Mechanism of Endoplasmic Reticulum Retention of Mutant Vasopressin Precursor Caused by a Signal Peptide Truncation Associated with Diabetes Insipidus*

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Autosomal dominant neurohypophyseal diabetes insipidus is caused by mutations in the gene encoding the vasopressin precursor protein, prepro-vasopressin-neurophysin II. We analyzed the molecular consequences of a mutation (G227) recently identified in a Swiss kindred that destroys the translation initiation codon. In COS-7 cells transfected with the mutant cDNA, translation was found to initiate at an alternative ATG, producing a truncated signal sequence that was functional for targeting and translocation but was not cleaved by signal peptidase. The mutant precursor was completely retained within the endoplasmic reticulum. The uncut signal did not affect folding of the neurophysin portion of the precursor, as determined by its protease resistance. However, formation of disulfide-linked aggregates indicated that it interfered with the formation of the disulfide bond in vasopressin, most likely by blocking its insertion into the hormone binding site of neurophysin. Preventing disulfide formation in the vasopressin nonapeptide by mutation of cysteine 6 to serine was shown to be sufficient to cause aggregation and retention. These results indicate that the G227 mutation induces translation of a truncated signal sequence that cannot be cleaved but prevents correct folding and oxidation of vasopressin, thereby causing aggregation and retention in the endoplasmic reticulum.

As illustrated in Fig. 1A, the precursor protein consists of four main segments (4): 1) a 19-amino acid signal sequence for targeting to the endoplasmic reticulum (ER); 2) the nonapeptide hormone vasopressin; 3) neurophysin II (NPII) consisting of 93 residues, which serves as the vasopressin transport protein in the circulation; and 4) a 39-amino acid glycopeptide of unknown function with a single N-glycosylation site. The signal sequence is cleaved off by signal peptidase upon translocation of the precursor into the ER lumen. After folding and disulfide bond formation, pro-vasopressin-NPII passes through the Golgi apparatus into secretory granules where the other segments are separated by proteolytic removal of the linker residues. The mutations identified in different kindreds of ADNDI are located in the sequences encoding the NPII moiety, the signal peptide, and in one case the vasopressin sequence (3, 5–9).

Typically, symptoms of ADNDI develop gradually over a period of months to years after birth (10–13). Postmortem studies revealed degeneration of the hypothalamic magnocellular neurons in the nucleus supraventricularis and paraventricular, which synthesize the vasopressin-NPII precursor (14–19). Based on these findings, it has been postulated that impaired transport and/or processing of the mutant precursor may result in its intracellular accumulation, eventually leading to degeneration of the vasopressinergic neurons and to the gradual manifestation of clinical symptoms. A recent study provided evidence for the toxicity of mutant precursors or their degradation products to cultured neuronal cells (20).

We have investigated the molecular mechanism of an unusual mutation associated with ADNDI recently identified in a Swiss kindred (21). Guanosine 227, the third nucleotide of the translation initiation codon of the vasopressin precursor gene, is deleted on one allele in affected family members. These heterozygous patients are completely vasopressin-deficient and show an abnormal appearance of the neurohypophysis in magnetic resonance imaging. Because loss of expression of one allele is not a plausible cause for the observed dominant phenotype, we hypothesized that a second, in-frame ATG present four codons downstream might serve as an alternative initiation site generating a mutant precursor with a truncated signal sequence (Fig. 1B, Vm). To test this hypothesis and to analyze how a signal sequence truncation might affect the fate of the protein, we transiently transfected mutant or wild-type prepro-vasopressin-NPII cDNA into COS-7 cells and characterized the effects of the guanosine 227 deletion on expression, membrane translocation, signal cleavage, polypeptide folding, and secretion of the precursor. The protein was found to be synthesized and translocated into the ER. However, the truncated signal sequence was effectively blocked by oxygen-free buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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¶ The abbreviations used are: ADNDI, autosomal dominant neurohypophyseal diabetes insipidus; NP, neurophysin; ER, endoplasmic reticulum; Endo H, endo-β-N-acetylglucosaminidase H; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The full-size cDNA of wild-type prepro-vasopressin-NPII was a gift from Dr. M. Ito (Northwestern University, Chicago, IL). To construct the full-size cDNA of the guanosine 227 deletion mutant, Vm, the 5' end of the precursor cDNA up to the SacI site was amplified by polymerase chain reaction using the mutagenic primer CAGGATCTGACACCACAGCTGC (the BamHI site produced by the deletion of guanosine 227 is underlined), and a second primer corresponding to a sequence in the plasmid vector The product was ligated at the SacI site to the unamplified 3' portion of the cDNA. The cDNA was confirmed by sequencing. In the process, a separate cDNA clone, VmΔ, was identified with an additional, accidental mutation, deletion of codons 28–36 of pro-vasopressin-NPII (residues 16–24 of NPII). 

