6 Discussion

Hereditary diseases such as NDI can cause large amounts of misfolded proteins. Cells have to find a way to cope with these misfolded proteins, because they constitute a fundamental threat for normal cellular homeostasis; otherwise apoptosis may be induced. Incompletely folded secretory and membrane proteins are retained by the quality control system in different compartments of the secretory pathway. This mechanism prevents misfolded proteins from moving along the secretory pathway and ensures that only functional proteins reach their site of action. An accumulation of incompletely folded proteins in the ER induces the UPR signal transduction pathway, a cellular stress response that up-regulates proteins necessary for protein folding and degradation. The UPR facilitates the clearance of misfolded proteins using the ERAD pathway that combines retrotranslocation of substrates from the ER and protein degradation by the cytosolic ubiquitin / proteasome system. On the other side, properly folded membrane proteins reach their site of action, such as the cell surface. Many proteins residing at the plasma membrane are internalized in endocytic vesicles and are eliminated in the second main compartment of turnover, the lysosomes.

The aim of this thesis was to clarify which degradation pathways are responsible for the turnover of human wild-type and mutant V2Rs retained by the quality control system in ER, ERGIC and Golgi apparatus. To date, degradative pathways of proteins that are retained in post-ER compartments have not been characterized in detail. Co-localization experiments between receptors and lysosomes using confocal laser scanning microscopy revealed that mature receptor forms of the wild-type and the mutant G201D were degraded in lysosomes. The core-glycosylated V2R mutants L62P and V226E were excluded from this compartment. The protein turnover was assessed in pulse-chase assays with inhibitors for the lysosomal or the proteasomal pathways, respectively. Using this method, the lysosomal degradation pathway for mutant G201D was confirmed. In addition, the pulse-chase experiments demonstrated that all immature V2R forms (wild-type and mutant) were degraded by a proteasome inhibitor-sensitive pathway. Co-immunoprecipitation experiments showed that all receptors pulled down the central ERAD component AAA ATPase p97/VCP. In an attempt to correlate the localization of the misfolded domain in the V2R (L62P, cytoplasmic; V226E, transmembrane; G201D, ER-luminal) with specific ERAD pathways, it could be demonstrated in a mammalian system that all investigated V2Rs were degraded via a Derlin-1 / p97/VCP-dependent ERAD pathway. Additionally, all receptors co-precipitated the 26S proteasome regula-

tory subunit Rpt1, which may be an important step in the unknown recognition mechanism between ERAD substrates and the regulatory subunits of the 26S proteasome.

6.1 Degradation pathways of misfolded proteins

Mammalian cells have evolved different mechanisms to clear their internal environment of unrequested proteins to maintain homeostasis and ensure their survival. Two main degradation pathways operate to prevent toxic accumulations of abnormal proteins. Lysosomes degrade long-lived proteins and acquire the material for turnover by endocytosis, macro- or microautophagy (Terman, Gustafsson et al. 2007). Proteasomes mainly catalyze the selective destruction of cytoplasmic, short-lived proteins and dislocation substrates of the ERAD. Beside turnover, clearance of non-native protein structures can also be achieved by formation of inclusion bodies or aggresomes.

This work showed that V2Rs were efficiently degraded and were not deposited in inclusion bodies. Furthermore, lysosomes as well as proteasomes contributed to the degradation of different receptor forms. The special pathway of turnover that is under control of the quality control system was determined by the cellular localization of the receptors. The mature wild-type V2R and the complex-glycosylated mutant G201D were shown to be degraded by lysosomes. Until now, it is not clear if complex-glycosylated receptors are directly transported from the Golgi apparatus to lysosomes for degradation, if the transport includes the plasma membrane or if both mechanisms operate. A suitable mechanism, which clears plasma membrane wild-type and mutant receptors deficient in signal transduction form the cell surface is the constitutive internalization into lysosomes (Schmidt, Lautz et al., unpublished data). Thus, lysosomes constitute a part of the cellular quality control system.

Lysosomes contribute to cellular protein balance by degrading internalized plasma membrane proteins. Many cell surface proteins, like GPCRs, ion channels and receptor tyrosine kinases are endocytosed in early endosomes. This compartment provides the first station of the endocytic pathway and determines if a protein is recycled to the plasma membrane or gets degraded in lysosomes. Ubiquitination, poly- or monoubiquitination, plays an important role in both receptor internalization and vesicular trafficking. Many components involved in this process carry ubiquitin-binding domains or ubiquitin-interacting motifs to transduce internalization signals and regulate vesicular transport (Di Fiore, Polo et al. 2003). Additionally, under certain circumstances not only the substrates for internalization but also the scaffolding protein β-arrestin are modified by ubiquitin chains (Shenoy and Lefkowitz 2005). In contrast to the β_2-adrenoceptor, which is deubiquitinated in early endosomes and recycles to the cell surface, the activated wild-type V2R is described to be stably connected with polyubiquitin and does not recycle to the plasma membrane (Martin, Lefkowitz et al. 2003). Two recently pub-

lished studies confirmed the lysosomal degradation pathway for the ligand-activated wild-type V2R. Using CLSM analysis, it could be shown that the co-localization of V2R.GFP with the lysosomal marker LAMP-2 increased from 26.8 % to 48.6 in presence of a V2R agonist (Robben, Knoers et al. 2004). The study supported lysosomal turnover of the wild-type V2R after internalization and at steady state. The localization analysis was in line with the findings in this thesis. Additionally, the results presented here showed that the wild-type V2R is polyubiquitinated at steady state (Fig. 15) and that AVP treatment enhances this receptor ubiquitination (App. Figure 6). This may hint to constitutive internalization of polyubiquitinated wild-type receptors, but this assumption cannot be encouraged by the present data, as the immunoprecipitation experiments were performed with whole cell lysates only. Furthermore, polyubiquitinated receptors may also represent the pool of ER-retained V2Rs that are substrates for the ERAD and modified with ubiquitin for proteasomal degradation.

