Degradation of wild-type vasopressin precursor and pathogenic mutants by the proteasome

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SUMMARY

Mutations in the gene encoding the antidiuretic hormone arginine vasopressin cause autosomal dominant neurogenic diabetes insipidus. Autopic data in affected individuals suggest that the neurons expressing mutant vasopressin undergo selective degeneration. Expression studies have shown that the mutants are retained in the endoplasmic reticulum, but how this trafficking defect is linked to neurotoxicity is unknown. One possibility is that unsecreted mutant precursors, or degradation products thereof, are cytotoxic. We therefore investigated the fate of endoplasmic reticulum-retained pathogenic mutants. Our data show that the mutants are retrotranslocated to the cytosol and degraded by the proteasome. In the presence of proteasomal inhibitors, three distinct un- or deglycosylated cytosolic species of vasopressin precursors were stabilized: pre-pro-vasopressin, pro-vasopressin, and an N-terminally truncated form. In addition to the retrotranslocated forms, a fraction of the newly synthesized precursor was not translocated, but synthesized into the cytosol due to inefficient function of the vasopressin signal peptide. As a result, cytosolic pre-pro-vasopressin and its degradation product were also recovered when wild-type vasopressin was expressed. Cytosolic forms of vasopressin might trigger cytotoxicity \textit{in vivo}, as has been proposed in the case of prion protein, which also contains an inefficient N-terminal signal peptide.

\textbf{Running title:} Proteasomal degradation of vasopressin precursors
The antidiuretic hormone, arginine vasopressin, is synthesized in vasopressinergic neurons of the hypothalamus as a precursor consisting of three moieties (Fig. 1): the 19 amino-acid signal sequence, the nonapeptide hormone, the vasopressin-associated carrier protein neurophysin II (NPII), and a 39 amino-acid glycopeptide (copeptide) with a single N-glycosylation site (1). The precursor is cotranslationally targeted to the ER, where the signal is cleaved off by signal peptidase and the copeptide is core glycosylated. The prohormone contains a total of eight disulfide bonds. After complex glycosylation in the Golgi apparatus, the matured precursor is cleaved into its three moieties and targeted to secretory granules at the distal end of the axons. From there, the hormone is released into the circulation upon osmotic and non-osmotic stimuli. Vasopressin binds to its receptor on cells of the renal collecting duct, initiating a signalling cascade which leads to the mobilization of aquaporin-2 water channels, allowing regulated water reabsorption. Through this mechanism, vasopressin mediates the conservation of as much as ~20 l of fluid per day, thereby playing an important role in water homeostasis.

Lack of circulating vasopressin causes diabetes insipidus. Affected individuals suffer from polyuria and polydipsia due to the inability to concentrate their urine. In rare cases, the condition is caused by mutations in the vasopressin gene and is inherited in an autosomal-dominant manner (2,3). Over thirty mutations have been reported which alter the signal peptide (4,5), the hormone (6,7), or the NPII moieties, respectively (8-14). Autosomal dominant neurohypophyseal diabetes insipidus (ADNDI) appears to be a neurodegenerative disease. Post-mortem histologic examinations revealed only few magnocellular neurons and scar tissue replacing much of the vasopressinergic nuclei (15-18). A degenerative process specific to cells expressing the mutant protein would also explain the complete penetrance in heterozygous individuals (19) and the delayed onset of the symptoms weeks to months after...
birth. The neurodegeneration hypothesis was further supported by a study which showed decreased viability of cultured cells stably expressing mutant vasopressin (20). A number of expression studies have shown that the mutant vasopressin precursors are retained in the ER (11,20-24). Together, the data suggest a cytotoxic effect of retained mutant precursors or of their degradation products. We therefore studied the degradation of vasopressin mutants associated with ADNDI and found it to occur by the proteasomal machinery following retrotranslocation into the cytosol. Analysis of the degradation intermediates furthermore showed that a significant portion of the primary translation products fails to enter the ER lumen. Both pathways of degradation, via the ER lumen and directly from the cytosol, were also found to some extent for the wild-type protein. The cytotoxic effect of mutant vasopressin prohormone may result from processes that are quantitatively, but not fundamentally different from those occurring in cells expressing the wild-type protein.
EXPERIMENTAL PROCEDURES

