Mutants of Neuroserpin That Cause Dementia Accumulate as Polymers within the Endoplasmic Reticulum*

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The dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB) is caused by the accumulation of mutant neuroserpin within neurons (Davis, R. L., Shrimpton, A. E., Holohan, P. D., Bradshaw, C., Feiglin, D., Sonderegger, P., Kinter, J., Becker, L. M., Lachawan, F., Krasnewich, D., Muenke, M., Lawrence, D. A., Yerby, M. S., Shaw, C.-M., Goopli, B., Elliott, P. R., Finch, J. T., Carrell, R. W., and Lomas, D. A. (1999) Nature 401, 376–379), but little is known about the trafficking of wild type and mutant neuroserpins. We have established a cell model to study the processing of wild type neuroserpin and the Syracuse (S49P) and Portland (S52R) mutants that cause FENIB. Here we show that Syracuse and Portland neuroserpin are retained soon after their synthesis in the endoplasmic reticulum and that their accumulation is linked to their inability to be secreted. This leads us to propose a model in which the limiting step in the plasma membrane, and regulating emotional behavior and memory (6–10). We have therefore established a cell model that recapitulates the secretion of neuroserpin and have used it to examine the effects of the Syracuse (S49P) and Portland (S52R) mutations that cause FENIB (11, 13). Our data show that both mutations cause the retention of neuroserpin as polymers.

Neuroserpin is a member of the serpin superfamily and is predominantly expressed by neurons of the developing and adult brain. This serpin is secreted from the axonal growth cones of the central and peripheral nervous system, where it inhibits the enzyme tissue plasminogen activator (1–5). The expression pattern of neuroserpin and its in vitro inhibitory activity implicate this serpin in regulating axonal growth, reducing seizure activity, controlling damage in cerebral infarction, and regulating emotional behavior and memory (6–10).

We have recently described an autosomal dominant demen-

tia, familial encephalopathy with neuroserpin inclusion bodies (FENIB), that is characterized by inclusions of mutant neuroserpin within cortical and subcortical neurons (11–13). This dementia is unusual in that the inclusions result from the retention of ordered polymers of neuroserpin, most likely within the endoplasmic reticulum (ER) of neurons (11). Moreover, the number of inclusions is directly related to the molecular instability caused by the mutation and inversely proportional to the age of onset of dementia (13). For example, the Syracuse mutation (S49P) causes dementia in middle age, whereas the more severe Portland mutant (S52R) causes more inclusions and an onset of dementia in the early 20s. Polymers of the serpins result from the sequential linkage between the reactive center loop of one molecule and the β-sheet A of a second serpin molecule (4, 11, 14–17). We have recently demonstrated that recombinant Syracuse neuroserpin (S49P) forms polymers 13-fold faster than wild type neuroserpin in vitro (4), but little is known about other neuroserpin mutants.

The polymers that form in FENIB are identical to those that are formed by a mutant of another member of the serpin superfamily, the Z variant of α1-antitrypsin, in the ER of hepatocytes in association with liver disease (14). This common mechanism of disease has allowed us to group these conditions and others that result from polymerization of serpin mutants, angio-edema (C1-inhibitor), thrombosis (antithrombin), and emphysema (α1-antichymotrypsin), as serpinopathies (18–20). The serpinopathies can be classified as “conformational diseases” that arise when proteins undergo self-association and tissue deposition (21). Serpinopathies differ from other conformational diseases in that the accumulated proteins are contained within the ER (22), which also qualifies the serpinopathies as “ER storage diseases” (23). It remains unclear how the ER handles ordered serpin polymers that, in contrast to nonpolymerogenic mutant secretory proteins, seem not to elicit the unfolded protein response (24).

