proteins were stabilized (Fig. 4, *MG 132* & Fig. 5 *B*), and that wild type and mutant receptors co-precipitated Derlin-1, p97/VCP, Rpt1/S7 and Rpn2/S2. These results indicate that newly synthesized misfolded or unfolded receptors are targeted to the ERAD by the quality control system. Therefore, we conclude that the ERAD is predominantly involved in the degradation of immature disease-causing V2R mutants, but also of immature, misfolded wild type V2Rs. The fact that also immature wild type proteins are degraded by the ERAD has been described for the δ opioid (Petäjä-Repo et al., 2001), luteinizing hormone receptor (Pietila et al., 2005) and the cystic fibrosis transmembrane regulator (CFTR) and ascribed to inefficient folding or structural instability of the proteins (Sun et al., 2006). These data show that only non- and core-glycosylated misfolded receptor proteins are degraded *via* ERAD and that complex-glycosylated misfolded receptor

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proteins are targeted to lysosomal degradation. Our results demonstrate that the ERGIC is the last compartment of quality control that participates in ERAD.

The wild type V2R has been described to be ubiquitinylated together with β-arrestin 2, but only after agonist induced internalization; both proteins are thought to be transported together to the lysosomal compartment for degradation (Martin et al., 2003). The ubiquitin ligases involved in the polyubiquitinylation of immature wild type V2Rs and NDI-causing mutants are still unknown. Most probable ubiquitin ligases like gp78/autocrine motility factor receptor (membrane-associated E3), Hrd1 (membrane-associated E3) and Ufd2 (E4 enzyme), that interact with p97/VCP and participate in the retrotranslocation of ERAD substrates (Ye, 2006), are involved in the polyubiquitinylation of immature forms of the V2R. In an attempt to determine the E3 that is responsible for the polyubiquitinylation of NDI-causing V2R mutants, immunoprecipitated samples were analyzed for the presence of Hrd1. It was not possible for us to detect specifically Hrd1 in these samples (data not shown).

In yeast, three distinct ERAD pathways have been proposed: the ERAD-L, -M and -C, which destroy proteins with misfolded luminal, intramembrane and cytosolic domains, respectively (Carvalho et al., 2006). These pathways use the cytosolic Cdc48 ATPase complex, whose membrane recruitment is facilitated by Ubx2. However, the ERAD-L and -M pathway require the ubiquitin ligase Hrd1, additionally ERAD-L needs Der1; both proteins have mammalian homologues (Hrd1, gp78 and Derlin-1, -2, -3) that are involved in the mammalian ERAD. ERAD-C requires the ubiquitin ligase Doa10, but not Hrd1 and Der1. Our results for the studied V2R mutants suggest that only the Derlin-1-mediated ERAD pathway is used in HEK293 cells. Regardless of their misfolded domain either cytosolic (L62P), luminal (G201D) or membrane (V226E), all mutants precipitated Derlin-1 and p97/VCP, the mammal homolog of Cdc48. We also investigated a second mutant with a mutation within the fourth transmembrane domain (S167L) that also interacts with Derlin-1 (data not shown). Our results demonstrate that at least the putative channel protein Derlin-1 and the AAA-ATPase p97/VCP are central components of the retrotranslocation machinery of the ERAD in a mammalian system.

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Sec61α and Derlin-1 (and its homologs, Derlin-2 and -3) are thought to be the channel or to be located at the site of retrotranslocation of misfolded proteins from the ER to the cytosol (Kalies et al., 2005; Ye et al., 2005; Ye et al., 2004). It is still unclear whether the proteasome is recruited to the ER membrane by binding to the retrotranslocation channel, so that substrates are directly released into the proteasome (Kalies et al., 2005) or if substrates are first released into the cytosol and then targeted to the proteasome (Meusser et al., 2005; Petäjä-Repo et al., 2001). It has been shown that the 26S proteasome disassembles in an ATP-dependent catalytic cycle, leading to 19S RP subcomplexes and free Rpn10/S5a (Babbitt et al., 2005). On the other hand, 19S RPs are able to interact with Sec61 at the ER membrane (Ng et al., 2007). Recently, Wahlman et al. demonstrated that ATP, the 19S RP, the ER luminal chaperone PDI and Derlin-1 are sufficient to retrotranslocate a soluble ERAD substrate *in vitro* (Wahlman et al., 2007). Interestingly, retrotranslocation of the substrate was ubiquitin, Sec61α, p97/VCP, and Ufd1 and Npl4 cofactors independent. However, the ERAD substrate used in this study was the fluorescence-labeled Δgpαf, a soluble, non-ubiquitinylated protein lacking disulfide bonds and transmembrane domains, which is derivative of the yeast pro-α factor mating pheromone. In our system, V2Rs were located in the ER-membrane, glycosylated, and/or polyubiquitinylated, which might explain the differences regarding the association with p97/VCP. Furthermore, we could identify de-glycosylated V2R (wild type and mutant) in the cytosolic fraction after proteasomal inhibition, indicating a complete retrotranslocation of the receptors to the cytosol. Our results support the idea that 19S RP plays a central role in retrotranslocation and recognition of ERAD substrates, prior to their degradation by the 26S proteasome. Since retrotranslocated V2Rs seemed not to be polyubiquitinylated, the interaction with 19S RP might induce their deubiquitinylation. Therefore, we propose that subunits of the 19S RP, like Rpt1/S7, Rpn1/S2 and Rpn10/S5a, function as scaffolds on the ER-substrate for the 19S RP and 20S CP, facilitating the assembly of the 26S proteasome at the substrate. Moreover, Derlin-1 is centrally implicated in the recognition and/or retrotranslocation of the V2R, independent of the location of the misfolded domain.

Until now, no effective pharmacological treatment of NDI has been developed. A promising therapeutic strategy is the use of pharmacochaperones that rescue transport defective receptors from the early secretory pathway to the plasma membrane (Bernier et al., 2006; Wüller et al., 2004).