**Plasmids and constructs** — cDNAs for the wild-type vasopressin precursor and the mutants A (-1)T, αE47, and G57S were a gift from M. Ito (Northwestern University, Chicago, IL). The signal peptide of enkephalin was fused to wild-type and αE47 pro-vasopressin and, to delete the vasopressin hormone sequence, to the wild-type neurophysin II-glycopeptide sequence (in the same manner as described in ref. 25). Point mutations D(-17)R, L(-9)R, and R(8)E/K(11)E/R(12)E, were generated by polymerase chain reaction. All cDNAs were subcloned into the pRc/RSV expression plasmid (Invitrogen) and verified by DNA sequencing.

**Cell culture and transient transfection** — COS-1 and CV-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine at 37°C in 7.5% CO₂. Neuro2A cells COS-1 cells were transiently transfected in 6-well plates using Lipofectine (Life Technologies, Inc.) and used 2–3 days after transfection. Neuro2a were grown in DMEM containing 4500 mg/l glucose in 5% CO₂. They were transfected using Metafectene (Biontex Laboratories). To produce stably expressing cell lines, the cDNA of the vasopressin precursor was subcloned into the expression vector pCB6 and transfected into CV-1 cells using calcium phosphate precipitation. Clonal cell lines resistant to 0.5 mg/ml G418-sulfate were isolated and screened for pro-vasopressin expression by immunofluorescence.

**Metabolic labeling and immunoprecipitation** — For labeling experiments, transfected cells were starved for 30 min in DMEM without cysteine and methionine (Sigma) supplemented with 2 mM L-glutamine. Cells were labeled for the times indicated with 100 µCi/ml [³⁵S] protein labeling mix (DuPont-NEN) in starvation medium and chased in starvation medium supplemented with excess cysteine and methionine. Cells were transferred to 4°C, washed with phosphate-buffered saline (PBS), lysed in 500 µl of lysis buffer (PBS, 1% Triton X-100,
0.5% deoxycholate, 2 mM phenylmethylsulfonyl fluoride), and scraped. After 10 min centrifugation in a microfuge, the lysate was subjected to immunoprecipitation using rabbit polyclonal anti-neurophysin II or anti-vasopressin antibodies (ICN). The immune complexes were isolated with protein A-Sepharose (Zymed) and analyzed by electrophoresis on 10% polyacrylamide Tris/tricine SDS-gels and autoradiography. For deglycosylation, immunoprecipitates were either boiled for 2 min in 50 µl 50 mM Na-citrate, pH 6, 1% SDS and incubated with 1 mU endo-α-N-acetylglucosaminidase H (Roche Biochemicals) for 2 h at 37°C, or they were boiled in 100 µl 0.1 M Na-phosphate, pH 6.8, containing 50 mM EDTA, 1% α-mercaptoethanol, 0.1% SDS and incubated with 0.25 U endoglycosidase F/N-glycosidase F (Roche Biochemicals) for 3 h at 37°C.

Protease inhibition — Stock solutions of 10 mM N-Acetyl-leucyl-leucyl-norleucinal (ALLN), 1 mM lactacystin, 1 mM pepstatin A (all from Sigma) in DMSO, and of 10 mM leupeptin (Roche Biochemicals) in water were prepared. For application to the cells, they were diluted into DMEM to final concentrations of 250 µM ALLN, 25 or 40 µM lactacystin, 100 µM leupeptin, and 5 µM pepstatin A. ALLN was added to the cells 90 min and lactacystin 10 min prior to the experiment and was freshly added to the starvation, labeling, and chase media. The lysosomal inhibitors leupeptin and pepstatin A were applied to the cells 16 h before the experiment and were present during all further incubations.