Although we mechanistically understand the biochemical abnormality that underlies the polymerization of neuroserpin, there is no information on the synthesis, transport, or degradation of either the wild type or the mutant proteins. The point at which mutant neuroserpin is retained within neurons in individuals with FENIB has been deduced from microscopic analysis of intraneuronal inclusions rather than from direct studies of trafficking of the wild type or the mutant protein (12). We have therefore established a cell model that recapitulates the secretion of neuroserpin and have used it to examine the effects of the Syracuse (S49P) and Portland (S52R) mutations that cause FENIB (11, 13). Our data show that both mutations cause the retention of neuroserpin as polymers.

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† The abbreviations used are: FENIB, familial encephalopathy with neuroserpin inclusion bodies; ER, endoplasmic reticulum; PBS, phosphate-buffered saline.
with the ER and that mutant and wild type neuroserpin are only partially degraded by proteasomes. Moreover, the faster aggregation and slower secretion of Portland neuroserpin, when compared with the Syracuse mutant, explains the difference in the severity of the clinical phenotypes.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—**Reagents, buffers, culture media, and serum for cell cultures were purchased from Sigma. Restriction enzymes and DNA molecular weight markers for cloning and analysis were from New England Biolabs (Wilbury Way, Hitchin, UK). The rabbit polyclonal anti-neuroserpin antiserum was produced by Abcam (Cambridge, UK). Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum. The anti-neuroserpin antiserum was detected by immunocytochemistry with an anti-neuroserpin polyclonal antibody. All three forms of neuroserpin pro-}

**Construction of Neuroserpin Expression Plasmids—**The cDNAs for human wild type and Syracuse (S49P) neuroserpin (4), including the secretion signal peptide, were cloned into the pTRE2-hyg vector from Clontech (BD Biosciences, Oxford, UK). pTRE2-hyg is part of the TetOn/TetOff system, but leaky expression from the tetracycline-sensitive promoter allows the use of this vector in COS-7 cells in the absence of the transactivator component of the system) using the BamHI and ClaI sites. The Portland mutation (S52R) was introduced into the wild type plasmid by site-directed mutagenesis with the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The cDNAs for wild type and both mutant neuroserpins contained in the expression plasmids were fully sequenced to ensure that there were no PCR errors.

**Cell Cultures and DNA Transfections—**COS-7 cells were maintained in a incubator and Yorkshire (Invitrogen) in serum-free Opti-MEM culture medium (Invitrogen) following the protocol recommended by Invitrogen. For the co-expression experiments, the cells were transfected in the same way with a mixture of 2 μg of wild type neuroserpin plus 2 μg of Syracuse or Portland neuroserpin, and parallel control cells were transfected with 2 μg of either wild type, Syracuse, or Portland neuroserpin alone.

**Immunocytochemistry for Fluorescence and Confocal Microscopy—**Cells were fixed in 6-well plates and dried on coverslips pre-treated with poly-L-lysine and transfected as described above. At 12, 24, 48, and 72 h after transfection, cells were washed, fixed in ice-cold 4% (w/v) paraformaldehyde for 30 min at room temperature, washed again, and blocked for 60 min with phosphate-buffered saline (PBS) containing 10% (w/v) bovine serum albumin, 0.1% (v/v) Triton X-100, and 0.1% (w/v) sodium azide, and then incubated in primary antibody for 90 min. Cells were washed again, incubated with the secondary antibody for 60 min, washed, and mounted in Fluorosave (Calbiochem, through CN Biosciences, Nottingham, UK), containing 2% (w/v) 1,4-diazabicyclo-[2.2.2]octane (Sigma) and 3 μg/ml 4,6-diamidino-2-phenylindole (Sigma). Cells for confocal microscopy were stained with anti-neuroserpin and donkey polyclonal anti-rabbit IgG (Texas Red), anti-goat IgG (fluorescein isothiocyanate), and anti-mouse IgG (fluorescein isothiocyanate) antibodies were used at 1.3 μg/ml.