The ERAD is the main and most important pathway of cells to deal with non-native protein structures destined for the secretory pathway, but the precise molecular mechanisms have yet to be elucidated. Many conformational diseases are due to improperly folded proteins in the ER and their retention by the quality control system. The cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel that is mainly expressed at the plasma membrane of secretory serous cells of submucosal glands of the respiratory tract, was one of the first transmembrane proteins that was ascribed to be degraded by the ERAD (Ward, Omura et al. 1995). The mutant ΔF508 CFTR which is widely spread in patients leads to cystic fibrosis by quality control retention of nearly 100 % in the ER. ΔF508 CFTR is subsequently retrotranslocated by the ERAD machinery and degraded by the proteasome. Until now, several diverse conformational disorders have been described and were reviewed by Aridor and Hannan (Aridor and Hannan 2000; Aridor and Hannan 2002).

In the present work, all investigated immature mutant and wild-type V2Rs were polyubiquitinated and stabilized by MG132-induced proteasome inhibition. Interestingly, Bouley et al. have shown lysosomal degradation for wild-type V2Rs and also stabilization of mature forms with chloroquine, but failed to detect stabilization of immature forms with the proteasome inhibitor lactacystin in LCC-PK1a cells (Bouley, Lin et al. 2005). To exclude that the stabilization of immature forms was due to treatment with MG132, the effect of lactacystin was also tested in the present thesis and revealed an obvious stabilization in the intensity of immature receptor forms. The differences could possibly be attributed to the usage of different cell lines.

The time course of wild-type and mutant V2R breakdown was assessed by pulse-chase experiments. HEK293 cell lines stably expressing receptor constructs were incubated with methionine and cysteine depleted medium overnight. The cells were labeled with [^{35}S]-methionine / cysteine for 45 min and samples were taken at 0, 2.5, 5 and 10 h after

end of the labeling process. Samples were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. The experiments revealed that mature wild-type receptors had a longer degradation half-live than 10 h at steady state and lysosomal inhibition did not affect their stability during the time of chase. Unglycosylated receptors were only present directly after the labeling process; at 2.5 h the majority of receptors were core-glycosylated. Most of the ER-retained V2Rs were either degraded or correctly folded and exported from the ER between 5 and 10 h.

Sadeghi et al. focused on maturation of HA-tagged V2Rs and compared transiently and stably transfected HEK293 cell lines (Sadeghi, Innamorati et al. 1997). A metabolic labeling study demonstrated a decay of non-glycosylated and core-glycosylated bands 20 min after the end of a 2 h labeling period. Stably expressed mature V2Rs were degraded between 6 and 8 h in HEK293 cells. Transiently transfected receptors in COS.M6 cells were found to have a calculated degradation half-life of 7 – 8 h for the core-glycosylated receptors and complex-glycosylated receptors, respectively. The results do not support the time course of degradation obtained in this work. The differences may be due to different expression levels of the stably expressing cell lines, different maturation levels or to the applied densiometric analysis.

The basal half-life of another GPCR, the δ opioid receptor was investigated by pulse-chase analysis in HEK293 cells (Petaja-Repo, Hogue et al. 2001). From 4 – 7 h the majority of core-glycosylated forms decayed: the densiometric analysis revealed a degradation half-life of 125 ± 11 min. The degradation half-life of mature receptors was not investigated, but there was no reduction in the intensity of the complex-glycosylated band apparent within 7 h of chase. The degradation half-life of the α_{2A}-adrenoceptor was investigated by a cell surface biotinylation assay and a half-life of 13 h in MDCKII cells was found (Wilson and Limbird 2000). Hughes et al. described the rates of disappearance of the α_1- and β_2-adrenoceptors in presence of cycloheximide (inhibition of synthesis of new proteins) in BC3H-1 cells. The α_1-adrenoceptor was cleared in ~25 h and the β_2-adrenoceptor in ~200 h in confluent cells (Hughes and Insel 1986). However, the estimated degradation half-lifes may vary between different cell types; the basal times of turnover of wild-type V2Rs may be in the range reported by others.

MDCK cells stably expressing V2R.GFP were treated with cycloheximide to block synthesis of new V2Rs by Robben et al. (Robben, Knoers et al. 2004). The stability of the wild-type receptor was analyzed for 8 h. The data indicated a degradation half-live of 11.52 ± 2.8 h for the mature receptor form. Thus, their deduced time for turnover was in the expected range. The same method was applied to investigate the degradation half-lives of mutant V2Rs (Robben, Knoers et al. 2005). The ER-retained mutant S167L showed an elimination half-life of 1.5 ± 0.4 h; the stability was strikingly decreased compared with the results of this thesis (approx. 10 h). One may argue that a block of

overall protein synthesis may also affect housekeeping proteins, proteins of the folding machinery or components of ERAD or the proteasome. In the same publication, results with inhibitors of lysosomes and the proteasome were presented. Data for the wild-type receptors were not shown, but the authors described that chloroquine-treatment prevented its degradation. Beyond this, the results shown for mutant G201D are in sharp contrast to this work, because no chloroquine-induced stabilization was described. However, the differences might be due to the experimental design or different cell lines used in the study.

In the present work it was found that the ER-retained mutant L62P and the ERGIC-retained mutant V226E were only expressed as immature receptor forms and were excluded from lysosomal degradation. Compared with the high-mannose forms of the wild-type receptor and the Golgi-localized mutant G201D, the stability of the core-glycosylated forms of L62P and V226E in the ER was enhanced. This may be due to the lack of anterograde transport along the secretory pathway and the exclusive degradation via the ERAD.

Several severe diseases are due to accumulations of abnormal proteins and are associated with aggresome (pericentriolar, microtubule-associated aggregates ensheathed by vimentin) or inclusion body formation. This pathway is used to degrade these accumulations of improperly folded proteins if the proteasome is overwhelmed (Johnston, Ward et al. 1998). Inclusion body formation may be toxic; it is generally accepted that aggresomes contribute to the pathogenesis of neurodegenerative disorders (Martinez-Vicente and Cuervo 2007). Aggresome formation has been shown for the first time for recombinantly expressed misfolded CFTR and presenilin-1, which are both transmembrane proteins with multiple domains. The mutations are linked to cystic fibrosis and Alzheimer's disease, respectively. Proteins are translocated from the ER to the cytosol, are deglycosylated and form stable aggresomes in a perinuclear region, the microtubule-organizing center (Johnston, Ward et al. 1998). The authors raised the possibility that aggresomes may be sequestered in autophagosomal structures and are eventually degraded by lysosomes. Recently published results support this hypothesis. It has been demonstrated for the L1341P mutant of dysferlin, a transmembrane protein responsible for limb girdle muscular dystrophy type 2B, that this mutant dysferlin formed aggregates in muscles (Fujita, Kouroku et al. 2007). Mis- or unfolded wild-type dysferlin was degraded by ERAD, as shown by co-immunoprecipitation of p97/VCP in C2C5 cells. Moreover, immunostaining experiments revealed that wild-type and mutant dysferlin aggregated in the presence of a proteasome inhibitor, whereas only the mutant dysferlin was sensitive to lysosomal inhibitors. Additionally, only aggregated mutant dysferlin was involved in the stimulation of autophagosome formation that was interestingly mediated by the PERK-eIF2α-pathway of the UPR. In another study Illing et al. found that a folding-defective GPCR, the blindness-causing ER-retained mutant rhodopsin P23H, was ubiq-