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Another possible approach to rescue intracellularly retained proteins may be the inhibition of the ERAD, as exemplary shown for the CFTR by reducing the expression of p97/VCP or blocking the proteasome with bortezomib (Vij et al., 2006). However, this new therapeutic strategy may be only valid for NDI-causing mutants that are transport defective, but still functional.

In summary, our data reveal that the quality control system of the cell determines the degradation pathway of NDI-causing mutants of the V2R. Immature receptor proteins that reach the ERGIC are polyubiquitinylated and degraded *via* the ERAD, all other mature receptor proteins located beyond the ERGIC are degraded *via* lysosomes. Furthermore, we demonstrate that a Derlin-1 and p97/VCP-mediated ERAD pathway is responsible for the degradation of the V2R and its disease-causing mutants, regardless of the location of the misfolded domain within the receptor.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. *A*, two-dimensional model of the V2R and three NDI-causing mutants. The amino acid sequence of the receptor is shown in one letter code, N-glycosylation at position N22, disulfide bond between C112 and C192 and palmitoylation of residues C341 and C342 are depicted. Substituted amino acids of mutants L62P, V226E, and G201D are shown in black. *B*, adenylyl cyclase activity assay with crude membranes of stably transfected HEK293 cells expressing the FLAG-tagged wild type and mutant V2Rs. Crude membranes were stimulated with 100 nM AVP, controls were performed in the absence of AVP. Data represent mean values of three independent experiments each performed in triplicate. Triplicates differed by less than 10%. SEM are depicted.

Fig. 2. Lysosomal localization of GFP-tagged wild type V2R and mutants L62P, V226E, and G201D analyzed in stably expressing living HEK293 cells. Cells were incubated with 150 nM LysoTracker® for 1 h and were examined by confocal laser scanning microscopy with horizontal (*xy*) scans. Receptor GFP fluorescence signals are shown in green (left panels) and LysoTracker® staining of the same cells in red (central panels). GFP and LysoTracker® Red fluorescence signals were computer-overlaid (right panels; overlap is indicated by yellow). The scans show representative cells. Scale bar, 10 μm. Similar data were obtained in five independent experiments.

Fig. 3. Inhibition of lysosomal degradation analyzed in living HEK293 cells stably expressing the GFP-tagged wild type V2R and mutants L62P, V226E, and G201D. Cells were incubated with 100 μM chloroquine for 3 h and 150 nM LysoTracker® for 1 h and were examined by confocal laser scanning microscopy with horizontal (*xy*) scans. Receptor GFP fluorescence signals are shown in green (left panels) and LysoTracker® staining of the same cells in red (central panels). GFP and LysoTracker® Red fluorescence signals were computer-overlaid (right panels; overlap is indicated by yellow). The scans show representative cells. Scale bar, 10 μm. Similar data were obtained in five independent experiments.

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Fig. 4. Degradation of wild type V2R and NDI-causing mutants in stably expressing HEK293 cells. Cells expressing the FLAG-tagged wild type V2R and receptor mutants L62P, V226E and G201D were starved in serum-free DMEM without methionine and cysteine for 16 h and metabolically labeled with 220 µCi [^{35}S] Easy tag protein labeling mix for 45 min without (left panel) or with 100 µM chloroquine (center) or 40 µM MG 132 (right panel). The metabolic labeling was stopped and at time points 0, 2.5, 5, and 10 h the cells were harvested, lysed and immunoprecipitated as described in experimental procedures and separated by SDS-PAGE. The gels were dried and exposed to X-ray film. Molecular mass markers are shown on the right. The results are representative of three independent experiments.

Fig. 5. Ubiquitinylation and immunoprecipitation of FLAG-tagged V2R receptor constructs. *A*, Immunoprecipitation of polyubiquitinylated receptors. HEK293 cells stably expressing wild type and mutant V2Rs were treated with 20 µM MG 132 for 16 h (+) or left untreated (-). Proteins eluted from α-FLAG M2 Affinity Gel were analyzed by SDS-PAGE and western blot analysis with a monoclonal mouse α-polyubiquitin antibody and a POD-conjugated α-mouse antibody. *B*, stably expressing HEK293 cells were lysed and receptors immunoprecipitated with a α-FLAG M2 Affinity Gel as in *A* and detected with a mouse monoclonal α-FLAG M2 antibody, a POD-conjugated α-mouse antibody and the ECL system. Control: untransfected HEK293 cells. (*) indicates presumed V2R multimers. Data are representative of six independent experiments.

Fig. 6. Total cell lysates analyzed for Rpt1/S7 expression by western blotting (top panel). HEK293 cells stably expressing wild type and mutant V2Rs were treated with 20 µM MG 132 for 16 h (+) or left untreated (-). Immunoprecipitation of FLAG-tagged V2R wild type and mutant receptors was performed and analyzed by immunoblotting in the two middle panels. Detection of co-precipitated subunits of the 26S proteasome was done with a polyclonal rabbit α-Rpt1/S7 antibody and a POD-conjugated α-rabbit antibody. V2R precipitation levels of the stripped membrane used for Rpt1 detection in the presence of MG 132 are shown in the lower panel. Controls were non-transfected HEK293 cells. Data are representative of four independent experiments.

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Fig. 7. Interaction of the V2R and NDI-causing mutants with p97/VCP. HEK293 cells stably expressing wild type and mutant V2Rs were treated with 20 μM MG 132 for 16 h (+) or left untreated (-). Total cell lysates analyzed for p97/VCP expression by western blotting are shown in the top panel. Immunoprecipitation of the V2R wild type and mutant receptors was carried out and analyzed by western blotting in the two middle panels. Detection of co-precipitated p97/VCP was done with a polyclonal rabbit α-p97/VCP antibody, POD-conjugated α-rabbit antibody, and the ECL system. V2R precipitation levels of the stripped membrane used for p97/VCP detection in the presence of MG 132 are shown in the lower panel. Controls were non-transfected HEK293 cells. Data are representative of four independent experiments.