Polymerase chain reaction amplification of the precursor cDNA using the mutagenic primer CCGGAATCTTGGATTGGCAGCTTGTCG in combination with a second primer in the flanking vector sequence was performed to generate the plasmid pVm(1–11S), which encodes the vasopressin precursor with an extended signal sequence and with Cys-11 of the signal peptide mutated to serine. 

Cysteine 6 was mutated to serine by polymerase chain reaction amplification of the 5' portion of the wild-type precursor cDNA using the mutagenic antisense primer CGGGAGCTTGTTTCTGAGGAGTTGACGC in combination with a second primer in the flanking vector sequence. Separately the 3' portion was amplified using the primer CAGGATCTGACACCACAGCTGC and a vector primer. The mutagenic primers mutated the codon TGC of cysteine 6 to the sequence AGC encoding serine. The sequence was not cleaved and interfered specifically with the

**RESULTS**

**Deletion of Guanosine 227 Results in a Truncated Signal Sequence That Is Functional but Not Cleaved**—Deletion of guanosine 227 in the prepro-vasopressin-NPII gene of the Swiss ADNDI kindred eliminates the original translation initiation codon. Expression of a mutant protein would depend upon initiation at an alternative in-frame ATG. There are only two internal ATG sequences in the entire prepro-vasopressin-NPII cDNA, encoding Met14 (the second codon of NPII) and Met15 (the fifth codon of the signal sequence; Fig. 1B). Translation initiation at Met14 would generate a cytosolic polyepitope, whereas a protein beginning with Met15 would still contain most of the signal peptide truncated by four residues (Fig. 1B). The wild-type signal sequence is unusual in that it contains a negatively charged aspartate and no positively charged residues in its N-domain (27, 28). In comparison, the truncated signal sequence appears rather more typical, because the negative residue is deleted.