**SDS and Nondenaturing PAGE and Western Blot Analysis—**The cell pellet from each well of 6-well plates was lysed in 20 μl of Nonidet lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% (v/v) Nonidet P-40, and 2 mg/ml protease inhibitor mixture (Roche Applied Science)) and sonicated. The lysates were centrifuged at 20,000 rpm and 4 °C for 15 min. The supernatants were recovered in SDS-PAGE loading buffer by heating for 5 min at 95 °C and then analyzed by 8% (w/v) acrylamide SDS-PAGE (25). Nondenaturing PAGE was performed on 7.5% (w/v) acrylamide non-denaturing gels using 2% loading buffer without SDS and β-mercaptoethanol. The proteins were transferred from the gels onto Immobilon P membrane (Millipore Corp., Bedford, MA) at 200 mA for 2 h for Western blot analysis. 20% (v/v) methanol was added to the transfer buffer for gels that had been run in SDS. After transfer, the membrane was washed in PBT (PBS plus 0.1% (v/v) Tween 20) and blocked overnight in PBT plus 5% (w/v) dried skimmed milk powder. The following day, the membrane was incubated with anti-neuroserpin antibody diluted 1:25,000 in PBT-milk for 105 min, washed six times for 5 min with PBT, and then incubated with 1:1,100 anti-rabbit IgG-horseradish peroxidase antibody in PBT-milk for 105 min. The membrane was washed a further six times for 5 min with PBT and 15 min in PBS before developing using the ECL Western Fermo maximum sensitivity substrate (Pierce) and exposed to film.

**Polymerization of Neuroserpin Mutants within the ER**

Metabolic Labeling and Immunoprecipitation—One day after transfection, cells were starved in 1 ml of methionine and cysteine-free Dulbecco’s modified Eagle’s medium for 1 h, pulsed with 15 min with 1 MBq/well RediVue Promix (Amersham Biosciences) containing 35S-labeled methionine and cysteine, and then harvested or washed with cold PBS and cultured in 1 ml of chase medium (Dulbecco’s modified Eagle’s medium containing 200 μM methionine and 200 μM cysteine) for 3 or 6 h. For the pulse-chase time course, the cells were chased for 0, 1, 3, and 6 h in the same chase medium supplemented with 10% (v/v) fetal bovine serum. For the protein degradation experiments, the cells were treated with 25 μM lactacystin (an irreversible proteasome inhibitor) or 200 μM proteasome inhibitors (Sigma) for 6 h (Sigma). The supernatants were recovered in SDS-PAGE loading buffer by heating for 5 min at 95 °C, and then analyzed by 8% (w/v) acrylamide SDS-PAGE using 10% (v/v) β-mercaptoethanol and boiled for 5 min at 95 °C, and then analyzed by 8% (w/v) acrylamide SDS-PAGE using 10% (v/v) β-mercaptoethanol and boiled for 5 min at 95 °C. The inhibitors were added to the cultures 1 h prior to starvation and kept in all subsequent steps until harvesting. After the chase period, the culture medium was collected and centrifuged at 2000 rpm and 4 °C for 5 min, and the cells were harvested by scraping with 0.15 ml/well Nonidet lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% (v/v) Nonidet P-40) containing a protease inhibitor mixture (Complete; EDTA-free protease inhibitor mixture tablets (Roche Applied Science)) plus 15 μg pepstatin A (Calbiochem, through CN Biosciences, Nottingham, UK), scarping and vortexing gently for 3 s, and then centrifuging at 16,000 rpm for 5 min. Samples of 0.5 ml of supernatant were used for trichloroacetic acid precipitation and scintillation counting of total incorporated radioactivity. The supernatants from culture medium and cell lysate samples were precleared with rabbit IgG bound to 45 μl of 50% (v/v) Protein A-Sepharose for 30 min at 4 °C, and then neuroserpin, cyclin B1, or calsepin D were immunoprecipitated overnight at 4 °C with their specific antibodies prebound to Protein A-Sepharose (45 μl of 50% of (v/v) Protein A-Sepharose plus 1 μg of purified antibody at 4 °C for 2 h). The following day, immunocomplexes were washed four times with cold washing buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% (v/v) Nonidet P-40) and once with cold PBS. For endoglycosidase H treatment, the immunocomplexes were resuspended in 5% (v/v) 1,4-diazabicyclo[2.2.2]octane (Sigma) and 3 μg/ml 4,6-diamidino-2-phenylindole (Sigma). Cells for confocal microscopy were stained with anti-neuroserpin and donkey polyclonal anti-rabbit IgG (Texas Red), anti-goat IgG (fluorescein isothiocyanate), and anti-mouse IgG (fluorescein isothiocyanate) antibodies were used at 1.3 μg/ml.