uitinated and subjected to proteasomal degradation in HEK293 cells (Illing, Rajan et al. 2002). Furthermore, the authors demonstrated by immunofluorescence analysis that P23H accumulated in aggresomes, identified by co-localization of the protein aggregations with the centrosomal marker γ-tubulin. Overexpression of receptors (determined by fluorescence resonance energy transfer in living HEK293 cells and immunofluorescence staining) or treatment with proteasome inhibitors (immunofluorescence study) caused inclusion body formation of wild-type proteins and increased the aggresome-disposal of mutant P23H. One may speculate that aggresome formation is a general feature if high amounts of transport-defective proteins charge the ER and lead to a saturation of the ERAD or somehow impair proteasomal degradation. However, results of microscopic analysis of living HEK293 cells expressing GFP-tagged wild-type and mutant V2R in the presence of MG132 do not support this hypothesis (Fig. 9). Although proteasomal turnover was blocked for 16 h and the intracellular distribution of immature V2R was changed because of retrotranslocation to the cytosol (Fig. 22), no receptor accumulations or aggregates were apparent. Therefore, aggresome formation is restricted to special, accumulation-prone proteins and is not implicated in the degradation of V2R.

6.2 Quality control and transport in the secretory pathway

Mutations in the *AVPR2* gene lead to improperly folded V2R mutants that are retained in all compartments of the secretory pathway. The ER retention and the ERGIC localization of mutants L62P and V226E were described with GFP-tagged V2Rs by Hermosilla et al. and were confirmed with FLAG-tagged mutants in the present work (Fig. 6). The Golgi apparatus location was assessed by immunofluorescence analysis and mutant G201D co-localized with the Golgi-marker GM110 (Fig. 6). The localization of mutant L62P was restricted to the ER, the wild-type V2R and mutants V226E and G201D were exported to post-ER compartments. Interestingly, the treatment with proteasome inhibitors caused an accumulation of all immature forms of the wild-type and mutant receptors in pre-Golgi compartments (Fig. 10, 15). The present receptor localization studies and MG132-promoted stabilization of immature forms in the pulse-chase assays demonstrated that a subset of immature proteins was recognized by the ER quality control system as unfolded or misfolded proteins. This may be due to different conformations of their tertiary structures (Hermosilla, Oueslati et al. 2004; Thielen, Oueslati et al. 2005). The exact properties of a protein that determine the decision for retention by the quality control system are still unknown, however a current hypothesis for the V2R and other membrane proteins is the conformational-induced exposure of hydrophobic clusters that are recognized and bound by luminal ER chaperones such as calnexin / calreticulin, BiP and GRP94 (Morello, Salahpour et al. 2001; Christianson,

Shaler et al. 2008), which causes retention of the protein in the ER. In the present mass spectrometry study, BiP was found to be associated with wild-type receptors under influence of proteasome inhibition.

It still remains to be determined if all or only subsets of ERGIC- or Golgi-localized receptor mutants are able to overcome the ER quality control system or if the escaped misfolded proteins of both compartments are transported back to the ER for degradation. One may argue that the ER-export of misfolded proteins may be due to an overwhelmed ERAD pathway. The ability to overcome the quality control system is not due to a saturated chaperone system or ERAD machinery, because mutant L62P is retained in the ER exclusively and is expressed in comparable amounts relative to the ERGIC located mutant V226E. In stably expressing cell clones of different fluorescence intensities it could also be shown that the localization of a highly expressed mutant L62P was restricted to the ER and a lower expressed ERGIC mutant still reached the ERGIC (Hermosilla, Oueslati et al. 2004). Furthermore, the co-expression of the GPCR rhodopsin with the nearly 100 % ER-retained mutant ΔF508 CFTR does not affect rhodopsin maturation (Rajan and Kopito 2005).

ERGIC-located proteins can only be detected in their retention compartment in the presence of bafilomycin A1 that blocks the retrograde transport from the ERGIC to the ER, which leads to an accumulation in this particular compartment. The ERGIC-localization of mutant V226E could be confirmed in bafilomycin A1-treated HEK293 cells by co-localization with the ERGIC-marker protein ERGIC-53 (Fig. 6). Additionally, the interaction of a wild-type V2R GST.ICL3 fusion protein with COPI transport vesicles was shown by pull-down experiments (Hermosilla, Oueslati et al. 2004). On the other hand, COPI was not detectable in co-immunoprecipitation studies of full-length receptors. However, anterograde transport for the ERGIC-retained mutant receptor was not observed (neither detection of mature receptors nor lysosomal localization), which supports the hypothesis that receptors are transported back to the ER for degradation. A retrograde trafficking from Golgi-located V2Rs to the ER still remains to be elucidated. Other proteins including the KDEL receptor, which functions as a transport protein, have been found to cycle between both compartments. For the misfolded soluble ERAD substrates carboxypeptidase Y and proteinase A, but not for membrane proteins, it has been reported that degradation by the proteasome requires ER to Golgi transport in yeast (Caldwell, Hill et al. 2001). The fact that mutant G201D is complex-glycosylated by enzymes of the Golgi apparatus and is expressed partially at the plasma membrane (Fig. 7, Fig. 11) suggests that only an anterograde transport takes place. Furthermore, G201D co-localizes with lysosomal vesicles in living HEK293 cells (Fig. 11, 12). However, an anterograde transport for mature G201D is most likely, because it reaches the plasma membrane and the lysosomal compartment.

