Mutations in the Vasopressin Prohormone Involved in Diabetes Insipidus Impair Endoplasmic Reticulum Export but Not Sorting*

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Familial neurohypophysial diabetes insipidus is characterized by vasopressin deficiency caused by heterozygous expression of a mutated vasopressin prohormone gene. To elucidate the mechanism of this disease, we stably expressed five vasopressin prohormones with a mutation in the neurophysin moiety (NP14G→R, NP47E→G, NP47E→A, NP57G→S, and NP65G→V) in the neuroendocrine cell lines Neuro-2A and PC12/PC2. Metabolic labeling demonstrated that processing and secretion of all five mutants was impaired, albeit to different extents (NP57G→V ≥ NP14G→R > NP47E→A ≥ NP47E→G > NP57G→S). Persisting endoglycosidase H sensitivity revealed these defects to be due to retention of mutant prohormone in the endoplasmic reticulum. Mutant prohormones that partially passed the endoplasmic reticulum were normally targeted to the regulated secretory pathway. Surprisingly, this also included mutants with mutations in residues involved in binding of vasopressin to neurophysin, a process implicated in targeting of the prohormone. To mimic the high expression of wild type prohormone or with the viability of the vasopressin-producing cells. Immunofluorescence displayed formation of large accumulations of mutant prohormone in the endoplasmic reticulum, accompanied by redistribution of an endoplasmic reticulum marker. Our data suggest that prolonged perturbation of the endoplasmic reticulum eventually leads to degeneration of neurons expressing mutant vasopressin prohormones, explaining the dominant nature of the disease.

Familial neurohypophysial diabetes insipidus (FNDI)1 is the best known inherited endocrine disease caused by prohormone defects (1, 2). In this disease, mutations in the vasopressin prohormone cause defects in the synthesis of vasopressin and hence result in a large increase in urine production (polyuria) and fluid intake (polydipsia) (1, 2). The vasopressin prohormone consists of vasopressin, its carrier protein neurophysin and a glycopeptide, which are separated by arginine/lysine rich cleavage sites (see Fig. 1A) (3, 4). It is synthesized in the magnocellular neurons of the supraoptic and paraventricular nuclei of the hypothalamus and is subsequently transported to the nerve terminals in the neurohypophysis via the regulated secretory pathway (5, 6). Binding of vasopressin to its carrier protein neurophysin has been implicated in sorting of the prohormone (4). In the secretory granules, processing of the vasopressin prohormone takes place, and upon stimulation of the nerve terminals, processing products are released (5, 7). In human FNDI, mutations have been observed in the signal peptide, the vasopressin moiety, and the neurophysin moiety of the vasopressin preprohormone (see Fig. 1B) (8–17). The disease displays two unexpected features for a deficiency caused by a defective prohormone. First, the disease is dominant, demonstrating that one mutant prohormone allele suffices to cause the defect. Second, the onset of disease symptoms is delayed to several months or years of age. These peculiarities suggest that the mutant human vasopressin prohormone somehow interferes with synthesis, transport, or processing of the wild type prohormone or with the viability of the vasopressin-producing cells.

To determine the mechanism of human FNDI, we investigated the intracellular fate of five vasopressin prohormones containing an established diabetes insipidus mutation. Because most FNDI mutations are present within the neurophysin moiety and concern substitution of amino acids implicated in determining protein structure, like cysteines involved in disulfide-bridge formation or prolines and glycines, which can make turns in polypeptide chains (see Fig. 1B) (8–17), we decided to analyze three mutations of glycine residues at different positions within the neurophysin moiety: the most N-terminal and most C-terminal mutations of a glycine residue (NP14G→R and NP57G→V) and the mutated glycine residue in the loop connecting the two β-strand domains (NP57G→S) (see Fig. 1). In addition, we analyzed both mutations identified in NP47E, which is the residue involved in vasopressin binding (NP47E→G and NP47E→A) (see Fig. 1B) (8). All five mutated residues display full interspecies conservation (4). To examine sorting and processing, we stably expressed the mutant prohormones in neuroendocrine cell lines. The results suggest the involvement of the endoplasmic reticulum in the pathogenicity of human FNDI and reveal that the structural requirements of the vasopressin prohormone for trafficking from endoplasmic reticulum to Golgi apparatus are much more strict than for sorting into the regulated secretory pathway.