Cytosol extraction — To separate the cytosol from microsomes, labeled cells were incubated at 4°C in swelling buffer (15 mM HEPES/KOH, pH 7.2, 15 mM KCl) supplemented with 2 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (from a 500-fold concentrated stock of 1 mg/ml each of pepstatin A, leupeptin, chymostatin, antipain, and 5 mg/ml benzamidine, dissolved in 40% DMSO and 60% ethanol) for 15 min at 4°C, scraped, and centrifuged for 30 min at 136000×g. The supernatant containing the cytosol and the resuspended organelle pellet were subjected to immunoprecipitation and analyzed as above.
RESULTS

Proteasome inhibitors stabilize mutant vasopressin precursors and degradation intermediates — To test the fate of wild-type and mutant pre-pro-vasopressin in COS-1 cells, transiently transfected cells were radiolabeled with $[^{35}\text{S}]$methionine/cysteine for 1 h and chased with excess unlabeled methionine/cysteine for 0 or 6 h. Cells and media were subjected to immunoprecipitation using an antibody directed against neurophysin II followed by SDS-gel electrophoresis and fluorography (Fig. 2A, lanes 1–8). Upon pulse-labeling, wild-type protein and the mutants ΔE47 and G57S were found as a major species of ~21 kDa corresponding to N-glycosylated pro-vasopressin. The mutant A(-1)T, in which mutation of the last residue of the signal sequence causes inefficient signal cleavage (5), appeared as two major products corresponding to glycosylated pre-pro-vasopressin and pro-vasopressin. In all cases, additional faint bands in the range of ~17–19 kDa were produced. After 6 h of chase, wild-type pro-vasopressin and the signal-cleaved fraction of the A(-1)T mutant were secreted into the medium. Since COS cells lack prohormone processing enzymes, intact glycosylated pro-vasopressin of 21 kDa was recovered. Hardly any protein could be detected in the cells, indicating that the mutants ΔE47, G57S, and the uncleaved fraction of A(-1)T had been retained and degraded.

To test for degradation via the ER-associated degradation pathway, the proteasomal peptide inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) was added to the medium 90 min before and during the pulse and the chase periods (Fig. 2A, lanes 9–16). ALLN stabilized the putative degradation intermediates of ~17–19 kDa for wild-type and mutant precursors, and to variable extent also the full-size, glycosylated band of the mutant precursors, consistent with proteasomal degradation of retained protein. This was confirmed by experiments using lactacystin, a more specific proteasomal inhibitor. Addition of 25 μM lactacystin stabilized low molecular weight forms that were indistinguishable from those seen
with ALLN treatment (Fig. 2B). In contrast, a mixture of leupeptin and pepstatin A, two inhibitors of lysosomal degradation, had no stabilizing effect on the mutant ΔE47 (Fig. 2C). These results indicate that mutant pro-vasopressin as well as a fraction of wild-type pro-vasopressin is degraded by the proteasome in a process that involves intermediates of 17–19 kDa.

Three cytosolic degradation intermediates are stabilized in the presence of proteasome inhibitors—In addition to the expected glycosylated pro-vasopressin and in the case of A(-1)T to glycosylated pre-pro-vasopressin, up to three different lower-molecular weight forms could be distinguished. To analyze potential precursor–product relationships, we performed a time-course of labeling of cells expressing either wild-type or mutant ΔE47 vasopressin precursor in the presence or absence of ALLN (Fig. 3A). In addition to an increasing signal of glycosylated pro-vasopressin (form 1), the three smaller species (forms 2–4) appeared with distinct kinetics. Form 2 appeared with highest relative intensity after the shortest pulse times of 5 min. Form 3, however, appeared and increased in intensity in parallel with glycosylated pro-vasopressin. Form 4 only accumulated after 30–60 min and in the presence of ALLN. The same bands were observed using the more specific proteasome inhibitor lactacystin (Fig. 3B). The patterns of products generated by wild-type and mutant precursors were qualitatively similar, indicating that they are not related to specific mutations.

To characterize the different forms, immunoprecipitates of ALLN-treated labeled cells expressing ΔE47 vasopressin precursor were incubated with endoglycosidase H or F (Fig. 4A, lane 1–3). The 21-kDa form 1 was deglycosylated to an apparent molecular weight of ~18 kDa corresponding to form 3. In contrast, the lower bands were insensitive to deglycosylation. This suggested that product 3 corresponds to un- or deglycosylated pro-vasopressin and product 4 to a subsequent degradation intermediate lacking a short segment of the polypeptide at the N- or C-terminus. Upon immunoprecipitation using an antibody directed
against the vasopressin hormone, form 4 was not recovered (Fig. 4A, lane 4), indicating that it lacks the hormone sequence at the N-terminus.