To test whether translation of the mutant cDNA initiates at one of the downstream ATGs and whether insertion into the ER occurs, wild-type and mutant cDNAs were transiently expressed in transfected COS-7 cells, labeled with [35S]methionine for 30 min at 37 °C, chased for 30 min at 37 °C with 7.5% CO2. The media were supplemented with 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection of COS-7 cells using DEAE-dextran and Me2SO was performed according to Cullen et al. (23) in 6-well plates. Transfected cells were incubated 48 h after transfection. For in vivo labeling, transfected cells were incubated for 30 min in Cullen (23) in 6-well plates. Transfected cells were processed 48 h after transfection. For in vivo labeling, transfected cells were incubated for 30 min at 37 °C with 100 μCi/ml [35S]methionine in starvation medium. Where indicated, cells were chased in complete medium containing excess methionine. Lysed cells and media were subjected to immunoprecipitation using a polyclonal rabbit anti-NPII antiserum at 37 °C with 7.5% CO2. The media were supplemented with 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection of COS-7 cells using DEAE-dextran and Me2SO was performed according to Cullen et al. (23) in 6-well plates. Transfected cells were incubated 48 h after transfection. For in vivo labeling, transfected cells were incubated for 30 min at 37 °C with 100 μCi/ml [35S]methionine in starvation medium. Where indicated, cells were chased in complete medium containing excess methionine. Lysed cells and media were subjected to immunoprecipitation using a polyclonal rabbit anti-NPII antiserum at 37 °C with 7.5% CO2. The media were supplemented with 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection of COS-7 cells using DEAE-dextran and Me2SO was performed according to Cullen et al. (23) in 6-well plates. 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The Mutant Precursor Is Efficiently Retained in the ER—To analyze their secretion properties, wild-type and mutant prepro-vasopressin-NPII precursors were expressed in COS-7 cells, pulse-labeled for 30 min with [35S]methionine, chased for up to 4 h, and then immunoprecipitated from the medium and from the lysed cells (Fig. 4). The wild-type precursor was rapidly secreted into the medium with a half-time of less than 1 h (lanes 1–8). Because COS cells lack a regulated secretory pathway and the corresponding processing proteases, pro-vasopressin-NPII was secreted intact. The secreted protein was resistant to Endo H digestion (lanes 9 and 10), indicative of conversion of the N-linked glycan from high mannos to complex type in the Golgi apparatus. In contrast, the mutant precursor was almost completely retained inside the cells in an Endo H-sensitive form (lanes 13–26). Localization of the mutant protein by indirect immunofluorescence showed strong staining with the typical reticular pattern of the ER extending through the entire cell (Fig. 5A). Double immunofluorescence staining for the ER resident protein p63 (Fig. 5D) (29) and for mutant vasopressin precursor (Fig. 5C) produced identical patterns in expressing cells. For comparison, the weak staining pattern of cells expressing the wild-type protein reflects the steady state distribution of the precursor in the secretory pathway, i.e., the network of the ER and a perinuclear spot typical of the Golgi apparatus (Fig. 5B).

An uncleaved, hydrophobic signal peptide could cause ER retention by anchoring the protein in the ER membrane. Membrane integration was tested by alkaline extraction (Fig. 2B). Microsomes of labeled cells were incubated at pH 11.5 to disrupt organelles and release peripherally associated and soluble proteins. As expected, wild-type vasopressin precursor was recovered in the soluble extract (lanes 1 and 2), whereas subunit H1 of the asialoglucoprotein receptor, a control integral membrane protein, was pelleted with the membranes (lanes 5 and 6). The mutant vasopressin precursor was as almost as efficiently extracted as the wild-type protein (lanes 3 and 4), suggesting that it was not anchored in the bilayer of the ER.

The Uncleaved Signal Causes Disulfide Aggregation but Does Not Prevent NPII Folding—The uncleaved hydrophobic signal peptide might cause ER retention by interfering with correct

![Image](https://example.com/image.png)
folding and disulfide bonding of the precursor. Pro-vasopressin-NPII contains eight intramolecular disulfide bridges. Formation of disulfide bonds is often reflected in the electrophoretic mobility of a protein under reducing versus nonreducing conditions. To address this, transfected COS cells were metabolically labeled and then incubated at 4 °C with iodoacetamide to alkylate free sulfhydryl groups to prevent post-lysis oxidation. Vasopressin precursor was immunoprecipitated and split into two samples that were boiled in SDS sample buffer with or without mercaptoethanol. Upon gel electrophoresis, the wild-type precursor migrated slightly faster in its nonreduced, more compact folded state than after reduction of the disulfide bonds (Fig. 6, lanes 1 and 3). In contrast, the nonreduced mutant precursor was hardly detectable at the corresponding position in the gel but appeared as aggregates that migrated more slowly or did not enter the gel at all (lane 4). Such aggregates are typically observed for misfolded proteins (28).