**EXPERIMENTAL PROCEDURES**

Construction of Expression Plasmids Encoding Wild Type and Mutant Human Vasopressin Genes—Point mutations were introduced by polymerase chain reaction with the enzyme Pfu (Stratagene) in either a...
178-base pair Kap1-SnaI fragment (mutant NP14G→R) or a 443-base pair Smal-EcoXI fragment (other mutants) of the cloned human vasopressin gene (GenBank™ accession number M11166) (18). The mutations made were exactly the same as described (8, 10, 11). The mutated fragments were completely sequenced and cloned back into the rest of the expression plasmid. In the expression plasmid obtained in this way, the vasopressin gene is expressed from its own TATA box and uses its own polyadenylation signal. An expression vector with hygromycin selection (pRSHyg) had been constructed by replacing the 2.3-kb PvuII fragment containing the G418 resistance gene of pRc/RSV (Invitrogen) containing the hygromycin resistance gene with the 2.1-kb NruI-SauI fragment of pREP4 (Invitrogen) containing the hygromycin resistance gene. Wild type and mutant vasopressin genes were cloned as 3.0-kb partial BglII-SacI fragments from the pRc/RSV plasmids to the pRSHyg vector.

Culture and Transfection of Cells—The mouse neuroblastoma cell line Neuro-2A (ATCC CCL 131) (19) was cultured in DMEM (Life Technologies, Inc.) supplemented with 4 mM glutamine, nonessential amino acids (Life Technologies, Inc.), 200 IU/ml penicillin, 200 μg/ml streptomycin, 5% fetal calf serum (Life Technologies, Inc.) in an atmosphere with 10% CO2. Neuro-2A cells were transfected with gene constructs cloned into the pRc/RSV vector with the calcium phosphate method (20). Stable clones were selected on medium supplemented with 1200 μg/ml G418 (Life Technologies, Inc.) and maintained in medium with 600 μg/ml G418.

The rat adrenal pheochromocytoma cell line PC12 transfected with mouse neurophysin convertase 2 (PC12/PC2) (21) was cultured in poly-l-lysine coated flasks in DMEM (Life Technologies, Inc.) supplemented with 4 mM glutamine, nonessential amino acids (Life Technologies, Inc.), 200 IU/ml penicillin, 200 μg/ml streptomycin, 5% fetal calf serum, and 10% horse serum (Life Technologies, Inc.) in an atmosphere with 10% CO2. PC12/PC2 cells were transfected with gene constructs cloned into the pRSHyg vector with Lipofectin (Life Technologies, Inc.) as described (21). Stable clones were selected on medium supplemented with 100 μg/ml hygromycin (Roche Molecular Biochemicals) and maintained on medium with 50 μg/ml hygromycin. Analysis of clones from independent transfection experiments gave similar results.

Antiserum—Rabbit antiserum D5 is raised against swine neurophysin and cross-reacts with human neurophysin (22). Rabbit antiserum HenryK was raised against rat neurophysin and cross-reacts with human neurophysin (23). Rabbit antiserum BoriaY2 recognizes the glycopeptide (24). Rabbit antisera W4 and W1 (25) are raised against vasopressin. W1 recognizes fully processed vasopressin, whereas W4 recognizes both processed and unprocessed vasopressin (25). Monoclonal D3 was raised against the C terminus of the ER protein protein-disulfide-isomerase and recognizes both protein-disulfide-isomerase (26). The monoclonal NM4 recognizes the cytosolic protein B50, which associates with the plasma membrane via palmitoyl chains (27).