**RESULTS**

**Mutant Syracuse and Portland Neuroserpin Accumulate within Transiently Transfected COS-7 Cells**—In order to establish a cellular model for FENIB, we cloned human wild type neuroserpin and the neuroserpin mutants Syracuse and Portland into the mammalian expression vector pTRE2-hyg. These plasmid DNAs were used for transient transfection of COS-7 cells, with an estimated transfection efficiency of 30–40%. Expression of wild type, Syracuse, and Portland neuroserpin was detected by immunocytochemistry with an anti-neuroserpin polyclonal antibody. All three forms of neuroserpin produced a reticular staining pattern 12 h after transfection in keeping with protein localization within the ER (Fig. 1, a–c and m), and wild type neuroserpin often also showed an additional and stronger perinuclear staining compatible with its presence in the Golgi complex (Fig. 1a). After 24 h, the signal was stronger, and neuroserpin could be clearly detected as punctate

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accumulations in cells transfected with either Syracuse or Portland, but not wild type, neuroserpin (Fig. 1, d–f and m). In the case of Portland neuroserpin, the level of accumulation after 24 h was already significantly greater than for wild type neuroserpin. The accumulation increased for both mutants after 48 and 72 h of culture (Fig. 1, h, i, k, and l) such that 70–90% of transfected cells contained retained neuroserpin. In comparison, wild type neuroserpin still gave the same ER staining pattern (Fig. 1, g, j, and m). The intensity of staining of wild type neuroserpin decreased after 72 h of culture, suggesting that a significant amount of the protein had been secreted.

**Syracuse and Portland Neuroserpin Are Secreted More Slowly than the Wild Type Protein**—Neuroserpin is a secreted glycoprotein. During biosynthesis, neuroserpin is translocated into the ER, where its signal peptide is cleaved and the protein moiety is N-glycosylated. Human neuroserpin has three potential N-glycosylation sites within its amino acid sequence (26). Core glycans are trimmed and further processed in the Golgi complex, resulting in a further increase in molecular mass. The predicted molecular mass of nonglycosylated neuroserpin is ~45 kDa, close to the size of the recombinant neuroserpin purified from *Escherichia coli* used in this study as a control. The addition and processing of N-linked glycans along the secretory pathway gradually increases the molecular mass of neuroserpin to 55 kDa (fully processed secreted form). The secretion of wild type, Syracuse, and Portland neuroserpin was assessed in our cell system by SDS-PAGE and Western blot analysis 12, 24, 48, and 72 h after transfection (Fig. 2, Wt, Sy, and Po). After 12 h, there were insufficient quantities of neuroserpin secreted by any of the transfected cells to be detected by Western blot analysis, although we could easily detect 20 ng of recombinant purified neuroserpin in the control lane (Fig. 2, Wt, Sy, and Po). After 12 h, there were insufficient quantities of neuroserpin secreted by any of the transfected cells to be detected by Western blot analysis, although we could easily detect 20 ng of recombinant purified neuroserpin in the control lane (Fig. 2, Wt, Sy, and Po).
Polymerization of Neuroserpin Mutants within the ER