Fig. 8. Interaction of the V2R and NDI-causing mutants with Derlin-1. *A*, HEK293 cells stably expressing wild type and mutant V2Rs were treated with 20 μM MG 132 for 16 h (+) or left untreated (-). Total cell lysates of untreated cells analyzed for Derlin-1 expression by western blotting are shown in the top panel. Actin was used as loading control. Immunoprecipitation of the wild type V2R and mutant receptors was carried out as described in Experimental Procedures and analyzed by western blotting. Detection of co-precipitated Derlin-1 was done with a polyclonal rabbit α-Derlin-1 antibody, POD-conjugated α-rabbit antibody, and the ECL system. V2R precipitation levels of the stripped membrane used for Derlin-1 detection in the presence of MG 132 are shown in the lower panel. Controls were non-transfected HEK293 cells. *B*, Co-immunoprecipitation study of the interaction between Derlin-1 and the V2R and its disease-causing mutants. Precipitations were done with a polyclonal α-Derlin-1 antibody (Santa Cruz) and detection was performed with a monoclonal α-FLAG M2 antibody. Specific bands, representing the immature forms of the V2R, could only be detected in the presence of MG 132. Controls were non-transfected HEK293 cells. Data are representative of four independent experiments.

Fig. 9. Cellular fractions analyzed for V2R expression. Stably expressing HEK293 cells were either treated with 20 μM MG 132 for 16 h (+) or left untreated (-). Isolated cytosolic (c) and membrane (m)

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fractions were analyzed by SDS-PAGE and immunoblotting. Detection of FLAG-tagged V2R constructs was done with a mouse monoclonal α-FLAG M2 antibody and a POD-conjugated α-mouse antibody. Controls were non-transfected HEK293 cells (control). The purity of cell fractions was confirmed by the detection of calnexin and GAPDH in all isolated fractions. Data are representative of three independent experiments.

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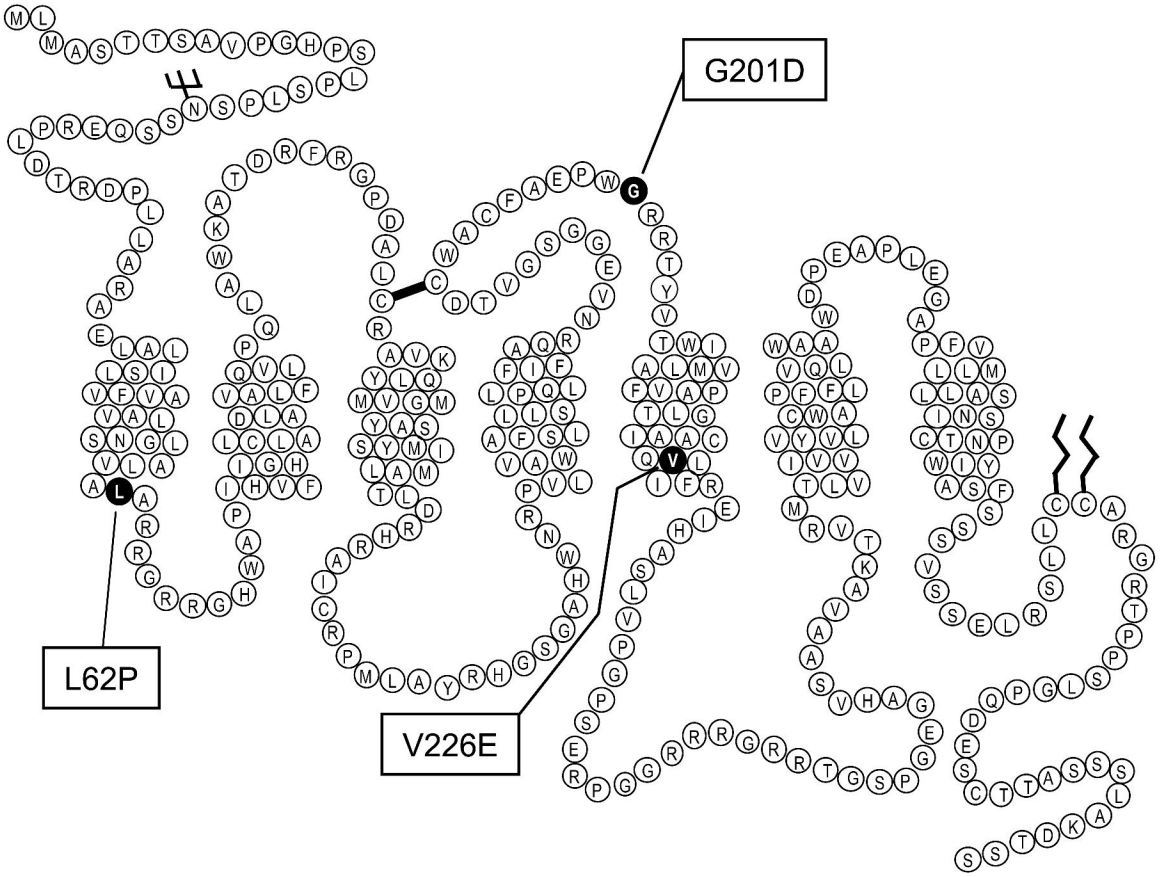
Table 1. V2R-associated proteins identified by NanoLC-ESI-MS/MS in the presence of MG 132

Protein	SwissProt	Molecular mass *Da*	Score	Peptides (MS/MS)
Immunoglobulin heavy chain-binding protein (GRP78/BiP)	P11021	72288	1399	23
26S proteasome ATPase regulatory subunit 7 (Rpt1/S7)	P35998	48472	352	5
26S proteasome non-ATPase regulatory subunit 2 (Rpn1/S2)	Q13200	100136	447	9
Elongation factor 1-a1 (eEF1A-1)	P68104	50109	621	13
Heat shock protein HSP 90-β (HSP 84)	P08238	83081	390	8
Heat shock protein HSP 90-α (HSP 86)	P07900	84476	253	5
Human V2 vasopressin receptor (V2R)	P30518	40253	246	5
Mouse Ig gamma-1 chain C region (IgG1)	P01869	43359	206	4

Table 1. V2R-associated proteins identified by NanoLC-MS/MS. V2Rs expressed in the presence of MG 132 were immunoprecipitated *via* their FLAG tag and separated by SDS-PAGE. Co-precipitated proteins were stained with Coomassie blue, excised from the gel and subjected to in-gel digestion by trypsin. NanoLC-MS/MS experiments were performed and peptides identified in a data-dependent mode (survey scanning) using one MS scan followed by MS/MS scans of the most abundant peak. The most abundant proteins identified are shown. Proteins listed were not found in cells that were not treated with MG 132 (with the exception of the V2R and IgG1). The complete set of proteins identified in the absence and presence of MG 132 is available in the Supplemental Data section (supp. Table 1 & 2).