Labeling of Cells, Immunoprecipitation, and Gel Electrophoresis—Cells were cultured in 6-well plates, washed with PBS, and starved for one hour in DMEM without cytoine (DMEM without cytoine, methionine, and glutamine (ICN) supplemented with 50 μg/ml chloroquine (Life Technologies, Inc.), glutamine, penicillin/streptomycin, and 10% fetal calf serum (Neuro-2A cells) or 5% fetal calf serum and 10% horse serum (PC12/PC2 cells)). Labeling was performed with 25 μCi of [35S]cysteine (ICN) for 18 h unless indicated otherwise. In case of a pulse-chase analysis, the chase was initiated by addition of 1/3 volume of DMEM supplemented with 4 mM cold cytoine (ICN) and serum. Cells were harvested by scraping and lysed in Triton X-100 lysis mix (50 mM Tris, pH 7.4, 5 mM MgCl2, 0.5% (v/v) TX-100, 1 mM phenylmethylsulfonylfluoride, 1 α,protinin, and 50 μg/ml soyan trypsin inhibitor), and immunoprecipitations were performed as described (28). Anti-serum D5 was used for immunoprecipitation of neurophysin-containing proteins. Where indicated, endoglycosidase H digests of the immunoprecipitated were performed with Endoglycosidase H (Boehringer Mannheim Biochemicals) for 32 h at 37 °C in digestion buffer (50 mM sodium citrate, pH 5.5, 0.2% SDS). Immunoprecipitated proteins were analyzed by 10% Tricine-SDS-PAGE (29). Routinely, nonreducing SDSPAGE was performed, because the vasopressin prohormone and its products gave sharper bands and thus a better separation under nonreducing conditions. Because the MW-SDS-17S marker (Sigma) used as a molecular mass marker was defined for reducing gels, we first show one gel run under reducing conditions to prove that the absence of reduction does not change the migration positions of the vasopressin prohormone products compared with the marker. In addition, because the separation between the vasopressin prohormone and the slightly slower migrating background band from the medium was better on the reducing gels, we used reducing SDS-PAGE when we wanted to focus at the vasopressin prohormone (in case of the endoglycosidase H digests). Protein gels were dried, and radioactive bands were detected by analysis on a BAS1000 phosphoimagier (Fujix).

Regulated Secretion of Neurophysin and Vasopressin—Regulated secretion was analyzed for radiolabeled and nonradiolabeled proteins. For analysis of radiolabeled proteins, cells were cultured and metabolically labeled for 24 h, the medium was removed, and cells were washed twice and incubated for 10 min at 37 °C in buffer (25 mM HEPES, pH 7.4, 125 mM NaCl, 4.8 mM KCl, 1.4 mM MgCl2, 10 mM glucose) with or without 3 mM barium chloride (21). Proteins were recovered from cells and incubation buffer by immunoprecipitation. For analysis of nonradiolabeled proteins, the regulated secretion assay was the same, but the incubation buffer from two wells was pooled, and vasopressin was extracted as described (30). The amount of vasopressin was determined by a radioimmunooassay using the W1 antiserum, recognizing primarily the processed form of vasopressin (25). Only in the absence of processed vasopressin, W1 also recognizes some vasopressin precursor (this paper).

To facilitate the use of the vasopressin prohormone, we used reducing SDS-PAGE when we wanted to focus at the vasopressin prohormone by W1 in a radioimmunoassay to be at least 45 times less (<2.2%) than that of processed vasopressin (data not shown).

Immunofluorescence—Cells were grown on coverslips and transiently transfected for 48 h. Fixation and immunofluorescence were performed as described (31, 32). Immunofluorescence was performed with a 1:1000 dilution of the anti-rabbit primary antiserum, followed by a 1:300 dilution of fluorescein-conjugated anti-rabbit immunoglobulins (Jackson ImmunoResearch laboratories) and with a 1:50 dilution of the 1D3 monoclonal hybridoma supernatant or 1:4000 dilution of NM4, followed by a 1:2400 dilution of Cy3-conjugated donkey anti-mouse immunoglobulins (Jackson ImmunoResearch Laboratories). The coverslips were mounted with DABCO/Mowial and examined with a 63× planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, The Netherlands) interfaced with a Leica TCS4D confocal scanning laser microscope (Leica, Heidelberg, Germany). For each photograph a series of eight recordings of one section through the cell was averaged. To detect the presence of nontransfected cells after single staining with fluorescein, auto-immunofluorescence was recorded in the cy3 channel at high detector amplifications.