The present results demonstrate that not only mutant V2Rs are retained by the quality control system and degraded by the ERAD, but the incompletely folded or misfolded wild-type receptors are also retained and degraded. One could argue that degradation of the wild-type V2R may be due to saturated ERAD machinery because of the high number of recombinant expressed receptor proteins. However, the phenomenon that wild-type proteins are degraded during or directly after synthesis has been observed in different studies. Schubert et al. addressed proper protein folding in a variety of cell types (Schubert, Anton et al. 2000). The study revealed that misfolded polypeptides in the ER constitute more than 30 % of all newly synthesized proteins and are substrates for breakdown by a proteasomal pathway. Up to 50 % of wild-type δ opioid receptors were reported to be retained in the ER (Petaja-Repo, Hogue et al. 2000) and were subsequently degraded by the ubiquitin-proteasome system (Petaja-Repo, Hogue et al. 2001).

In line with these findings are studies concerning the CFTR; nearly 70 % of nascent wild-type CFTRs undergo inefficient folding and rapid turnover (Ward and Kopito 1994). The degradation process was also observed in cell lines expressing low levels of CFTR (Lukacs, Mohamed et al. 1994; Ward and Kopito 1994). It is conceivable that the low folding efficiency results in ER overload and ERAD is due to the heterologous overexpression of wild-type proteins in immortalized cell lines. Varga et al. tackled this question in a recent publication determining the efficient maturation of genomic CFTR in Calu-3 and T84 cell lines. These epithelial cells endogenously express wild-type CFTR; interestingly, the folding process of the protein was highly efficient. The CFTR was completely processed in its complex-glycosylated form and the ERAD pathway was inactive. A parallel approach in HeLa and COS-7 cells with recombinant expressed CFTR revealed diminished maturation and increased turnover by the ERAD (Varga, Jurkuvenaite et al. 2004). In contrast, Sun et al. found that genomic CFTR co-precipitates the ERAD components p97/VCP and Derlin-1 in Calu-3 cells, albeit it was mainly expressed in its mature form (Sun, Zhang et al. 2006).

Nearly all studies concerning mammalian ERAD were done in recombinant expressing systems and the data obtained with genomic proteins led to contrary results. Thus, it cannot be ruled out that the observation that the ERAD pathway is responsible for the degradation of immature wild-type proteins may only be due to expression or overexpression of recombinant proteins in artificial systems. Furthermore, until now there is no human cell line available that endogenously expresses detectable amounts of wild-type V2R, much less disease-causing mutant V2Rs. Rat inner medullary collecting duct (IMCD) cells endogenously express V2Rs, but these receptors have a different behavior in comparison to human receptors, their cell surface expression is reduced and the extent of intracellular retention increased (Fenton, Brond et al. 2007). Moreover, NDI may be a disorder that affects mainly humans, since only two natural occurring cases of inherited NDI were reported in animals (dog and horse).

A second objection that may argue against the physiological occurrence of ERAD of wild-type proteins is the influence of proteasome inhibitors. It has been shown that blocking the catalytic sites of the proteasome enhances cell stress and the tendency for aggregation. But the data in this thesis revealed that the ERAD component Derlin-1 and also the proteasomal subunit Rpt1 were co-precipitated without influence of proteasome inhibitors. The ER-retained mutant L62P was also able to pull-down p97/VCP under control conditions.

A major open question in the ERAD field concerns the connection between the recognition of misfolded substrates by the quality control system and their retrieval for cytoplasmic degradation. The ER membrane contains unfolded proteins, folding intermediates, misfolded proteins and correctly folded proteins. This raises the question of how terminally misfolded proteins are recognized, distinguished from structurally similar forms and targeted by the quality control system.

It is generally accepted that nascent glycoproteins interact with the lectins calreticulin (soluble form) or calnexin (membrane-bound form) during processing and undergo several cycles of N-glycan binding and release to facilitate proper protein folding. The ER-retained V2R mutant L62P co-localizes with the membrane-bound chaperone calnexin (Fig. 6). Co-immunoprecipitations of V2R mutants and calnexin suggest a physical interaction (Morello et al., 2001; Lautz et al.; unpublished data). In addition, the data acquired by mass spectrometry experiments demonstrated that the ER-residing Hsp70 chaperone BiP was co-precipitated by the wild-type receptor. On one hand, the chaperone always assist in the folding process of nascent proteins, but on the other hand, BiP is up-regulated under ER stress conditions and the recruitment of BiP to misfolded proteins is increased.

In the present work, the subcellular fractionation of cells into membrane and cytosolic fractions demonstrated that V2Rs are completely retrotranslocated to the cytosol before degradation (Fig. 22). However, microscopic analysis revealed no formation of inclusion bodies or aggresomes. Thus, there must be cytosolic interaction partners that solubilize the hydrophobic transmembrane proteins. A possible explanation may be the support of cytosolic chaperones. Members of the Hsp70 and Hsp90 families were present in the co-immunoprecipitated mass spectroscopy samples, indicating a possible interaction between retrotranslocated receptors and cytosolic chaperones. In addition to ER luminal and cytoplasmatic Hsp chaperones, other components of the ERAD machinery, which are interacting with V2Rs, are supposed to display chaperone activity. p97/VCP (Song, Wang et al. 2007) and the ATPases of the regulatory particle (Braun, Glickman et al. 1999) are described to facilitate chaperone functions. So they may contribute to the solubility of cytoplasmatic V2Rs and to efficient degradation.

The E3 enzyme carboxyl terminus of Hsp70-interacting protein (CHIP) monitors degradation of cytoplasmic exposed misfolded patches of target proteins recognized by heat shock proteins (Connell, Ballinger et al. 2001). Therefore, CHIP and Hsp70 / Hsp90 chaperones provide a checkpoint in quality control. The first substrate identified for the ubiquitin ligase CHIP was the CFTR channel (Meacham, Patterson et al. 2001). The CHIP-mediated pathway of turnover may not be the only way of elimination of misfolded CFTR, because Gnann et al. demonstrated the influence of both E3 enzymes Hrd1 and Doa10 on channel degradation in yeast (Gnann, Riordan et al. 2004). Yet it is unknown if CHIP plays also a role in the ubiquitination and degradation of V2Rs.

A diverse group of proteins with no sequence similarity is eliminated by the ERAD pathway indicating that there must be a mechanism shared by all of them. Accordingly, proteins with defects in luminal, transmembrane or cytoplasmic domains may be targeted to the same or to different ERAD pathways. It remains unclear whether one pathway is sufficient for all substrate proteins. This question was addressed for V2R mutants with different misfolded domains in this work. All other studies addressing this theme were done with different dislocation substrates. Disease-causing mutants of the V2R are excellent tools for the study of the connection between misfolded domains of ERAD substrates and their road of destruction, because the disease-causing mutations are present in all major receptor domains.