Fig. 1

A


G201D
L62P
V226E

B

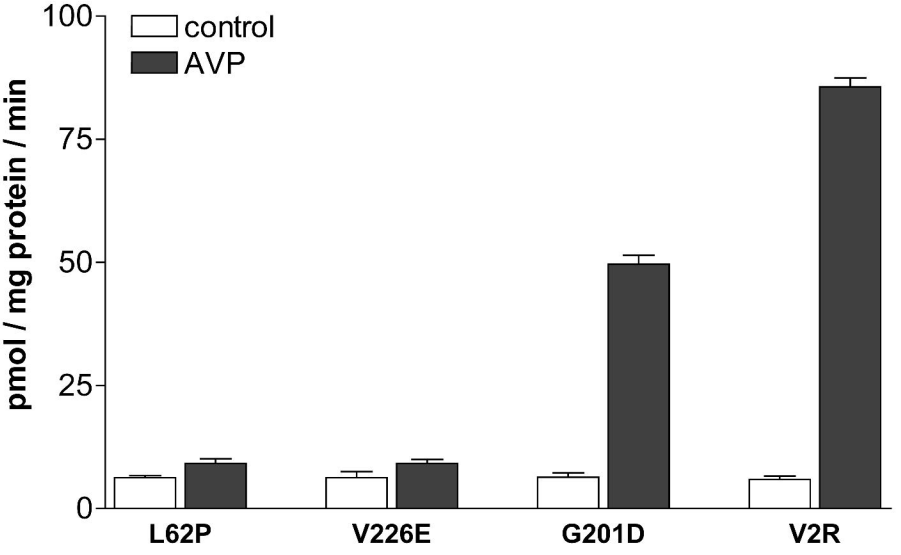

100
75
50
25
0
control
AVP
pmol / mg protein / min
L62P
V226E
G201D
V2R

Fig. 2

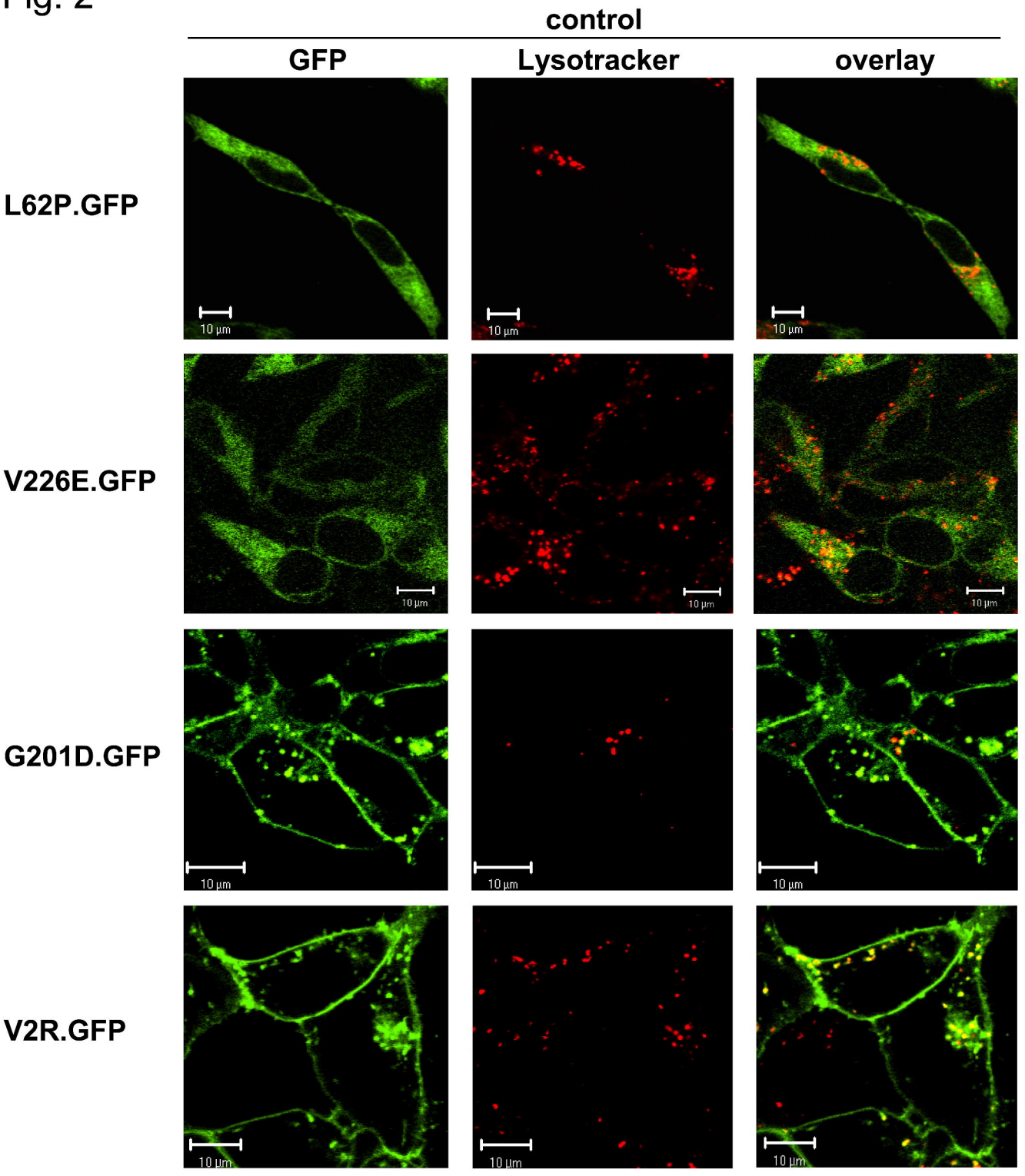

control
GFP
Lysotracker
overlay
L62P.GFP
V226E.GFP