RESULTS

Mutant Vasopressin Prohormones Exhibit Reduced Processing and Secretion—We selected five familial neurohypophysial diabetes insipidus mutations (two mutations of the Glu47 residue of neurophysin implicated in binding of vasopressin (NP47E→G and NP473E)) and three mutations of glycine residues in neurophysin (NP14G→R, NP57G→S, and NP65G→V) (Fig. 1B) and stably expressed wild type and mutant prohormone genes in the neuroblastoma cell line Neuro-2A. After metabolic labeling and immunoprecipitation with an antiserum against neurophysin, we obtained wild type proteins of 20 and 10.6 kDa (Fig. 2A, lanes 3 and 4), correlating well with the calculated molecular masses for the glycosylated vasopressin prohormone (approximately 20 kDa) and the intermediate processing product vasopressin-neurophysin (11.2 kDa). Indeed, immunoprecipitation with a panel of antibodies against different moieties of the prohormone, demonstrated that the 20-kDa protein contained vasopressin (VP), neurophysin (NP), and glycopeptide (GP), whereas the 10.6-kDa protein contained only VP and NP. In addition, a minor labeled protein of 9 kDa, correlating well with the calculated mass for neurophysin (9.8 kDa), was recovered for the wild type (Fig. 2A, lane 3). Whereas the prohormone and VP-NP were present in both cells and medium, the 9.8-kDa protein was retained intracellularly.

The data show that in Neuro-2A cells, the wild type human vasopressin prohormone was efficiently processed to VP-NP but...
Retention and Sorting of Mutant Vasopressin Prohormones

FIG. 1. Schematic representation of the vasopressin prohormone and the established diabetes insipidus mutations. A, schematic representation of the vasopressin prohormone structure. The three moieties of the prohormone (VP, NP, and GP) as well as the sequences of the cleavage sites (GKR and R) and the position of the glycan (lollipop symbol) are indicated. The disulfide organization and known secondary elements are included (respectively, on top and below the bar representing the prohormone) (40, 41). →, β-strand; ⋄, α helix; ——, loop. B, the established diabetes insipidus mutations and their position within the vasopressin preprohormone. The 28 different FNDI mutations identified in man (8–17) are positioned in the preprohormone. The five mutations studied in this paper are in italics and marked with asterisks. According to convention in nomenclature of the FNDI mutations (8), amino acids of each moiety of the vasopressin preprohormone were numbered separately. Thus, the abbreviation G14R underneath the NP moiety indicates the substitution of the G at position 14 of NP by an R. The Δ indicates the deletion of the indicated residue(s). SP indicates the signal peptide.

only to a very limited extent to fully processed neurophysin. As for many cell lines, the prohormone and the incompletely processed product were partially secreted via the constitutive pathway.

The fate of the mutant prohormones differed considerably from that of the wild type prohormone in two aspects. First, processing of the mutant prohormones to VP-NP was severely impaired. Second, the amount of neurophysin-containing protein secreted was strongly reduced (Fig. 2A, even lanes). As a result, most of the mutant prohormone remained in the cells in an unprocessed form.

Processing and secretion were impaired to different extents among the different mutants. The reduction in processing and secretion was largest for the NP14G→R and NP65G→V mutations in which both processing to VP-NP and secretion of prohormone into the medium were virtually absent (Fig. 2A, lanes 5, 6, 13, and 14). The NP57G→S mutant displayed a relatively efficient processing and secretion (Fig. 2A, lanes 11 and 12), and NP47E→G and NP47ΔE showed an intermediate phenotype (Fig. 2A, lanes 7–10).