To more directly assess the folding state of the protein, wild-type and mutant precursors were subjected to a protease sensitivity assay. Labeled protein was immunoprecipitated using anti-NPII antiserum and protein A-Sepharose and was incubated with increasing concentrations of trypsin. The Sepharose beads were washed, and bound material was analyzed by gel electrophoresis and fluorography (Fig. 7). From the wild-type precursor (lanes 1–5), a fragment of ~12 kDa (fragment 3) was generated that was completely resistant to trypsin at up to 5000 μg/ml. Its size corresponds to that of the NPII domain, indicating that the flanking segments (vasopressin and glycopeptide) were cleaved off at the connecting peptides normally hydrolyzed by processing enzymes in secretory granules. Intermediates of ~21 and ~13 kDa (fragments 1 and 2) were weakly detectable at the lowest trypsin concentration (lane 2). Their sizes suggest that these intermediates correspond to NPII-glycopeptide and vasopressin-NPII fragments, respectively. This was confirmed by performing the same experiment using an antiserum directed against the vasopressin nonapeptide (data not shown). Only the full-size precursor and the 13-kDa intermediate (fragment 2) were recovered, indicating that they contain the vasopressin sequence.

Upon incubation of the mutant precursor with trypsin (Fig. 7, lanes 6–10), the 12-kDa NPII fragment was generated as well, although it was slightly less resistant to the highest protease concentrations. Intermediate fragments of ~21 kDa (fragment 1) lacking the uncleaved signal and vasopressin, and of ~14 kDa (fragment 2*) lacking the glycopeptide portion were also produced. The latter intermediate was the only one to be recovered using anti-vasopressin antibody (not shown), supporting the notion that it consists of the uncleaved signal, vasopressin, and NPII. It is more abundant than the corresponding fragment of the wild-type precursor (even considering that it contains an additional methionine in the signal sequence). The uncleaved signal thus appears to affect the acces-

**Fig. 3.** Radiosequencing of translocated mutant vasopressin precursor. A, mutant vasopressin precursor (Vm) was translated in vitro with [35S]methionine in the absence (–) or presence of microsomes (M) and compared with the wild-type protein translated into microsomes in the presence (M+I) or absence of signal peptidase inhibitor (as described under "Experimental Procedures"). The fluorograph of an SDS gel is shown. B, mutant precursor translated in vitro in the presence of [3H]leucine and inserted into microsomes was subjected to automated Edman degradation. Radioactivity released at each cycle is plotted. The only compatible precursor sequence, corresponding to residues –15 to –2 of the precursor, is shown below.
sibility of the peptide connecting vasopressin to NPII. Most importantly, it did not interfere with the folding of NPII itself.

In contrast, the precursor VmΔ, a cloning artifact of Vm with an additional small deletion of codons 28–36 of pro-vasopressin-NPII (residues 16–24 of NPII), did not generate specific trypsin-resistant fragments (Fig. 7, lanes 11–15). VmΔ thus serves as a control showing that protease resistance depends on NPII folding.

The Uncleaved Signal Interferes with Disulfide Formation in Vasopressin—In the wild-type pro-vasopressin-NPII, all cysteines are engaged in disulfide bonds, seven in NPII and one in
vasopressin. The mutant precursor, however, contains an additional cysteine, Cys\(^{11}\), in the uncleaved signal peptide (Fig. 1B). To test whether this cysteine is responsible for the formation of disulfide aggregates and retention, we constructed the mutant Vm(C–11S), in which Cys\(^{11}\) in the truncated signal sequence of Vm was mutated to serine. As shown in Fig. 8, mutation of this cysteine did not significantly affect the properties of the protein. It was synthesized by COS cells as a 23-kDa glycosylated, Endo H-sensitive protein (Fig. 8A, lanes 1 and 2), indicating that the truncated signal without cysteine was functional and uncleaved. The majority of the protein was retained in an Endo H-sensitive form within the cell (lanes 5–8). After 2 h of chase, ~7% was recovered from the medium as Endo H-resistant protein, which is slightly more than for Vm. Trypsin resistance of the 12-kDa NPII fragment indicates correct folding of NPII (Fig. 8B). Most notably, elimination of Cys\(^{11}\) did not prevent most of the protein from forming disulfide-linked aggregates (Fig. 8C), indicating that covalent aggregation is not, or not solely, caused by Cys\(^{11}\). The extraordinary protease resistance of the NPII portion in Vm and Vm(C–11S) argues that its seven disulfide bonds were correctly formed. Thus, the disulfide bond in the vasopressin segment may not be correctly formed in the mutant precursors and cause aggregation.