G201D.GFP
V2R.GFP
10 µm

Fig. 3

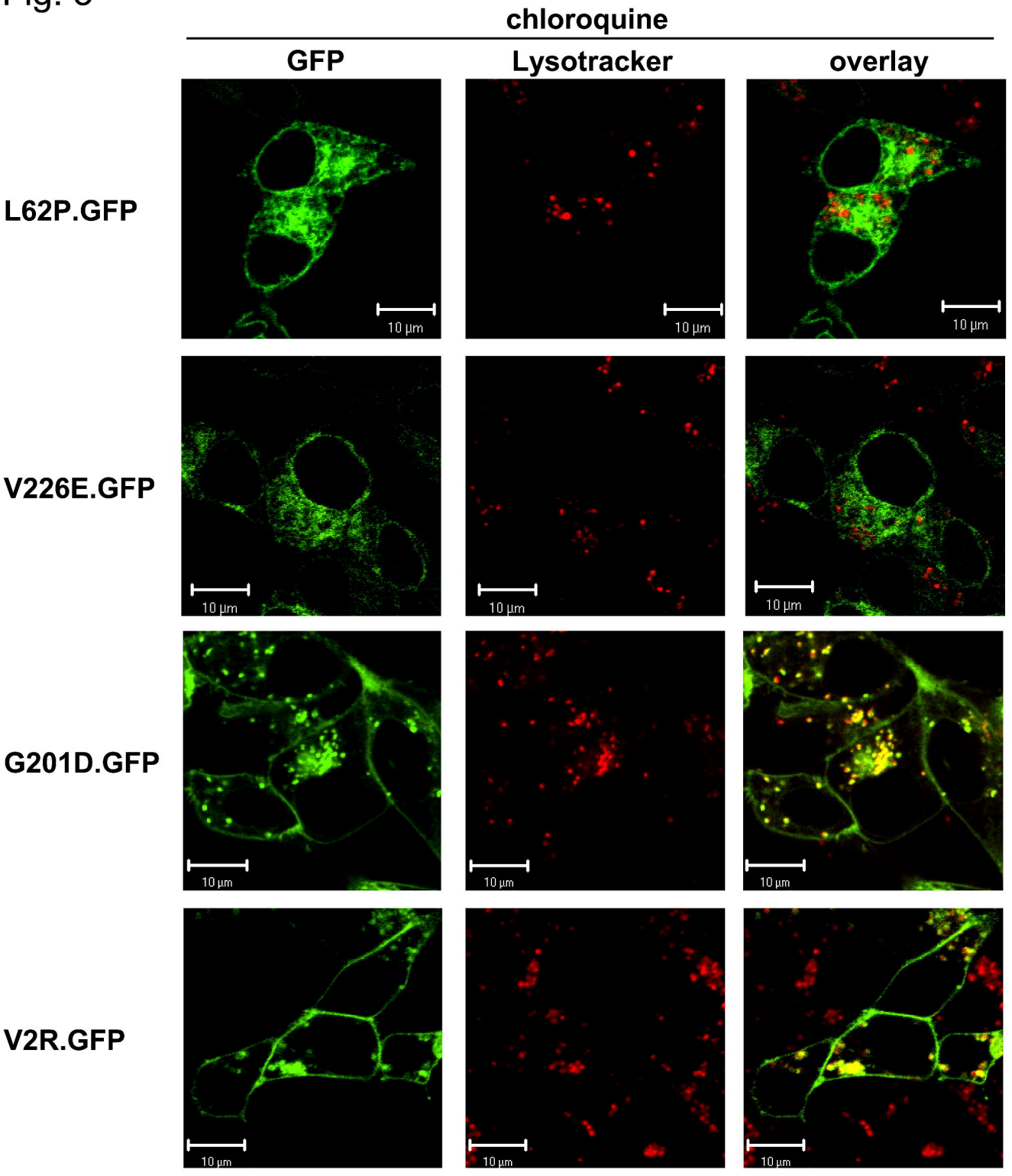

chloroquine
GFP
Lysotracker
overlay
L62P.GFP
V226E.GFP
G201D.GFP
V2R.GFP
10 µm

Fig. 4

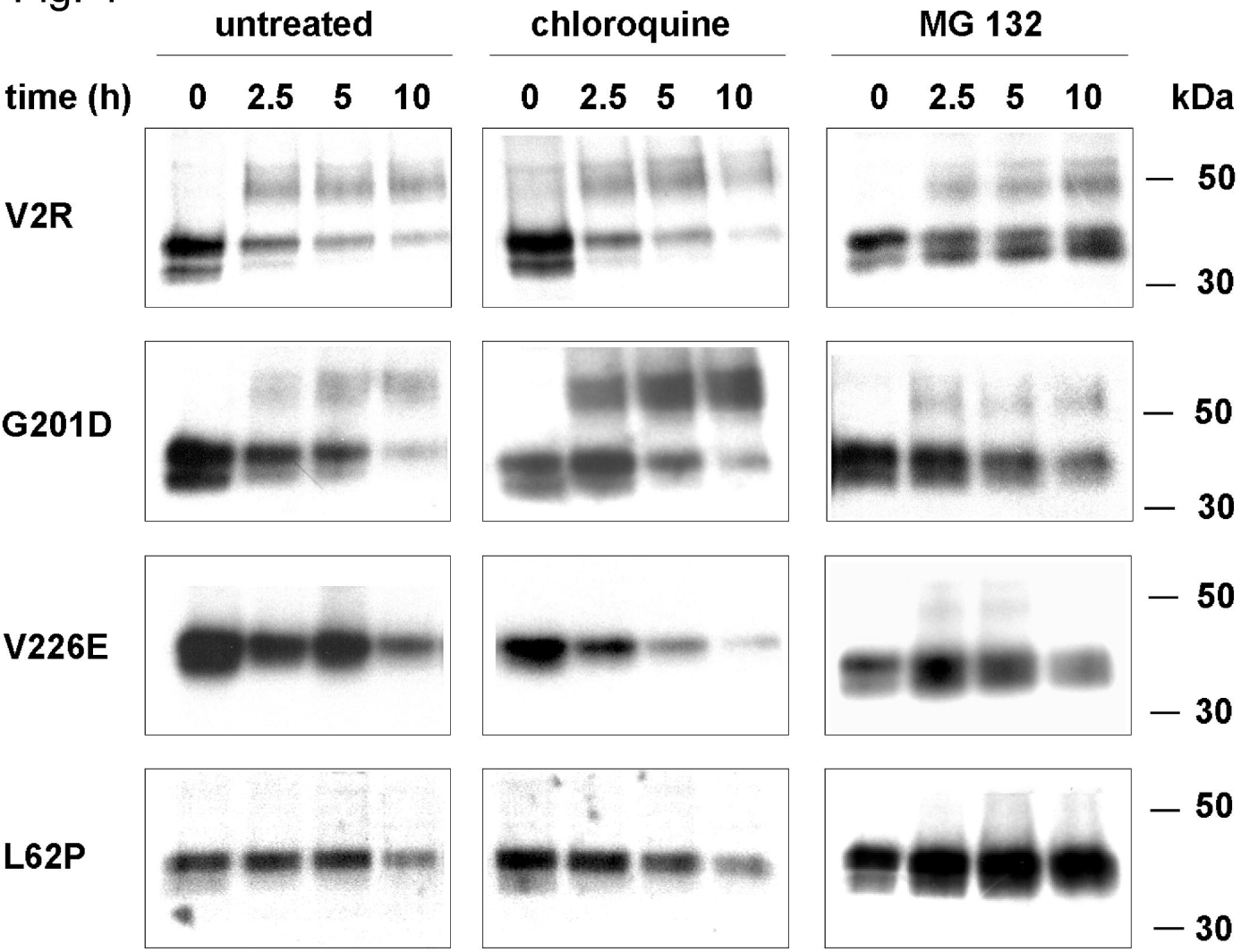

untreated
chloroquine
MG 132
time (h)
0