6.3 ERAD and different ERAD pathways

The ERAD combines a series of events eventually leading to proteasomal turnover of ER-retained, potentially toxic, non-native protein structures. Terminally misfolded proteins selected by quality control components are delivered to the retrotranslocation machinery and are translocated from the ER to the cytosol through an unknown protein-conducting channel. To date, it is unknown if this translocon is also involved in dislocation of membrane-embedded proteins. ER-membrane or cytosolic-located ubiquitin ligases tag luminal-located ERAD substrates during retrotranslocation with polyubiquitin chains: thus, aberrant membrane proteins may be modified directly. The AAA ATPase p97/VCP participates in the membrane extraction of polyubiquitinated dislocation substrates. This signal directs the majority of dislocation substrates to proteasomal breakdown. Despite an extensive body of data, the molecular mechanisms of ERAD are not well understood. A growing amount of evidence suggests the presence of different ERAD routes depending on the substrate proteins, since it is unlikely that one single mechanism can facilitate the recognition and targeting of all classes of dislocation substrates.

The data obtained here demonstrated that V2Rs were retrotranslocated to the cytosol prior to degradation (Fig. 22). Glycosylated receptor forms were restricted to membrane

fractions and non-glycosylated receptor forms were only present in the cytosolic fraction. Using this method no ubiquitinated receptor forms could be detected. The glycosylation state was in line with findings reported for other ERAD substrates. The deglycosylating enzyme of the V2Rs was not identified, but there is a strong assumption that PNGase F might provide a general deglycosylation mechanism of ERAD substrates. PNGase F associates with components of the ERAD complex that are co-immunoprecipitated with V2Rs in this work. The glycosidase may be recruited by the membrane-associated Derlin-1 and is able to interact with one subunit of the regulatory particle of the 26S proteasome in HeLa cells (Katiyar, Li et al. 2004; Katiyar, Joshi et al. 2005). A recent study also shows interaction of PNGase F with p97/VCP (Zhao, Zhou et al. 2007). The lack of ubiquitination of retrotranslocated V2Rs may be due to the activity of proteasome-associated or cytoplasmatic DUBs.

Ubiquitinated V2Rs were isolated by immunoprecipitation from whole cell lysates and the polyubiquitin signal strongly increased in presence of proteasome inhibitors (Fig. 15). The V2Rs may be ubiquitinated directly, which may provide a new binding site for interacting proteins or indirectly via an interacting protein of the ERAD machinery that is ubiquitinated. To demonstrate a direct interaction with polyubiquitin, all lysines of the V2R were substituted by arginine (Fig. 17, *C*). The substitution should abrogate polyubiquitin signals, but it did not affect the ubiquitination state of wild-type V2R. The receptors may also be ubiquitinated at cysteine residues or N-terminal amino acids. This was not investigated and therefore direct ubiquitination cannot be ruled out. An attempt to identify ubiquitin-modified fragments of V2Rs by mass spectrometry led to receptor fragments in 200 kDa bands, but failed to detect the ubiquitin modification. The V2Rs co-precipitate a series of proteins associated with the ERAD complex, such as Derlin-1 and p97/VCP. Post-translational modifications regulate the activity of ERAD proteins, so there might be a co-precipitated protein that is ubiquitinated (such as Hrd1 (Shen, Ballar et al. 2007)) and thereby increases the V2Rs ubiquitin signal. A possible explanation for the unknown co-precipitated protein may also provide PDE4 (Fig. 23), which is suggested to be modified by ubiquitin (Enno Klussmann, personal communication). The assumption that V2Rs co-precipitate ubiquitinated proteins is supported by the observation that the polyubiquitinated V2Rs form high molecular mass complexes. The ubiquitin smear starts from 250 and stops at 60 kDa, but complex-glycosylated V2R have a molecular weight of 50 kDa; modification with 4 ubiquitin chains may lead to ~ 80 kDa proteins. However, the high molecular smear may be due to aggregated, polyubiquitinated V2Rs or to a stable complex with components of the ERAD machinery. The findings presented here support the second hypothesis that an ubiquitinated protein is associated with the V2Rs, but cannot rule out a direct modification by ubiquitin or that both pathways operate.

Two interesting new studies revealed that SUMOlyated substrates (SUMO is a small ubiquitin-like modification) are polyubiquitinated by SUMO-dependent E3 ligases of the human RNF4 family to promote degradation (Tony Hunters lab). Tatham et al. described this in association to the disease acute promyelocytic leukaemia, where the promyelocytic leukaemia (PML) protein is polysumolyated and subjected to RNF4-mediated degradation (oral communication 2008, unpublished data). The formation of polyubiquitinated polySUMO chains is also a mechanism which may contribute to high molecular protein complexes.

Two ERAD complexes (HRD1 and DOA10) embedded in the ER membrane have been reported to provide the central machinery for ERAD in yeast. The cores of these translocation machineries form the multispanning, membrane-bound E3 ligases Hrd1 and Doa10 with cytosolic RING finger domains in their structure. The ubiquitin ligases have to recognize misfolded dislocation substrates in the ER membrane or proteins that transit through the membrane to the cytoplasm and specifically target them with polyubiquitin chains. Despite the multiplicity of E3 enzymes participating in the ubiquitin system, only five E3 ligases have been associated with ERAD until now. An attempt to identify Hrd1 in V2R polyubiquitination failed because of co-precipitation of Hrd1 in all samples including the negative control. However, these results do not rule out the participation of the E3 enzyme in V2R turnover. All mutant V2Rs (and the wild-type V2R that is not misfolded because of mutations) co-precipitated the ERAD components Derlin-1 and p97/VCP independent of the misfolded domain in the receptors.