The first residue of vasopressin, Cys\(^1\), forms a disulfide bridge with Cys\(^6\). It is conceivable that lack of signal cleavage in Vm and Vm(C–11S) interferes with the formation of this disulfide bond, leaving its thiol groups potentially available for oxidation to form covalent aggregates. To test whether unpaired cysteines of vasopressin might cause ER retention, we constructed the mutant V(C6S). Cys\(^6\) was mutated to serine in the context of the wild-type precursor sequence, leaving Cys\(^1\) without its normal disulfide partner, even if the signal is removed. Upon expression in COS cells, V(C6S) showed an electrophoretic mobility of 22 kDa like the wild-type precursor (Fig. 9A, lanes 1 and 2), indicative of signal cleavage. Nevertheless, it was not secreted from the cells and remained sensitive to Endo H digestion (lanes 6–9). The mutation did not interfere with folding of NPII, because the trypsin-resistant NPII fragment of ~12 kDa was generated in the protease sensitivity assay (Fig. 9B). However, V(C6S) was also found largely as aggregates when analyzed under nonreducing conditions (Fig. 9C). These results indicate that failure to form the disulfide bond in vasopressin is sufficient to cause aggregation and ER retention of the precursor protein.

The guanosine 227 deletion in Vm thus acts indirectly. By eliminating the normal translation initiation site, it induces translation of a truncated signal sequence that, because it cannot be cleaved, prevents the correct folding and oxidation of vasopressin. This is ultimately sufficient to cause aggregation and ER retention.

**DISCUSSION**

In this study, we have analyzed the mechanism by which a mutation in the translation initiation codon of prepro-vasopressin-NPII causes ADNDI in a Swiss kindred. The mutant precursor was expressed and glycosylated, indicating that translation initiated at an alternative ATG codon and produced a functional signal for ER targeting and translocation (Fig. 2). The only possible initiation site is codon –15 generating a signal sequence truncated by four residues. This shortened signal is in better agreement with the typical characteristics of
The uncleaved mutant vasopressin precursor was efficiently retained in the ER in a high mannose glycosylated form (Figs. 4 and 5) and could be recovered largely in disulfide-linked aggregates (Fig. 6) typical of unfolded or misfolded proteins (28). All the cysteines in the precursor are normally involved in intramolecular disulfide bonds, except for one in the signal sequence. However, folding of the NPII domain, which contains 7 of the 8 disulfide bonds in the precursor, was not significantly affected by the uncleaved signal, because the NPII domain was found to be almost as resistant to trypsin digestion as in the wild-type protein (Fig. 7). Based on the positions of the N and the C termini of NPII in the crystal structure (35), the small, C-terminal glycopeptide domain is not in contact with the signal vasopressin segments and is thus unlikely to be disturbed during folding. In addition, it does not contain any cysteines and thus cannot be responsible for disulfide aggregation. Hence, retention and aggregation is caused by the uncleaved signal and/or by misfolded vasopressin. Cys\(^{-11}\) of the signal sequence is not solely responsible, because its mutation to serine did not eliminate aggregation and did not allow secretion (Fig. 8). Our results therefore suggest that the disulfide bond in vasopressin is not formed in the uncleaved mutant protein, leaving the free thiols available for incorrect intermolecular disulfide formation. Consistent with this model, we found that preventing disulfide formation in vasopressin by mutating one of its two cysteines is sufficient to cause ER retention and aggregation, even if the signal is removed correctly (Fig. 9).