Based on its size, product 2 likely represents pre-pro-vasopressin, the primary translation product that had not been translocated to the ER lumen. For comparison, we expressed various mutant precursors to serve as size markers (Fig. 4B). A mutant with a nonfunctional signal sequence (L(-9)R; lane 2), in which an arginine disrupts the hydrophobic core, comigrated with form 2. Only a very small fraction was glycosylated but not processed by signal peptidase (arrowhead). A mutant lacking the signal peptide entirely (ΔSP), i.e. pro-vasopressin synthesized into the cytosol, migrated like band 3 (lane 3). In a further construct the hormone domain was deleted (ΔVP) by fusing NPII-glycopeptide to the signal sequence of pre-pro-enkephalin. In addition to a glycosylated product of ~21 kDa, this construct also produced a 17-kDa form comigrating with band 4 (lane 4). Interestingly, this product of 17 kDa was generated by all constructs, indicating that N-terminal clipping occurred independently of whether the protein was initially inserted into the ER or synthesized directly into the cytosol, and whether a signal sequence was still attached or not.

ER-associated degradation involves the retrotranslocation of unfolded or misfolded proteins from the ER lumen back to the cytosol where they are exposed to cytosolic N-glycanase (26,27). To determine the localization of the low-molecular weight forms, cells expressing wild-type or ΔE47 vasopressin precursor were labeled for 1 h in the presence of ALLN, broken by swelling and scraping, and subjected to ultracentrifugation. We then analyzed the immunoprecipitated products in the membrane pellet (M) and the cytosol fraction (C) in comparison to the unfractionated total cell lysate (L; Fig. 4C). The experiment was performed with cells labeled for 5 min (lanes 1–3), producing predominantly form 2, or for 60 min (lanes 4–9), generating forms 3 and 4 in addition to glycosylated pro-vasopressin. The membrane fraction contained almost all of the glycosylated wild-type and mutant pro-vasopressin, whereas the smaller products were predominantly recovered in the cytosolic
fraction. These products were therefore either retrotranslocated from the ER lumen or had never been targeted into the ER.

The native signal peptide of vasopressin precursor is inefficient in ER targeting — The occurrence of unglycosylated pre-pro-vasopressin indicates that the native signal sequence is inefficient in mediating translocation across the ER membrane. To test this, we expressed wild-type and ΔE47 mutant precursor with the native signal sequence in parallel with the same proteins containing the signal peptide of pre-pro-enkephalin. Upon labeling for 3–60 min, form 2 was only produced by the constructs with the native vasopressin signal sequence, but not with the enkephalin signal (Fig. 5A). In contrast, forms 3 and 4 were generated with either signal, indicating that they are derived from form 1, glycosylated pro-vasopressin in the ER lumen, after retrotranslocation. The fact that wild-type and ΔE47 mutant proteins behaved identically indicates that the mutation is not responsible for the phenomenon and that the native signal of the vasopressin precursor is inherently inefficient.

The signal of the vasopressin precursor is unusual in that it contains a negative charge near the N-terminus (D(-17)) and is C-terminally followed by a cluster of positive charges (Fig. 5B). The enkephalin signal, in contrast, has a positive N-terminus and a longer hydrophobic core. To test whether the unusual charge distribution is responsible for inefficient translocation of the vasopressin precursor, D(-17) was mutated to R, or the residues R(8), K(11), and R(12) were mutated to E. However, upon expression of these charge mutants in COS-1 cells, the pre-pro-vasopressin form (form 2) was still detected (Fig. 5C). Inefficiency to translocate the protein is thus not, or not solely, due to the unusual charge distribution.