2.5
5
10
0
2.5
5
10
0
2.5
5
10
kDa
V2R
G201D
V226E
L62P
50
30
50
30
50
30
50
30

Fig. 5

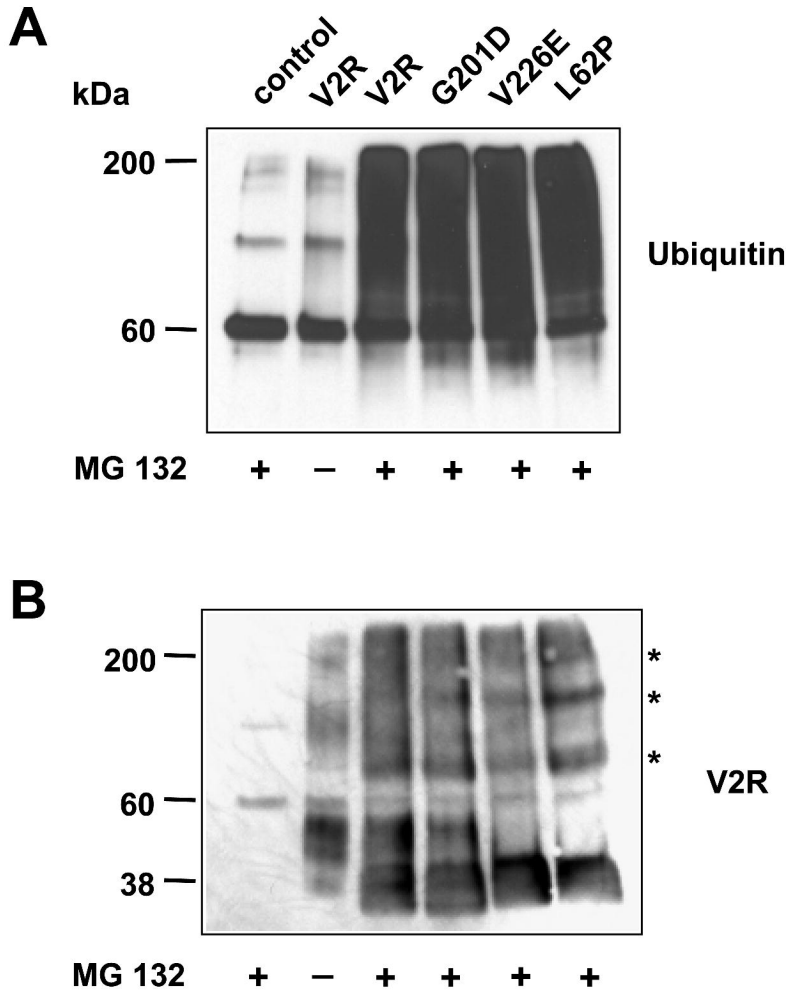

A
kDa
control
V2R
V2R
G201D
V226E
L62P
200
60
Ubiquitin
MG 132
+ – + + + +
B
200
60
38
*
*
*
V2R
MG 132
+ – + + + +