Biosynthesis of Neurophysin Is Impaired for Mutant Vasopressin Prohormones—Neuro-2A cells express low levels of the (neuro)endocrine-specific prohormone convertases PC1 and PC2 (33), both of which are expressed in the supraoptic and paraventricular nuclei of the hypothalamus (34, 35). Because this might be the cause of the incomplete processing observed for the wild type vasopressin prohormone, we stably transfected wild type and mutant prohormones in PC12/PC2 cells, stably expressing mouse PC2 (21). Three wild type neurophysin-containing proteins, migrating with apparent molecular masses of approximately 20, 10.5, and 8.5 kDa, were obtained (Fig. 3A, lane 3) and identified as respectively the prohormone, VP-NP, and NP (Fig. 3B). Although processing of the wild type prohormone was not complete in the PC12/PC2 cells, a significant amount of neurophysin was produced (Fig. 3A, lane 1 and 2). In contrast to the prohormone and the VP-NP intermediate product that were efficiently secreted via the constitutive secretory pathway, neurophysin was mainly stored intracellularly, suggesting its transport to the regulated secretory pathway.

For the mutant prohormones, constitutive secretion was reduced (Fig. 3A, even lanes), and the severity of this reduction followed the same order as observed in the Neuro-2A cells (NP65G→V > NP14G→R > NP47ΔE > NP47E→G > NP57G→S). With the exception of the NP14G→R and NP65G→V mutants, processing to neurophysin was observed for the mutant prohormones, and this fully processed product was selectively retained intracellularly. However, the efficiency of neurophysin formation was diminished, and a clear
The biosynthesis and storage of the fully processed neurophysin proteins (Fig. 4, A and B) suggested a delay in secretion, with the maximum amount of neurophysin occurring rapidly in the absence of a stimulus, consistent with this secretion being constitutive. 

Processing and Secretion Are Delayed for Mutant Prohormones—To establish the time course of processing and secretion, pulse-chase experiments were performed in PC12/PC2 cells for the wild type, one moderately deficient (NP47E→G) and one severely deficient (NP65G→S) prohormone. The wild type prohormone was rapidly processed to VP-NP, which was followed by conversion to neurophysin (Fig. 4A). Secretion of prohormone and VP-NP, and to a smaller extent neurophysin, into the medium occurred rapidly in the absence of a stimulus, consistent with this secretion being constitutive. 

The moderately deficient NP47E→G prohormone demonstrated a delay in secretion, with the maximum amount of prohormone in the medium appearing only after 4 h of chase as compared with 1 h for the wild type (Fig. 4, A and B). In addition, processing of this mutant prohormone displayed a similar delay. Whereas the amount of wild type VP-NP in cells and medium is maximal after 1 h of chase, for the NP47E→G prohormone this point is only reached after 4 h of chase. Even so, the first neurophysin appeared after 20 h of chase for the mutant, as compared with 4 h for the wild type (Fig. 4, A and B).

The severely deficient NP65G→V prohormone was neither secreted nor processed during the 20-h chase period, as indicated by the lack of appearance of prohormone in the medium and the lack of biosynthesis of the mutant VP-NP and VP proteins (Fig. 4C). Despite this, only a small amount of prohormone remained after 20 h of chase, indicating a slow intracellular degradation of the mutant prohormone. Based on the data it is concluded that the investigated mutations either delay or abrogate processing and secretion of the mutant prohormones.

Evoked Secretion of Mutant Neurophysin-containing Proteins—The biosynthesis and storage of the fully processed neurophysin of several mutants suggested that the mutant prohormones were sorted at least in part to the regulated secretory pathway in PC12/PC2 cells. To test this, regulated secretion was stimulated in metabolically labeled cells expressing either the wild type or the NP47E→G mutant prohormone. Because PC12 cells do not always contain sufficient amounts of voltage-gated calcium channels (36), secretion was induced by 3 mM BaCl2. Barium ions permeate the cell easier than calcium ions and once inside can substitute for calcium in inducing release of secretory granules (37). BaCl2 evoked secretion of approximately 50% of wild type prohormone, VP-NP and NP (Fig. 5A,
Mones are secreted upon stimulation.

...of BaCl$_2$.

...secretory granules, indicating that the mutant prohormone (the VP-NP intermediate and neurophysin) had reached the... secretory granules, indicating that the mutant prohormone...retained elsewhere in the cell.