Missorting of pre-pro-vasopressin is not due to overexpression and also occurs in neuronal cells. — To exclude the possibility that mistargeting of pre-pro-vasopressin is simply a consequence of high-level expression in COS cells, we examined the polypeptides produced in stably transfected CV-1 cells, the parental cell line of COS-1 cells lacking the large T antigen driving the SV40 promoter/origin of replication present in our expression plasmids. In
labeling and immunoprecipitation experiments, the stable CV-1 cell line expressing wild-type vasopressin precursor yielded a somewhat lower signal from the same number of cells than COS cells of which only 5–10% were transfected. The CV-1 cells are therefore producing at least 10–20 fold less of the protein per cell. Even in this situation, all three low molecular weight forms were made (Fig. 6A). In particular, form 2, pre-pro-vasopressin, was made as well. The inefficiency of the vasopressin signal is thus also apparent at moderate rates of synthesis.

In vivo, the vasopressin precursor is expressed by hypothalamic secretory neurons. We tested whether the products observed in fibroblasts were also generated in Neuro2a cells, a mouse neuroblastoma cell line that endogenously expresses secretogranin II and that had been shown to sort exogenous pro-opiomelanocortin into dense-core granules (28). In transfected Neuro2a cells expressing wild-type or ΔE47 mutant vasopressin precursor and labeled for 5–60 min, pre-pro-vasopressin (form 2) and unglycosylated pro-vasopressin (form 3) were detected and stabilized by ALLN as in COS-1 and CV-1 cells (Fig. 6B). However, form 4 corresponding to N-terminally truncated pro-vasopressin could not be detected. Whereas the inefficiency of the vasopressin signal is observed in all cell types tested, the cytosolic protease responsible for N-terminal truncation of vasopressin precursors in COS-1 and CV-1 cells, is missing in Neuro2a cells.
DISCUSSION

Degradation of vasopressin precursor occurs via proteasomes — In cells expressing mutant vasopressin precursor, products of lower molecular weight than full-size glycosylated pro-vasopressin were stabilized by proteasomal inhibitors. No stabilization was observed with inhibitors of lysosomal proteases. This confirmed the expectation that the mutant proteins retained in the ER by the lumenal quality control system are subject to ER-associated degradation (ERAD), i.e. proteolysis by the cytosolic proteasome. Interestingly, significant stabilization of the same types of intermediates was also observed in cells expressing the wild-type protein. This may be due to a considerable number of polypeptides that never attained the native structure owing to errors in translation or post-translational processes necessary for proper protein folding. It has previously been estimated that about one third of newly synthesized total protein is rapidly degraded (29).

Proteins targeted for proteasomal degradation are often, but not always, tagged by ubiquitin (30-34). We were unable to demonstrate ubiquitination of vasopressin polypeptides using multiple approaches, including immunoblotting of immunoprecipitated vasopressin precursor with anti-ubiquitin antibodies, increasing the cells' ubiquitin pool by overexpressing a ubiquitin cDNA, or coexpression of dominant-negative ubiquitin constructs (K48R and K48RG76A). It is unclear whether we did not reach sufficient amounts of ubiquitinated material, whether the ubiquitinated form might not be recognized by our antibodies, or whether the vasopressin mutants are targeted to the proteasome through alternative pathways, such as neddylation or sumoylation (35-37). To detect ubiquitinated proteins is notoriously difficult. In general only a small amount of the protein is detectable in ubiquitinated forms, which furthermore are heterogeneous in size. In addition, rapid deubiquitination may occur in cell lysates.
With proteasome inhibitors three unglycosylated forms accumulate in the cytosol — Upon incubation with proteasome inhibitors, three vasopressin precursor forms of molecular weights in the range of 17–19 kDa were stabilized (forms 2–4). All three of them were unglycosylated and cytosolic. Comparison of their electrophoretic mobility with that of different mutant vasopressin precursors, and immunoreactivity with antibodies directed against the hormone domain indicate that these forms correspond to unglycosylated pre-pro-vasopressin (form 2), deglycosylated pro-vasopressin (form 3), and N-terminally truncated pro-vasopressin (form 4). Small amounts of form 3 have previously been observed in untreated cells (22), but have been interpreted to be the product of incomplete glycosylation in the ER lumen. That this form is largely released into the supernatant of broken cells indicates that it has been retrotranslocated and deglycosylated.