Fig. 6

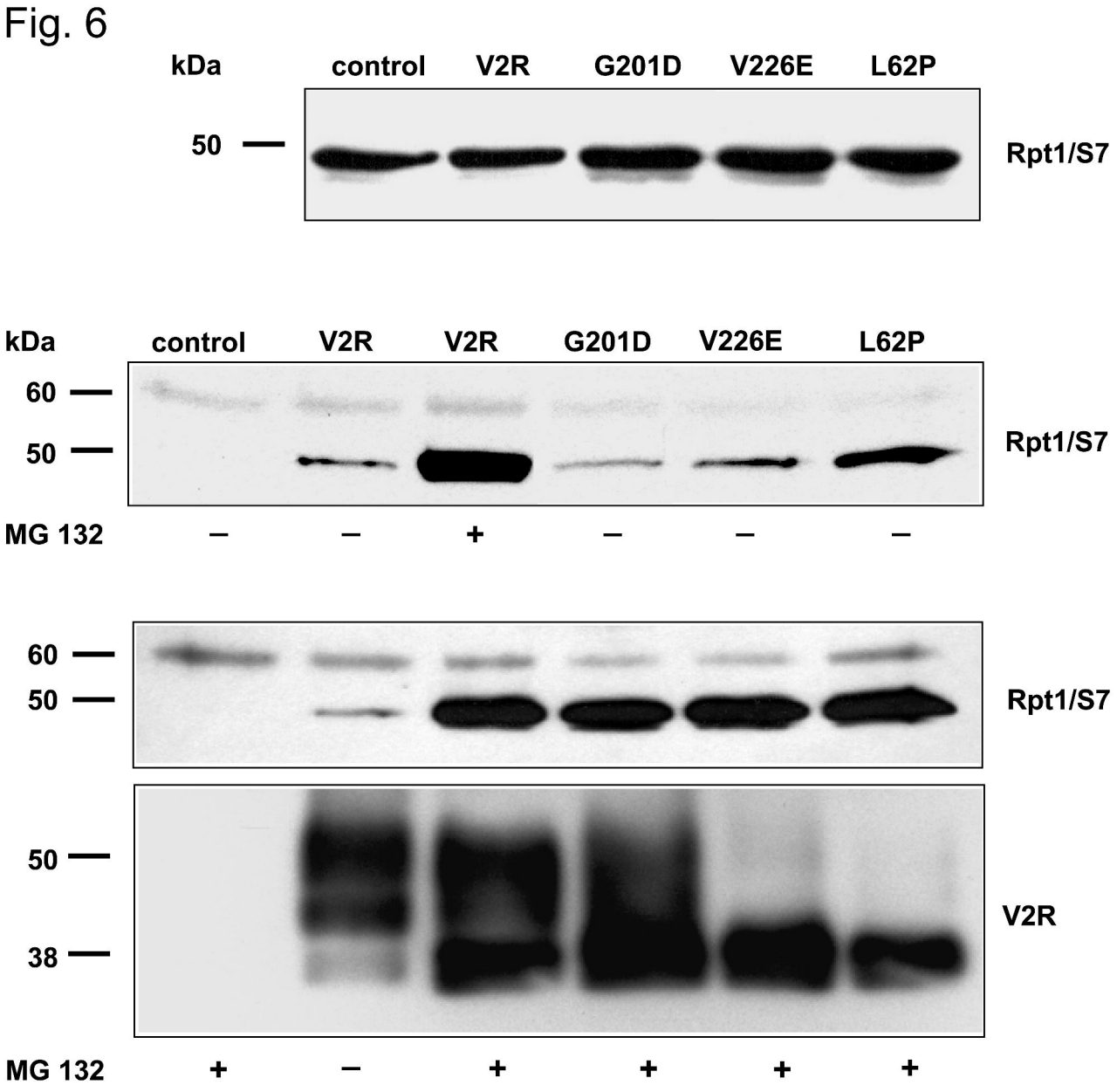

kDa
control
V2R
G201D
V226E
L62P
50
Rpt1/S7
kDa
control
V2R
V2R
G201D
V226E
L62P
60
50
Rpt1/S7
MG 132
–
–
+
–
–
–
60
50
Rpt1/S7
50
38
V2R
MG 132
+
–
+
+
+
+