...processed VP are very low, are VP-containing proteins also recognized (compare lane 6 with lanes 8, 10, and 12). Iodinated rat NP and VP were used as additional molecular mass markers (lanes 13 and 14).

These results demonstrate that all three wild type neurophysin-containing proteins were present in secretory granules after 24 h of labeling of PC12/PC2 cells. However, in cells expressing the NP47E→G mutant, only the processed products (the VP-NP intermediate and neurophysin) had reached the secretory granules, indicating that the mutant prohormone was retained elsewhere in the cell.

**Biosynthesis and Evoked Secretion of Vasopressin**—The processing of VP-NP to neurophysin after sorting of wide type and mutant prohormones to the secretory granules of the PC12/PC2 cells (Figs. 3A and 5A) strongly suggested the biosynthesis of correctly processed vasopressin hormone in the regulated secretory pathway of these cells. To investigate this, metabolically labeled cells were subjected to stimulated secretion. Cells expressing wild type prohormone responded by release of considerable amounts of vasopressin (Fig. 5B, lane 6). This release reflected regulated secretion of secretory granules as indicated by the failure of the cells to secrete vasopressin without stimulation (Fig. 5B, lane 4). In addition, smaller quantities of vasopressin were secreted upon stimulation of cells expressing the NP57G→S mutant prohormone (Fig. 5B, lane 12). No evoked secretion of vasopressin could be detected for the NP47 mutant prohormones with this method (Fig. 5B, lanes 8 and 10).

To lower the detection level for vasopressin, a radioimmunooassay was employed (Fig. 6). Confirming our previous results, the wild type prohormone gave rise to evoked secretion of considerable amounts of vasopressin (Fig. 6, left panel). For all mutant prohormones, secretion of detectable amounts of vasopressin could be evoked. However, the levels of secretion were strongly reduced (Fig. 6). These data demonstrate that the mutant prohormones give rise to the biosynthesis and secretory granule storage of (low amounts of) the vasopressin hormone.

**Mutant Vasopressin Prohormones Are Retained in the Endoplasmic Reticulum**—Our results demonstrated that the mutant vasopressin prohormones were retained intracellularly (Figs. 2A and 3A) in compartments other than the secretory granules (Fig. 5A). To identify the retention compartment, prohormones were analyzed by digestion with endoglycosidase H. The majority (81%) of the intracellular form of the wild type vasopressin prohormone was resistant to endoglycosidase H digestion (Fig. 7A, lanes 1 and 2), demonstrating that most of the wild type prohormone in the cell resided in post-endoplasmic reticulum compartments after an 18-h labeling period. In contrast, the intracellular forms of all mutant prohormones were present in the ER, as evidenced by their sensitivity to endoglycosidase H digestion (Fig. 7A, lanes 3–12). Only for the least deficient mutant (NP57G→S), a minority of the prohormone (23%) was resistant, demonstrating partial exit from the ER. The endoglycosidase H resistance of the small amounts of mutant prohormones that reached the medium (Fig. 7B) excluded that the observed sensitivity of the intracellular mutant prohormones was due to an aberrant folding of the prohormones, preventing maturation of the glycan. Based on these data, it is concluded that mutant vasopressin prohormones are retained in the endoplasmic reticulum.

**Mutant Vasopressin Prohormones Display an Enhanced Tendency to Form Large Clusters of Accumulation in the Endoplasmic Reticulum**—The cells stably expressing a mutant vasopressin prohormone did not exhibit a decreased viability or cell growth, nor did ER retention of the prohormone result in any obvious abnormalities of this organelle (data not shown). To mimic the very high expression levels of the vasopressin prohormone in the magnocellular neurons of the hypothalamus, wild type, and mutant prohormones were overexpressed by transient transfection of Neuro-2A cells. Immunostaining with an antiserum against NP resulted in a diffuse staining throughout the cell body for the wild type prohormone (Fig. 8A). In contrast, in approximately half of the cells expressing the NP14G→R, the NP47E→G, the NP47E, or the NP57G→S mutant prohormone, the NP immunoreactivity concentrated in large oval structures within the cytoplasm of the cells (Fig. 8A). Similar accumulations were uncommon for the wild type prohormone, occurring in only 1–5% of the transfected cells. Expression of the NP65G→V mutant protein resulted in an intermediate phenotype with 25% of the transfected cells displaying accumulations of a smaller size (Fig. 8A).