The data support a scenario (illustrated in Figure 7) in which pre-pro-vasopressin is inserted into the ER, cleaved by signal peptidase, and modified to glycosylated pro-vasopressin (form 1). Mutant proteins which are unable to fold into the native structure, but to some extent also wild-type polypeptides, are retrotranslocated to the cytosol and rapidly deglycosylated (form 3). In addition, a fraction of the translation products is not transported into the ER (form 2), based on the finding that the signal sequence was not cleaved and the protein remained unglycosylated. In the presence of proteasomal inhibitors, both forms 2 and 3 may be N-terminally clipped to produce form 4 before degradation. The protease responsible for this slow clipping is unknown and appears to be expressed in a cell type specific manner, since form 4 was not detectable in Neuro2a cells. Cytosolic nonproteasomal proteases are known to be involved in the processing of antigenic peptides to be presented by MHC class I molecules (e.g. ER aminopeptidase associated with antigen processing, ERAAP; ref. 38).

The vasopressin signal functions inefficiently — The production of pre-pro-vasopressin suggests that the native vasopressin signal is inefficient. This is not due to incomplete signal
cleavage, since no glycosylated pre-pro-vasopressin could be detected. In ADNDI mutants affecting the cleavage efficiency of the signal, such as A(-1)T (mutation of the cleavage site; ref. 5) and ΔG227 (truncation of the signal; ref. 22), glycosylated pre-pro-vasopressin is easily detected. A(-1)T also revealed increased form 2 (Fig. 2A, lane 15, and Fig. 2B, lane 11), since retrotranslocated polypeptides add to those that were primarily synthesized into the cytosol. Therefore, the defect in the native vasopressin signal is due to inefficient targeting or translocation. The phenomenon is not an artefact of overexpression and potential saturation of the secretory route, since it is also observed in CV-1 cells expressing at least ten times less protein per cell. Moreover, it is detected in Neuro2a cells which have characteristics of neuroendocrine cells and is thus likely to occur also in vasopressinergic cells in vivo.

The charges flanking the hydrophobic core of signal sequences largely determine signal orientation in the ER translocation machinery (39). Typically, the N-terminal portion of signal peptides is positively charged (the positive-inside rule; ref. 40), or at least more positive than the C-terminal flanking sequence (41). This is not the case for the vasopressin signal where the charge difference Δ(C–N) calculated according to the rules by Hartmann et al. (41) is +2. Surprisingly, however, the unusual charge distribution is not responsible for the translocation inefficiency: mutation of D(-17) to R or of R(8), K(11), and R(12) to E did not reduce the production of pre-pro-vasopressin despite an improved charge difference of 0 and -4, respectively. The efficiency of the enkephalin signal fused to pro-vasopressin is thus also not just due to the positive N-terminus. The hydrophobic core of the enkephalin signal is longer and more hydrophobic (in total and on average) than that of the vasopressin signal. This might account for more efficient recognition by signal recognition particle (42).

Inefficient function of signal sequences is observed rarely. Plasminogen activator inhibitor-2 (PAI-2) is found as a secreted and a cytosolic form because of an internal, uncleaved signal that is inefficient both in binding signal recognition particle and in subsequent interaction with the translocation complex (43). Parathyroid hormone-related
peptide (PTHrP) precursor is in part found as pre-pro-PTHrP in the cell and accumulates in nucleoli due to a nucleolar targeting signal (44). Although this signal has a positive N-terminus, its C-terminal sequence is even more positively charged. In both cases, dual localization may reflect separate functions in different compartments.