Fig. 7

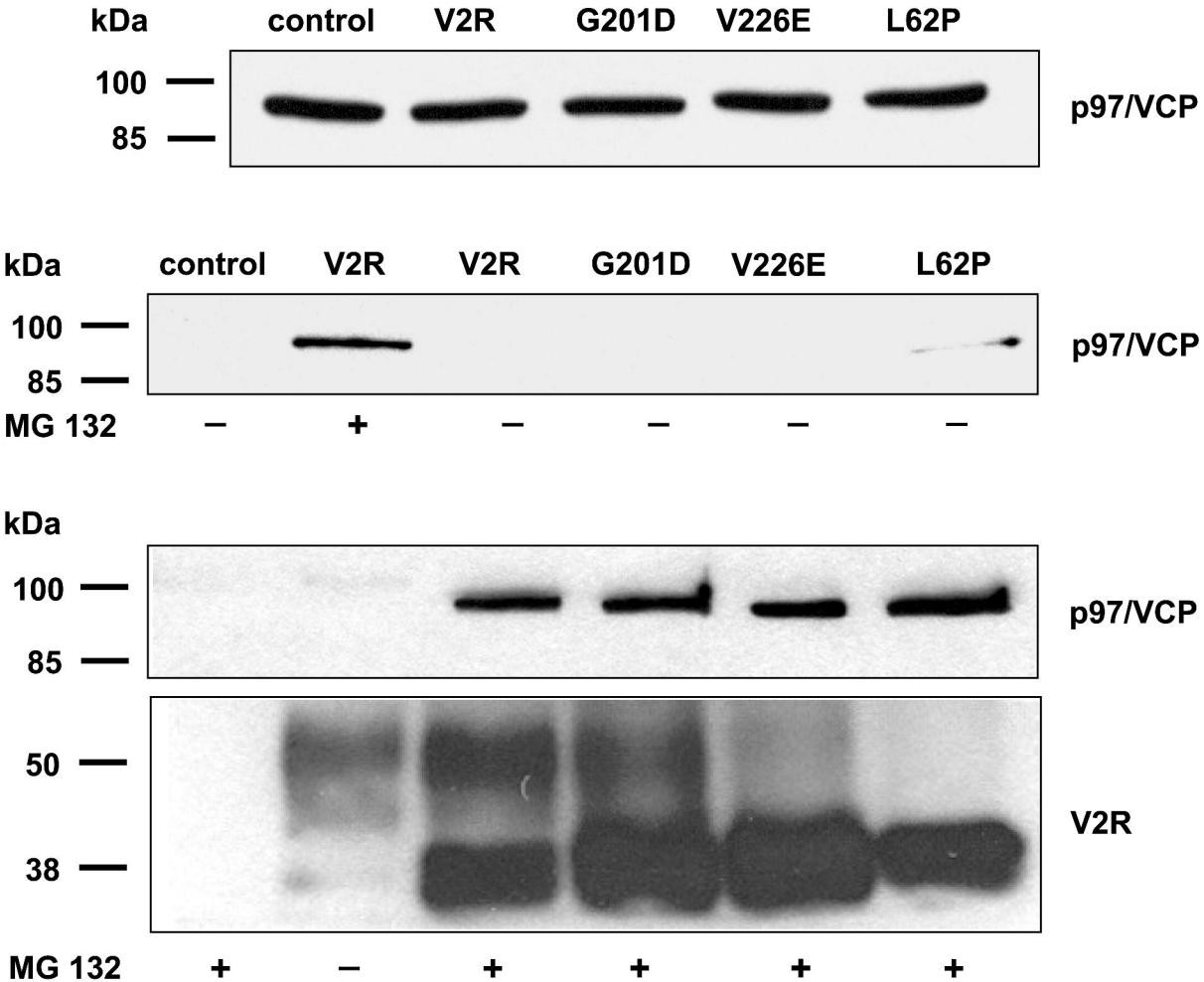

kDa
control
V2R
G201D
V226E
L62P
100
85
p97/VCP
kDa
control
V2R
V2R
G201D
V226E
L62P
100
85
p97/VCP
MG 132
–
+
–
–
–
–
kDa
100
85
p97/VCP
50
38
V2R
MG 132
+
–
+
+
+
+

Fig. 8

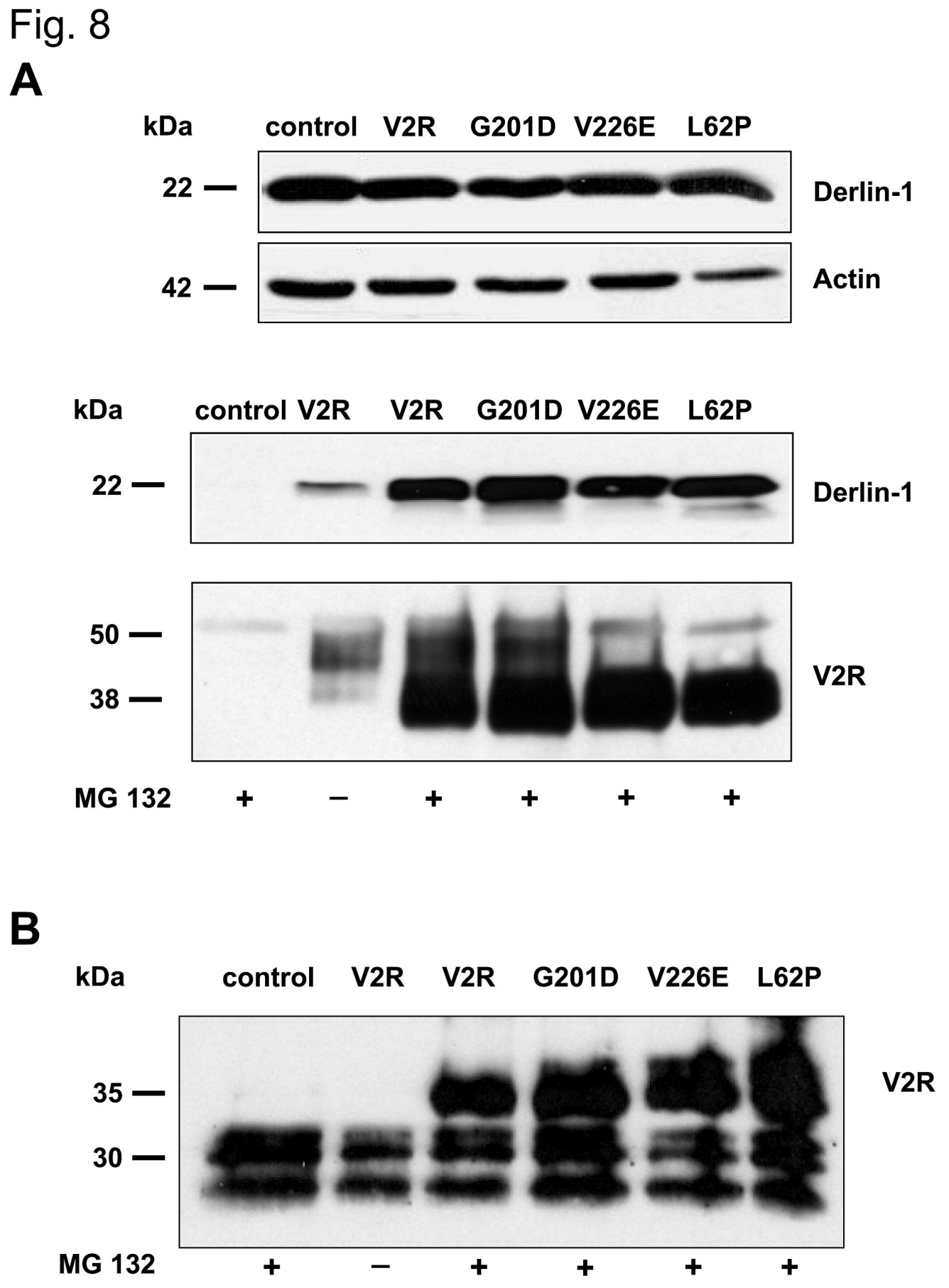

A
kDa
control
V2R
G201D
V226E
L62P
22
Derlin-1
42
Actin
kDa
control
V2R
V2R
G201D
V226E
L62P
22
Derlin-1
50
38
V2R
MG 132
+
–
+
+
+
+
B
kDa
control
V2R
V2R
G201D
V226E
L62P
35
30
V2R
MG 132
+
–
+
+
+
+

Fig. 9