Degradation of Wild-type Vasopressin Precursor and Pathogenic Mutants by the Proteasome*

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Mutations in the gene encoding the antidiuretic hormone arginine vasopressin cause autosomal dominant neurogenic diabetes insipidus. Autoptic 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 unseparated 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 was 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 mutants associated with ADNDI and found it to degrade products thereof, are cytotoxic. We therefore studied the degradation of cytosolic 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.

EXPETIMENTAL PROCEDURES

Plasmids and Constructs—cDNAs for the wild-type vasopressin precursor and the mutants A1T, ΔE47, and G57S were a gift from M. Ito (Northwestern University, Chicago, IL). The signal peptide of enkephalin was fused to wild-type and ΔE47 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.
Proteasomal Degradation of Vasopressin Precursors

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 [35S]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 pre-vasopressin. The mutant A–1T, 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–1T mutant were secreted into the medium. Since COS cells lack prohormone-processing enzymes, intact glycosylated pre-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–1T had been retained and degraded.

To test for degradation via the ER-associated degradation pathway, the proteasomal peptide inhibitor 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 a variable extent also, the full-sized, 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 are 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–1T 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 the 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 pre-
cursor were incubated with endoglycosidase H or F (Fig. 4A, lanes 1–3). The 21-kDa form 1 was deglycosylated to an apparent molecular size 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 that product 4 corresponds 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 non-functional signal sequence (L9R; 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 (SP), i.e. pro-vasopressin synthesized into the cytosol, migrated like band 3 (lane 3). In a further construct, the hormone domain was deleted (VP) by fusing neurophysin II-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 (Fig. 4C, M) and the cytosol fraction (C) in comparison with the unfractionated total cell lysate (L). 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 therefore either were 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 polypeptides produced in stably transfected CV-1 cells, the parental cell line of COS-1 cells lacking the large T antigen. 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 test the possibility that mis-targeting 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

The signal of the vasopressin precursor is unusual in that it contains a negative charge near the N terminus (Asp€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, Asp€17 was mutated to Arg, or the residues Arg8, Lys11, and Arg12 were mutated to Glu. 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.
Degradation of Vasopressin Precursor Occurs via Proteasomes—In cells expressing mutant vasopressin precursor, products of lower molecular weight than full-sized glycosylated pro-vasopressin were stabilized by proteasomal inhibitors. No stabilization was observed with inhibitors of lysosomal proteases. This confirmed the observation that the mutant proteins retained in the ER by the lumenal quality control system are subject to ER-associated degradation, 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 ubiquitin pool of the cells 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 ubiquitylated 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 sizes 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, as well as 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 been observed previously 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 Fig. 7) in which pre-pro-vasopressin is inserted into the ER, 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 translated 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).
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 non-proteasomal 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; Ref. 38).

The Vasopressin Signal Functions Inefficiently—The production of pro-pre-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–1T (mutation of the cleavage site; Ref. 5) and ΔG227 (truncation of the signal; Ref. 22), glycosylated pro-pre-vasopressin is easily detected. A–1T also revealed increased form 2 (Fig. 2, A, lane 15, and B, lane 11) since retro-translocated 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 artifact of overexpression and potential saturation of the secretory route since it is also observed in CV-1 cells expressing at least 10 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, in which 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 Asp17 to Arg or of Arg8, Lys11, and Arg12 to Glu did not reduce the production of pro-pre-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 sequence (41) is found as a secreted and a cytosolic form because of an internal, uncleaved signal sequence (41). This is not the case for the vasopressin signal, in which 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 Asp17 to Arg or of Arg8, Lys11, and Arg12 to Glu did not reduce the production of pro-pre-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).

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