Of special pathophysiological interest is the case of the prion protein (PrP). A particular misfolded conformation of PrP (PrP\(^{\text{Sc}}\)), which is favored by certain mutations in the protein, causes neurodegenerative disorders (prion diseases). The N-terminal signal of PrP is inefficient and membrane targeting of a fraction of the protein is rescued by a C-terminal hydrophobic sequence (45). PrP can adopt multiple membrane topologies, including a fully translocated form (\(^{\text{sec}}\)PrP), two transmembrane forms with either the N- or the C-terminal portion of the polypeptide translocated into the ER lumen (\(^{\text{Nim}}\)PrP and \(^{\text{Cmp}}\)PrP, respectively), and a cytosolic form (46,47). Again, the charge difference of the signal is the opposite of that typical for secretory signal peptides. It has been shown that mutant PrP as well as a significant portion of wild-type PrP is degraded via the proteasome, since cytosolic, unglycosylated forms accumulate upon treatment with proteasomal inhibitors (48,49). It has recently been proposed that the cytosolic forms of PrP trigger at least some neurodegenerative prion diseases, because expression of a cytosolic form of PrP lacking the N-terminal signal and the C-terminal glycosylphosphatidylinositol anchor sequence was toxic in Neuro2a cells and rapidly caused neurodegeneration in transgenic mice (50). In addition, conversion to a PrP\(^{\text{Sc}}\)-like conformation was found to be increased for a mutant PrP causing heritable prion disease correlating with its increased transport into the cytosol (51). To which extent the situation of mutant vasopressin precursor parallels that of PrP remains to be determined.

It is not known at present whether ADNDI results from a toxic product in the cytosol or in the ER lumen. Several factors that are induced by ER stress have been shown to mediate cell damage. The transcription factor CHOP has been shown to induce an apoptotic response
in renal tubular cells of mice injected intraperitoneally with tunicamycin (52), and
downstream effectors of CHOP have been characterized (53). In a mouse model of non-
autoimmune diabetes mellitus, hyperglycemia occurs as a consequence of apoptotic
destruction of pancreatic α-cells induced by ER stress due to ER retention of a mutant insulin
precursor. Targeted deletion of CHOP delays the onset of diabetes in these mice (54).
Tunicamycin-induced apoptotic destruction of renal tubular cells is significantly alleviated in
mice deficient in caspase-12, which is located at the ER and mediates ER-associated apoptosis
in a specific manner (55). Caspase-12 also mediates amyloid-α-induced toxicity in primary
cortical neurons cultured ex vivo, but is not activated by non-ER-associated apoptotic signals,
such as cycloheximide, tumor necrosis factor, or anti-Fas, again illustrating its specific role in
ER-associated cell damage.

In ADNDI, cytotoxic pathways triggered by ER-retention directly and/or by
retrotranslocated degradation intermediates in the cytosol may be involved in the
degeneration of vasopressinergic neurons. Our findings indicate that proteasomal
degradation of mistargeted precursors occurs even when two wild-type alleles are expressed.
In heterozygous individuals, a critical concentration of toxic molecules may be exceeded,
leading to neuronal degeneration.

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REFERENCES


FIGURE LEGENDS

FIG. 1. **Wild-type and mutant vasopressin precursor and its signal sequence.** The domain organization of the vasopressin precursor is shown with disulfide bridges as line connections and the glycosylation site as a diamond. The positions of mutations in mutant vasopressin precursors used in this study are indicated by an arrow. Below, the sequence of the signal peptide and the N-terminal portion of pro-vasopressin is shown. To prevent ER targeting, L(-9) of the signal peptide was mutated to R as indicated.

FIG. 2. **Effect of proteasomal inhibitors on the stability of wild-type and mutant vasopressin precursors.** Wild-type (wt) vasopressin precursor and the mutants G57S, ΔE47, and ΔA(-1)T were expressed in COS-1 cells, labeled with $[^{35}\text{S}]$methionine/cysteine for 1 h, and chased for 0 or 6 h without inhibitors (control) or in the presence of 250 µM ALLN (panel A), 25 µM lactacystin (panel B), or of the lysosomal inhibitors leupeptin and pepstatin A (LP; panel C), as described in *Experimental procedures*. Cells and media were subjected to immunoprecipitation using an antibody directed against neurophysin II, and immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiography. The apparent molecular weight of glycosylated pro-vasopressin of 21 kDa is indicated. Products of lower apparent molecular weight of ~17–19 kDa are pointed out by asterisks.