Because our data demonstrated retention of mutant vasopressin prohormones in the ER of stably transfected cells (Fig. 7), we investigated whether the accumulations were present in the ER. Double staining of cells transiently expressing mutant prohormone with an antiserum against NP and an antibody against the ER marker protein-disulfide-isomerase (PDI), revealed colocalization of both immunoreactivities (Fig. 8B, left three panels). Furthermore, colocalization of immunoreactivity...
was absent when co-staining was performed with an antibody against a plasma membrane-associated protein (Fig. 8B, right three panels), excluding that the accumulates bind any antibody specifically. Thus, the data shown in the upper two panels of Fig. 8B, demonstrated that mutant prohormones form large accumulations in the ER of highly expressing cells. In addition, not only the distribution of the mutant prohormone within the ER was aberrant but also the distribution of the ER marker PDI changed. In untransfected cells, the PDI immunoreactivity was digested with endoglycosidase H, and treated and untreated immunoprecipitates were analyzed by reducing 10% Tricine-SDS-PAGE.

FIG. 7. Mutant vasopressin prohormones are retained within the endoplasmic reticulum. PC12/PC2 transfectants were metabolically labeled, and neurophysin-containing proteins were immunoprecipitated from cell lysates (A) and media (B). Half of the immunoprecipitate was digested with endoglycosidase H, and treated and untreated immunoprecipitates were analyzed by reducing 10% Tricine-SDS-PAGE.

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FIG. 6. PC12/PC2 cells expressing mutant vasopressin prohormones synthesize vasopressin and secrete it in a regulated manner. PC12/PC2 transfectants were washed and incubated in buffer without BaCl₂ (− stimulation) followed by incubation in buffer with BaCl₂ (+ stimulation). Levels of vasopressin hormone secreted were determined by radioimmunoassay. The figure gives the amount of VP relative to the total amount of cellular protein in the incubated wells. The bars represent averages ± S.D. for two (wild type) or three (mutants) independent experiments. Note the large difference in scale of the y axis for the wild type and mutant prohormones.

FIG. 7. Mutant vasopressin prohormones are retained within the endoplasmic reticulum. PC12/PC2 transfectants were metabolically labeled, and neurophysin-containing proteins were immunoprecipitated from cell lysates (A) and media (B). Half of the immunoprecipitate was digested with endoglycosidase H, and treated and untreated immunoprecipitates were analyzed by reducing 10% Tricine-SDS-PAGE.

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DISCUSSION

Human FNDI displays two features that are unexpected for a disease caused by a defective prohormone: the disease is dominant and the onset of the symptoms is delayed. In order to investigate the cause of these peculiarities, we examined the intracellular transport and processing of five different vasopressin prohormones containing an established diabetes insipidus mutation. Our data demonstrate, first, that mutant diabetes insipidus prohormones are largely retained in the ER (see Fig. 9 for a schematic summary) (38, 39). Second, sorting of the small amount of mutant prohormone that escapes the ER was not impaired (Fig. 9) (38). Release of vasopressin and neurophysin from cells expressing mutant prohormones was normally evoked by stimulation of secretion, indicating that the mutant vasopressin prohormones entered secretory granules and did not disturb the regulated secretory pathway of the cells. Third, very high expression of diabetes insipidus mutant prohormones in neuroblastoma cells resulted in large accumulations (accretions) of prohormone in the ER of the cells and in an aberrant morphology of this organelle. We propose that these accretions and the consequent disturbance of the ER are deleterious to the cell and will decrease functionality and/or viability of the magnocellular neurons, which express high amounts of vasopressin prohormone in vivo. This hypothesis would explain both the dominant inheritance of human FNDI and the delayed onset.