Central to the HRD1 complex in mammals are the ubiquitin ligase Hrd1 and the potential channel component Der1 (Ye, Shibata et al. 2005). The Sec61 channel interacts with Hrd1 (Plemper, Bordallo et al. 1999) and is additionally implicated in ER-import of proteins and may also be involved in retrotranslocation of ERAD substrates. Der1 is proposed to contribute to retrotranslocation and associates with VIMP, an adaptor protein for VCP recruitment (Ye, Shibata et al. 2004). Cytosolic factors involved in mammalian ERAD are Herp (protein of unknown function, Herp is up-regulated under UPR conditions), Ubc7 (ubiquitin-conjugating enzyme), VIMP and p97/VCP / Ufd1/Np14. The complex of p97/VCP / Ufd1/Np14 mediates the membrane extraction of retrotranslocated ERAD substrates (Meyer, Shorter et al. 2000). p97/VCP coprecipitates both Derlin-1 and transiently overexpressed ubiquitin ligases Hrd1 and gp78 in 293T cells (Ye, Shibata et al. 2005). The luminal components BiP, EDEM, Hrd3, and YOS9 are presumably involved in recognition of terminally misfolded proteins.

The yeast DOA10 complex (TEB4 is the proposed homolog in mammals) has only membrane-residing components like the multispanning E3 ligase Doa10 and the ubiquitin-conjugating enzymes Ubc6 und Ubc7. p97/VCP is recruited to the cytoplasmatic site of the DOA10 complex. Nakatsukasa et al. proposed a model for a Doa10-mediated ERAD pathway implicated in degradation of transmembrane proteins in yeast

(Nakatsukasa, Huyer et al. 2008). They investigated the well-characterized ERAD substrate Step6p. In this pathway, Step6p assembled different chaperones (the Hsp70 chaperone Ssa1p and the Hsp40 co-chaperones Ydj1p and Hlj1p) and was ubiquitinated by the E4 ubiquitin chain extension enzyme Ufd2p. It is unknown if this pathway is a general rule.

The first difference determined between ERAD substrates was the presence of terminally misfolded ER-luminal and integral membrane proteins. Vashist et al. investigated two substrates of each group and observed different rates of disappearance in yeast (Vashist and Ng 2004). The membrane-embedded proteins showed a half-life of 8 – 12 min; ER luminal-located substrates diminished in 27 – 35 min. They demonstrated with chimerical molecules that the site of lesion but not the localization was the determining factor for Golgi-transport before ERAD. From this data, they hypothesized the existence of two ERAD pathways: ERAD-C for folding intermediates with cytoplasmic defects (Doa10-dependent) and ERAD-L for all soluble proteins and transmembrane substrates with mutations in ER-luminal domains (Hrd1- and Der1-dependent). However, the authors compared only a limited subset of dislocation substrates and their results need further investigation; there may be other requirements of the system that are yet unknown and therefore the findings can not be described as a general model.

On the basis of this data and a mass spectroscopy screening in yeast, Carvalho et al. proposed three distinct ERAD pathways that degrade translocation substrates with misfolded domains in the ER lumen, membrane and cytosol (Carvalho, Goder et al. 2006). Doa10 was used to co-precipitate associated proteins of the translocation complex. The authors identified Ubc7p, Cue1p (membrane adaptor of the E2 Ubc7), Cdc48 (p97/VCP) and cofactors and Ubx2p (VIMP), the known components of the DOA10 complex. A similar approach with Hrd1 yielded Hrd3p, Ubx2p and Cdc48, YOS9p, Der1p and Usa1. Usa1 is a new interacting protein identified in the translocation apparatus. The authors concluded that they might have identified most or all components of the ubiquitin ligase complexes. Furthermore, they analyzed the complexes by gradient centrifugation and found components of the proteasomal 19S particle, but did not reveal which subunits were identified. Hrd1, Hrd3, Usa1p and Der1p were associated in a complex named Hrd1p core complex. Interestingly, they did not find the Sec61 translocon in their samples. They proposed three different ERAD pathways: ERAD-L facilitates turnover of soluble and membrane proteins with mutations in luminal domains; it requires the Hrd1 core complex (Hrd1, Hrd3, Usa1p and Der1p) and Cdc48, Ubc2p and Yos9p. ERAD-C degrades proteins with cytoplasmic lesions and consists of the components Doa10, Ubc7p, Cue1p, Cdc48 and cofactors, as well as Ubx2p. The third pathway, ERAD-M, is the pathway predicted for turnover of proteins with mutations in transmembrane domains. It requires the same component as ERAD-C with the excep-

tion that not Doa10 but Hrd1 is responsible for ubiquitination. The authors hypothesize that the three pathways may also operate in mammals.

This is in striking contrast to the results presented here, because only one Derlin-1 and p97/VCP-dependent ERAD pathway moves wild-type and V2R mutants with misfolded luminal (G201D), transmembrane (V226E) and cytosolic (L62P) domains to the cytoplasm (Fig. 18, 19). Additionally, a second transmembrane mutant V2R (S167L) was investigated, which also pulled down Derlin-1 and p97/VCP. The results presented here demonstrate that at least Derlin-1 and p97/VCP are part of the mammalian ERAD complex that degrades V2Rs. It was shown for the first time that mutations of different domains of the same substrate protein do not determine association with different ERAD complexes in a mammalian system. Therefore misfolded wild-type V2R and three mutants with different located conformation changes might either be recognized by the same adaptor / ERAD component or by different yet unknown signals that may activate the same ERAD complex.

Because only 5 E3 enzymes (Hrd1, gp78, TEB4, Rma1 and RFP2) are known to polyubiquitinate ERAD targets, the first hypothesis may be true. E3 ligases are enzymes with a high specificity; they modify not every lysine in substrate proteins, but specific lysines. Enzymes need specific conformations of substrates to dock on and also the correct orientation of the enzyme / substrate complex to catalyze their action. Since E3 enzymes such as Hrd1 ubiquitinate structural different targets (CPY, alpha(1)-antitrypsin, Pae1 receptor (substrate of parkin)) it is to assume that an unknown adaptor protein binds to misfolded substrates to facilitate the interaction with the ubiquitin ligase. This suggests that different E3 ligases may be the key to different ERAD pathways.

Some investigators hold on to the assumption that luminal and cytosolic factors discriminate between folding defects in different domains of dislocation substrates. Molecular chaperones reside in both compartments ER (lumen and membrane) and cytosol. They prevent protein aggregation and assist in protein folding in an ATP-dependent manner (Young, Agashe et al. 2004). Chaperones bind to hydrophobic exposed peptide stretches of partially folded substrates to ensure their solubility via a C-terminal polypeptide-binding domain. Similar exposed residues of different ERAD substrates may promote chaperone interactions (several structural unrelated proteins bind Hsp70 chaperones). Misfolded integral membrane proteins with folding defects at the luminal part of the protein may interact with luminal chaperones (BiP, Grp94), or in the cytosolic exposed domain (Hsp70, Hsp90) leading to different conformations. Moreover, proteins with lesions in transmembrane regions may expose the conformational change to one preferred site of the ER membrane depending on the place of the mutation. As demonstrated in this thesis, immature wild-type V2Rs that may have different conformations can interact with both kinds of chaperones (BiP, Hsp70, Hsp90).