All of the patients with FENIB so far identified are heterozygous for a neuroserpin mutation (13). Thus, wild type neuroserpin is co-expressed with the mutant allele that causes the disease. We therefore assessed the effect of heterozygous co-expression on the secretion of neuroserpin in our COS-7 model by co-transfecting the cells with a 1:1 mix of plasmids encoding either wild type and Syracuse neuroserpin or wild type and Portland neuroserpin. The results were very similar for both co-expression combinations (Fig. 2, Wt + Sy and Wt + Po). The bands for intracellular neuroserpin in co-expressing cells were always stronger than the intracellular signal from cells expressing an equivalent dose of wild type neuroserpin alone. This was particularly striking at the 24-h time point, when cells transfected with 2 μg of wild type neuroserpin DNA showed only a very faint intracellular band, whereas cells co-transfected with 2 μg of wild type plus 2 μg of mutant neuroserpin showed a strong intracellular signal. In the culture medium of cells co-expressing wild type and mutant neuroserpin, the neuroserpin band was clearly detectable after 24 h, at which point it was still difficult to detect a signal in the medium of cells transfected with 2 μg of either Syracuse or Portland neuroserpin alone (data not shown). After 48 and 72 h, more neuroserpin had been secreted into the medium of cells co-expressing wild type and mutant neuroserpin than in the medium of cells expressing only Syracuse or Portland neuroserpin.

We next studied the secretion of wild type and mutant neuroserpin in more detail by pulse-chase experiments (Fig. 3, a and b), transfecting the cells with wild type, Syracuse, or Portland neuroserpin alone or co-transfecting them with wild type plus Syracuse or wild type plus Portland neuroserpin. After a 15-min pulse with [35S]methionine/cysteine, an anti-neuroserpin antibody immunoprecipitated the same three bands from all of the cell lysates (Fig. 3a, chase 0 h). These bands had molecular masses ranging between 45 and 50 kDa and most likely represent singly, doubly, and triply N-glycosylated forms of neuroserpin within the ER, with the largest of the three being the most abundant species. In the successive chase time points at 1, 3, 6, and 15 h after the end of the pulse, immunoprecipitation analysis of the cell lysates showed mainly the same 50-kDa intracellular band for all transfected cells, although its intensity varied depending on the neuroserpin transfection and the chase time. In the case of the cells transfected with mutant neuroserpin alone, a faint and slightly higher molecular weight band could be consistently detected in the cell lysates (most clearly after 1 h of chase), probably corresponding to mutant neuroserpin contained in the Golgi complex. The analysis of the culture media showed a 55-kDa band that corresponded to neuroserpin with Golgi-processed N-glycan chains, which also changed in intensity depending on the DNA transfected and the chase time. In contrast to the Western blot analysis (Fig. 2), the 55-kDa band of secreted neuroserpin can be easily detected for all neuroserpin variants after pulse-chase due to the recovery of all of the neuroserpin protein from the samples by immunoprecipitation and to the higher sensitivity of the detection method for radiolabeled protein. Fig. 3b shows the quantitation of the bands shown in Fig. 3a. In most of the cell lysates (Fig. 3b, upper graph), the amount of labeled neuroserpin was higher after the 1-h chase than after the 15-min pulse, probably because the radiolabeled methionine present within the cells was still incorporated into newly synthesized neuroserpin protein during the first 1 h of chase. We therefore calculated the half-times for intracellular disappearance and extracellular appearance using the sum of neuroserpin present in the cells and the media after the 1-h chase as the 100% radiolabeled neuroserpin. In cells expressing only wild type neuroserpin, the intracellular levels of neuroserpin decreased with a half-time of 1 h, corresponding to the initial fast increase in secreted wild type neuroserpin (half-time 1 h, Fig. 3b, lower graph). After 3 h, the amount of wild type neuroserpin decreased in the culture medium, probably due to extracellular degradation in the serum-containing chase medium, as suggested by the presence of degradation bands after the 15-h chase. The half-time for intracellular clearance of Syracuse neuroserpin was 5.5 h, and the half-time for Portland neuroserpin was around 3 h, whereas the half-times for secretion were around 4.5 h for Syracuse and 11 h for Portland neuroserpin (Fig. 3b). In the samples of secreted mutant neuroserpin, we did not detect any degradation bands even after the 15-h chase (Fig. 3a, Sy and Po M lanes). In the case of
the cells co-expressing wild type and mutant neuroserpin, both conditions (wild type + Syracuse and wild type + Portland) gave similar results. The half-times for intracellular clearance of neuroserpin were 2 h for wild type + Syracuse-expressing cells and 3 h for wild type + Portland-expressing cells (Fig. 3b, CULTURE MEDIA), and the half-times for secretion in both cases were close to 1.5 h (Fig. 3b, CULTURE MEDIA).