Large differences were observed between the five investigated mutant prohormones with respect to exit from the ER. Mutation of residues NP14G or NP65G, which are both located in one of the β pleated sheets of the neurophysin moiety (Fig. 1), virtually abolished exit from the ER. A similar result was obtained for the mutant NP17G→V (38). Mutation of NP47E, which is located in the α helix of neurophysin (Fig. 1), exhibited a less severe phenotype, and the least efficient ER retention was observed after substitution of residue NP57G, which is located in the loop connecting the α helix with the C-terminal β pleated sheet of neurophysin (Fig. 1) (39). The order of severity of ER retention for the different vasopressin prohormones suggests that mutations in the β-strands of neurophysin are most deleterious for folding of the vasopressin prohormone, followed by mutations in the α-helix and then by mutations in less ordered structures like the loop from residues 50–58. The high impact of mutation of the β-strands might be due to the high β pleated sheet content of neurophysin and thus the large impor-
The NP47E residue binds the protonated N terminus of vasopressin (40, 41). Because this association enhances dimerization of the neurophysin molecule (42, 43), it has been implicated in sorting of the vasopressin prohormone to the regulated secretory pathway, which is accompanied by prohormone aggregation in the trans-Golgi network (42, 44). Indeed, vasopressin-neurophysin association can also occur within the unprocessed prohormone and has an pH optimum close to the pH of the trans-Golgi network (3, 4). Despite the crucial importance of NP47E in binding of vasopressin and in this manner enhancing the dimerization (and multimerization) of neurophysin, the small percentage of NP47

\[ \text{NP47E} \]

and NP47E

\[ \text{NP47E} \]

prohormone that escaped ER retention was correctly sorted to the regulated secretory pathway. Both processing to VP-NP and NP and regulated secretion of these processing products were observed,
indicating location of these products in secretory granules. We conclude that efficient association of vasopressin with neurophin is not an absolute prerequisite for targeting of the vasopressin prohormone. This suggests that (homo)aggregation is not absolutely required for sorting. Alternatively, however, dimerization or multimerization of the prohormone might not be tightly coupled to aggregation in the trans-Golgi network. A discrepancy between the ability to multimerize and the ability to aggregate has been observed for proinsulin. A proinsulin mutant that failed to form dimers or hexamers was able to aggregate at high concentrations in the presence of Ca\(^{2+}\) and was correctly sorted to the regulated secretory pathway in the presence of extracellular Ca\(^{2+}\) (45).

Expression of mutant vasopressin prohormones to very high levels induced large accumulations in the ER, which effected an aberrant ER morphology (Fig. 8). Similar accretions have been observed in vivo in the magnocellular neurons of rat expressing a mutant prohormone resulting from the nonhomologous crossing over of the vasopressin and oxytocin genes and have been established to consist of accumulations of globular aggregates in dilated sacculles of the rough ER (46, 47). This indicates that the accretions can form not only in cells in culture but also in the intact animal. We hypothesize that these accretions are deleterious to the magnocellular neurons that highly express the accretions can form not only in cells in culture but also in a mutant prohormone resulting from the nonhomologous crossing over of the vasopressin and oxytocin genes. A discrepancy between the ability to multimerize and the ability to aggregate has been observed for proinsulin. A proinsulin mutant that failed to form dimers or hexamers was able to aggregate at high concentrations in the presence of Ca\(^{2+}\) and was correctly sorted to the regulated secretory pathway in the presence of extracellular Ca\(^{2+}\) (45).

other neurodegenerative diseases in which protein aggregates are present as a consequence of altered protein structure, e.g., Alzheimer's disease and prion disease.

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FIG. 9. Schematic representation of the intracellular transport and secretion of wild type and mutant vasopressin prohormones expressed in cell lines. Different transport/processing steps are indicated by arrows. The large difference observed in efficiency of exit from the ER for wild type and mutant prohormones is represented by two separate arrows for this process. The boundaries of the cell are indicated by a double line. The partial sorting of regulated secretory proteins to the constitutive secretory pathway is only observed in cell lines and not in vivo. Note that the only transport/processing step for which we could find a clear difference between mutant and wild type vasopressin prohormones is the export from the ER.