Similar results have been obtained for wild-type and mutant ΔF508 CFTR that associate with Hsp70 (Choo-Kang and Zeitlin 2001) and Hsp90 during ERAD (Fuller and Cuthbert 2000). The mutant CFTR is additionally bound by the small chaperone alphaA-crystallin; the authors propose that this chaperone specifically targets ΔF508 CFTR for ERAD (Ahner, Nakatsukasa et al. 2007). This association may be important evidence for the assumption that some chaperones can discriminate between mutant and wild-type proteins.

The recognition mechanism of misfolded dislocation substrates is under intensive investigation, but there is a second major open question in the ERAD field. How does the proteasome identify ERAD substrates?

6.4 Recognition by the 26S proteasome

Proteasomes are composed of the 20S catalytic core, where the protein cleavage takes place, and one or two 19S regulatory particles, which facilitate the recognition and unfolding of degradation substrates. Additionally, the 19S regulatory particles are implicated in gating of the catalytic chamber to protect cells from uncontrolled proteolysis. A growing field of research aims to unravel the underlying molecular mechanisms of the regulatory particle functions, the involvement of single subunits and their arrangement into different pathways participating in the complex interplay.

The 19S regulatory particle is constructed of base and lid components. The base is build of six ATPases Rpt1-6 and the non-ATPase subunits Rpn1, Rpn2 and Rpn13. Rpn10 is located between both subcomplexes. The lid comprises eight non-ATPase subunits.

It was shown in this work that wild-type and mutant V2Rs interact with the 19S regulatory particle ATPase subunit Rpt1 (Fig. 21). A second non-ATPase subunit Rpn1 was identified in the mass spectrometry data. The Rpt1, Rpn5 and Rpn10 subunits were previously described to bind to polyubiquitinated proteins (Raasi and Wolf 2007). Therefore, the Rpn10 subunit was also tested for a physical interaction with V2Rs, but could not be identified in co-immunoprecipitation studies (App. Figure 4).

Binding of regulatory particle subunits may imply that the whole regulatory particle is co-precipitated by V2Rs. However, since the Rpn10 subunit was not detected, it can be hypothesized that ubiquitinated V2Rs bind to free subunits of the regulatory particle. The occurrence of free proteasome subunits was reported by van Nocker et al (van Nocker, Sadis et al. 1996) and supports the important assumption that non-bound Rpt1 facilitates substrate selection. There is evidence that proteasomes are recruited to the ER membrane before substrate degradation (Hirsch and Ploegh 2000). In addition, assembly of proteasomes is reported to take place at the ER membrane (Fricke, Heink et al. 2007). V2Rs are retrotranslocated from the ER membrane into the cytoplasm, where

they are deglycosylated and deubiquitinated. The Rpt1-6 subunits are part of the 26S proteasome which is predicted to interact with p97/VCP (Hirsch, Jarosch et al., 2004). This supports the hypothesis that Rpt1 recognizes misfolded V2Rs presented by p97/VCP during retrotranslocation. From the data described in this thesis a possible mechanism of substrate recognition of the proteasome can be proposed, in which the Rpt1 subunit of the regulatory particle binds to polyubiquitinated, dislocated receptors and the proteasome might assemble directly on the receptor / Rpt1 complex. Recruitment of regulatory particle subunits, especially Rpn11, may also explain the cytoplasmic deubiquitination of V2Rs.

Recently published results revealed the participation of proteasomal subunits in dislocation of ERAD substrates (Lipson, Alalouf et al. 2008). This process is also described for many ERAD substrates to be facilitated by the ATPase p97/VCP. But some ERAD substrates seem to require the 19S regulatory particle for retrotranslocation (Lee, Liu et al. 2004). The authors concluded that the driving force for dislocation is subjected to the ATPase activity of the base Rpt subunits. The hypothesis was raised that the ATPase domain of p97/VCP interacts with the ATPases of the regulatory particle. Recruitment of regulatory subunits may be facilitated by p97/VCP in this model. One possibility suggested by the present work is that Rpt1 functions as an adaptor protein in the recognition of ERAD substrates; one domain of Rpt1 may bind to p97/VCP and the other to polyubiquitinated V2Rs to target the receptors for proteasomal degradation.

Lipson et al. investigated the participation of unique Rpt subunits in ERAD and described the Rpt4 subunit as implicated in retrotranslocation, whereas the Rpt2 subunit was involved in proteasomal degradation (Lipson, Alalouf et al. 2008). The data confirm the hypothesis of the present work that unique regulatory particle subunits facilitate different functions in ERAD.

In yeast, Rad23 and Dsk2 are proposed to interact with p97/VCP and deliver ubiquitinylated proteins to the proteasome (Medicherla, Kostova et al. 2004). Rad23 was reported to interact with the proteasomal subunit Rpn1 (Elsasser, Gali et al. 2002). However, there may be different pathways to the proteasome, since not all substrates are able to interact with Rpt1 or are dislocated independent of p97/VCP.

Recently, Okuda-Shimizu and Hendershot reported a role for the ERAD component Herp (Usa1 in yeast) in the degradation of non-glycosylated ERAD substrates (Okuda-Shimizu and Hendershot 2007). Herp bound to ubiquitinated substrates and additionally interacted with the regulatory particle component Rpn2 and a catalytic core subunit. One may speculate that Herp is the unknown adaptor protein, which interacts with ubiquitinated V2Rs. However, Herp was only associated with non-glycosylated ERAD substrates and the assumed mechanism may not play a role for the glycosylated V2Rs.