The crystal structures of bovine NPII complexed with a dipeptide analog of vasopressin (35) and of the closely homologous bovine neurophysin I-oxytocin complex (36) have been determined. They show that the \(\alpha\)-amino group of the hormone is hidden in the binding pocket, forming three hydrogen bonds and a salt bridge, and that the disulfide bond of the hormone is facing into the binding site. Binding of the N-terminal vasopressin segment of pro-vasopressin-NPII into the hormone binding site may be essential to stabilize the intramolecular disulfide bond between Cys\(^2\) and Cys\(^6\) by protecting it from further disulfide isomerization. If the signal sequence is not removed, this is impossible, and the signal and vasopressin will remain exposed. The observation that the NPII domain of the wild-type precursor is somewhat more stable at high trypsin concentrations than that of the mutants Vm or V(C6S) (Figs. 7 and 9B) may reflect stabilization of NPII when the correctly oxidized hormone is bound to its binding site. A stabilizing effect of the ligand on NPII has previously been documented in \textit{in vitro} folding studies (37).

In the prepro-vasopressin-NPII precursor sequence, close to 30 different mutations causing ADNDI have been identified (3, 5–9). Besides the mutation studied here, three localized to the signal sequence. Ala\(^{-1}\) was mutated to threonine in six kindred (3, 8, 13, 38, 39), representing the most frequent ADNDI mutation known so far. In addition, substitution of Ala\(^{-1}\) by valine (3, 9) and of Ser\(^{-19}\) by phenylalanine (3) have been identified. These signal mutations involve the residues that are most critical for signal peptidase activity. Reduced signal cleavage was experimentally demonstrated \textit{in vitro} for the alanine-to-threonine mutation (38). These cleavage site mutants are very likely to cause ER retention by the same mechanism as the guanosine 227 deletion mutant analyzed in this study. There is only one known case of ADNDI with a mutation in vasopressin itself (Tyr\(^2\) to His) (6). Because Tyr\(^2\) is essential for vasopressin binding to NPII, Cys\(^3\) and Cys\(^6\) of the mutant protein are likely to remain exposed to the ER lumen, with the same consequences as observed for the signal mutant analyzed here. All other mutations were distributed throughout the NPII se-
ER Retention of Mutant Vasopressin Precursor

quence and include missense, deletion, and nonsense mutations that most likely cause ER retention by disturbing the correct folding of the NPII domain.

The gradual development of the dominant phenotype of ADNDI and postmortem studies suggest that the disease is caused by the degeneration of the hypothalamic cells producing mutant hormone precursor. In a recent study, reduced viability of neuro2A neuroblastoma cell lines expressing different vasopressin precursor mutants (a signal cleavage site mutant and several NPII mutants) was observed (20), indicating that the retained precursor (or its degradation products) has a cytopathic effect. ER retention, first shown for the glycine 17-to-valine mutation of NPII (40), and the resulting cytotoxicity might be a common feature of many, if not all, prepro-vasopressin-NPII mutations. This is reminiscent of α1-antitrypsin deficiency, which causes liver cirrhosis in susceptible individuals. Mutant antitrypsin was found in hepatocytes in characteristic insoluble inclusions that were associated with hepatocellular damage (41). Several mutant proteins, including the relatively frequent Z variant, were shown to be retained in the ER in a misfolded form producing large noncovalent homopolymers of a specific structure (loop-sheet polymerization) (42, 43). In contrast to this mechanism, mutant vasopressin precursors were not found in inclusions but rather evenly distributed throughout the ER (Fig. 4 and Ref. 20). The disulfide aggregates appear to be heterogeneous, suggesting that disulfide linkage occurs to other still unfolded proteins in the ER rather than by oxidative homooligomerization. In addition, the mutations that cause ADNDI are quite different. For example, truncation of the C-terminal third of NPII is very likely to cause gross misfolding of the rest of the precursor, whereas lack of signal cleavage in the guanosine 227 deletion mutant did not impede NPII folding (Fig. 7). Elucidating the mechanism of cytototoxicity by retained vasopressin precursors is the main challenge in understanding the molecular pathogenesis of ADNDI and may provide important insights into the mechanisms underlying neurodegenerative diseases.

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