FIG. 3. **Time-course of appearance of different vasopressin precursor forms.** COS-1 cells expressing wild-type (wt) or ΔE47 mutant vasopressin precursor were labeled for 5–60 min with $[^{35}\text{S}]$methionine/cysteine. Cells were incubated with proteasome inhibitor (+) before and during labeling as described in *Experimental procedures* or were untreated (–). ALLN (250 µM) was used as inhibitor in panel A, and lactacystin (40 µM) in panel B. Vasopressin products were immunoprecipitated and analyzed by SDS-gel electrophoresis and autoradiography.
FIG. 4. **Characterization of low molecular weight vasopressin products.** Panel A: COS-1 cells expressing the vasopressin precursor mutant ΔE47 were incubated with ALLN, labeled with $[^{35}S]$methionine/cysteine for 1 h, subjected to immunoprecipitation using anti-neurophysin II (αNP) or anti-vasopressin (αVP) antibodies and analyzed either directly (−) or after deglycosylation using endoglycosidase H (H) or endoglycosidase F (F). Panel B: COS-1 cells expressing the following mutant precursors were labeled for 30 min in the presence of ALLN and analyzed as above: ΔE47, the signal peptide mutant L(-9)R, the signal peptide deletion mutant ΔSP, and the mutant ΔVP which lacks the vasopressin hormone sequence. Panel C: COS-1 cells expressing wild-type or ΔE47 mutant precursor as indicated were incubated with ALLN and labeled for 5 min to generate (besides form 1) predominantly form 2, or for 60 min to generate predominantly forms 3 and 4. The labeled cells were then broken by swelling and centrifuged at high speed. The supernatants containing cytosolic proteins (C) and the membrane pellets (M) were analyzed in parallel to unfractionated aliquots of total cell lysates by immunoprecipitation, gel electrophoresis and autoradiography.

FIG. 5. **Signal dependence of form 2 generation.** Panel A: COS-1 cells expressing wild-type or mutant ΔE47 precursor with either the native vasopressin signal (V) or the signal sequence of pre-pro-enkephalin (E) were incubated with ALLN and labeled for 3–60 min with $[^{35}S]$methionine/cysteine before immunoprecipitation, gel electrophoresis and autoradiography. Panel B: The sequence of the enkephalin signal (E) fused to pro-vasopressin (gray) is shown in comparison to the native vasopressin signal (V). Mutation of the charges flanking the hydrophobic core of the signal in constructs V(D>R) and V(RKR>EEE) are indicated. Panel C: COS-1 cells expressing the vasopressin precursor with the native signal (V), with the enkephalin signal (E), or with the charge mutants V(D>R) and V(RKR>EEE) were labeled for 5 min and analyzed as in A.
FIG. 6. Generation of unglycosylated pre-pro-vasopressin (form 2) in a stable CV-1 cell line and transfected Neuro2a cells. A CV-1 cell line stably expressing the wild-type vasopressin precursor (panel A) and transiently transfected Neuro2a cells expressing wild-type (wt) or ΔE47 mutant precursor (panel B) were labeled for 5–60 min with [35S]methionine/cysteine with (+) or without (−) incubation with ALLN. Vasopressin products were immunoprecipitated and analyzed by SDS-gel electrophoresis and autoradiography. Open arrowheads indicate the absence of form 4 production in Neuro2a cells.

FIG. 7. Schematic summary of the products and degradation intermediates of vasopressin precursor. The majority of vasopressin precursor is translocated into the ER lumen, cleaved by signal peptidase and glycosylated to form 1 (glycosylated pro-vasopressin). Molecules unable to fold are retrotranslocated into the cytosol and deglycosylated to form 3 (deglycosylated pro-vasopressin). A fraction of precursor proteins (even of the wild-type) is not translocated and is found as cytosolic pre-pro-vasopressin (form 2). Forms 2 and 3 are stabilized by proteasomal inhibitors. In COS-1 and CV-1 cells, but not in Neuro2a cells, they give rise to an N-terminally processed form 4. Primarily forms 2, 3, and 4 accumulate upon addition of proteasome inhibitors indicating that they are substrates of the proteasome (shown on the right).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Degradation of wild-type vasopressin precursor and pathogenic mutants by the proteasome
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