6.5 Pharmacological influence on the UPS and ERAD

Until now, no effective pharmacological treatment of NDI has been developed. In vitro, the action of pharmacological chaperones facilitated the rescue of intracellular retained receptor mutants (Wüller, Wiesner et al. 2004). In this study, substances (V2R antagonists) that were expected to display pharmacological chaperone activity were tested on diverse NDI-causing receptor mutants. The different substances enabled the cell surface expression of different mutants that were expressed in the correct conformations due to pharmacochaperone association. Therefore, this approach may not lead to a common therapy for NDI (caused by functional intracellularly retained mutants), but may be an option for individual patients with certain receptor conformations. Recently, the first clinical study with the chaperone SR49059 was performed in 5 patients with NDI. SR49059 lead to a decrease of the 24-hour urine from 12 l to 8 l (Bernier, Morello et al. 2006). The inefficient function of rescued receptors observed in the study may be due to the occupancy of V2Rs with pharmacochaperones, which might influence their binding and activation by vasopressin.

In 2003, the first inhibitor of proteasomal activities was approved by the Food and Drug Administration for treatment of patients with multiple myeloma. The substance, bortezomib, is the first in the class of proteasome inhibitors and induces cell death in both solid and haematological malignancies. Bortezomib blocks activation of NF-κB resulting in increased apoptosis, decreased angiogenic cytokine production and inhibition of tumor cell adhesion to stroma. However, inhibition of overall protein turnover causes severe side effects, such as peripheral neuropathy, fever, vomiting and decreased platelet blood count. Therefore, bortezomib is only used as a third-line therapy for patients who have already obtained two different chemotherapies before.

Inhibition of general ERAD pathways may also result in problems since it is part of the cellular quality control system, which protects cells from apoptosis and 30 % of all newly synthesized, secretory proteins are subjected to ERAD. In addition, it may contribute to severe diseases: the prion protein PrP^{c} is dislocated from the ER to the cytoplasm. Inhibition of proteasomal turnover leads to cytosolic protein aggregates and conversion to the pathogenic scrapie form PrP^{sc} (McCracken and Brodsky 2003). In contrast, it was demonstrated for the ΔF508 mutant of the CFTR that blocking of proteasomal degradation with bortezomib resulted in rescue of the ER-retained protein to the cell surface and may contribute to therapy of CF (Vij, Fang et al. 2006). In addition, inhibition of the CFTR / p97/VCP complex by small interfering RNAs complementary to p97/VCP led to an increased rescue of ΔF508 CFTR to the plasma membrane. However, a more selective inhibition of specific ERAD components, such as E3 ligases, may reduce toxicity. Therefore, selective inhibition of ERAD components may facilitate the

development of a new kind of NDI treatment. However, this therapeutic strategy may be only valid for NDI-causing mutants that are transport defective but still functional.

Finally, the results obtained in this thesis demonstrate that the cellular quality control system determines the pathway of turnover of V2Rs. Immature receptors (wild-type and mutants) are retained in ER and ERGIC and are subjected to degradation by the ERAD. Receptors that reach the Golgi apparatus and compartments beyond are transported to lysosomes. Thus, lysosomes are part of the cellular quality control system. For the first time it could be demonstrated in a mammalian system that all immature receptors, independent of the location of the misfolded domain in the receptor, are degraded by a Derlin-1 / p97/VCP dependent ERAD pathway. In addition, the data of this work reveal a putative mechanism by which V2Rs may be recognized by the 19S regulatory particle of the proteasome.

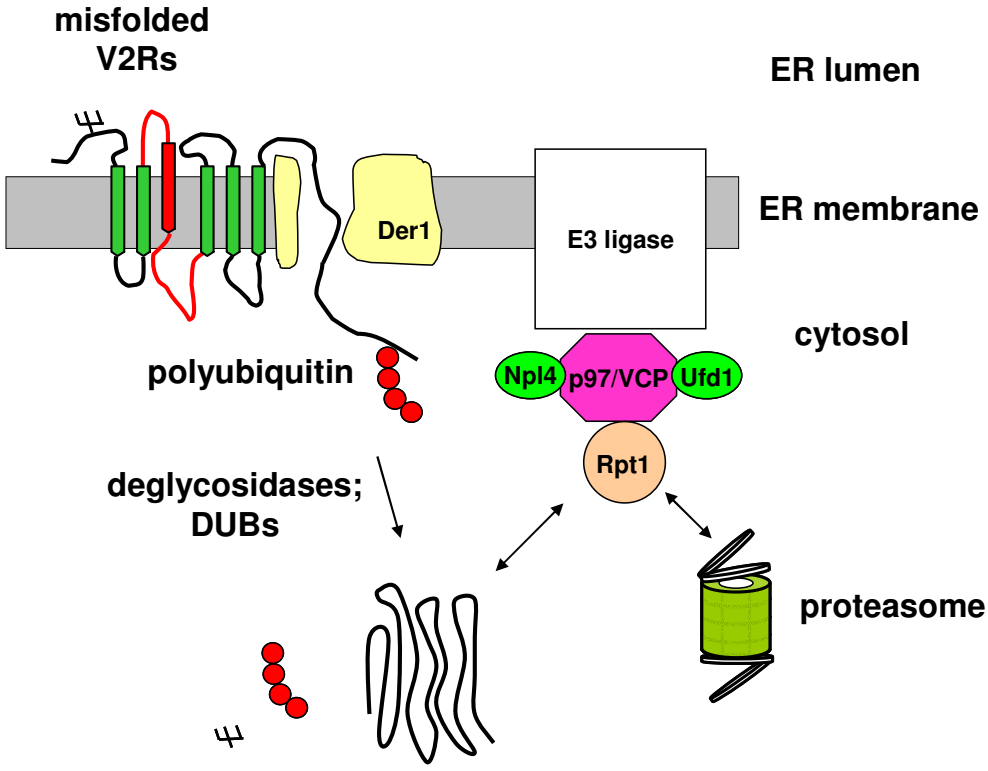

Fig. 24: Model of the mammalian ERAD pathway used by wild-type and mutant V2Rs in HEK293 cells. V2Rs with misfolded luminal, transmembrane or cytosolic domains are polyubiquitinated during retrotranslocation to the cytosol by an unknown ubiquitin ligase. They interact with the putative channel protein Derlin-1 and the AAA ATPase p97/VCP, which is supposed to act as a "molecular gearbox" in the ERAD pathway in recruitment of factors important for membrane extraction and degradation of dislocation substrates. Cytosolic-located glycosidases and DUBs facilitate the deglycosylation and deubiquitination of translocated V2Rs. The V2Rs show a physical interaction to the Rpt1 subunit of the regulatory particle of the 26S proteasome, which also interacts with p97/VCP. This process provides a possible mechanism of ERAD substrate recognition by the 26S proteasome.