In two different sets of experiments, three independent repetitions of the pulse chase with either a 3- or 6-h chase showed that after 3 h, wild type neuroserpin was secreted faster than the mutant forms, but there was no significant difference between Syracuse and Portland neuroserpin after this time (data not shown). However, after 6 h, the difference between the quantities of intracellular and extracellular neuroserpin were significant for all three species (Fig. 3c), with the Portland mutant showing the slowest secretion. In these experiments, an additional lower band was usually detected after the chase both in the cell lysates (47 kDa) and the culture medium (48 kDa) of cells transfected with wild type neuroserpin (Fig. 3c, asterisk). This band probably corresponds to cleaved or partially processed neuroserpin.

**Mutant Neuroserpins Are Retained within the Endoplasmic Reticulum**—Syracuse and Portland neuroserpin were retained longer within the cells and appeared as discrete punctate accumulations on immunocytochemistry (Figs. 1–3). In order to study their cellular localization, the neuroserpin that was immunoprecipitated after a 3-h chase was digested with endoglycosidase H. This enzyme removes N-linked glycans that are still in the high mannose ER form but does not affect oligosaccharide chains after the addition of sialic acid in the Golgi apparatus. Endoglycosidase H treatment did not affect the extracellular neuroserpin bands but reduced all intracellular neuroserpin to a single 45-kDa band (Fig. 4), demonstrating that all intracellular neuroserpin was retained within the ER. The additional 47–48-kDa band seen for wild type neuroserpin (Fig. 4, asterisk) also collapsed to the single 45-kDa band after endoglycosidase H treatment, suggesting that the difference in size between the two wild type bands was due to differences in glycosylation rather than proteolytic cleavage of the 50-kDa band.

To confirm that Syracuse and Portland neuroserpin accumulated within the ER, we performed confocal microscopy on 24-h post-transfection cells using antibodies against the ER-resident protein calreticulin and the Golgi-resident protein GM130 for comparison. All three forms of neuroserpin co-localized with calreticulin (Fig. 5a), demonstrating that wild type and mutant neuroserpin are located within the endoplasmic reticulum 24 h after transfection. In the merged images of Syracuse and Portland neuroserpin, the overlapping neuroserpin/endoplasmic reticulum signal appears orange rather than yellow due to the strong anti-neuroserpin staining of the accumulated mutant.
neuroserpin. Only wild type neuroserpin co-localized also with GM130 (Fig. 5b), whereas the mutant neuroserpins did not co-localize with GM130, suggesting that the transport from the ER to the Golgi is the limiting step in the processing and secretion of Syracuse and Portland neuroserpin.

Mutant Neuroserpins Form Intracellular and Extracellular Polymers—Syracuse and Portland neuroserpin readily form polymers in vivo and in vitro (4, 11). The detection of protein accumulation by immunocytochemistry in cells transfected with mutant neuroserpin prompted us to investigate whether they contained polymers. Nondenaturing PAGE and Western blot analysis of the cell lysates and culture media showed clearly that polymers of Syracuse and Portland neuroserpin were present both in the cells and the culture media after 24, 48, and 72 h of culture (Fig. 6). When either of the mutants was co-expressed with the wild type protein, we detected the same bands seen with cells expressing wild type neuroserpin alone together with polymers identical to those found in cells expressing only mutant neuroserpin (Fig. 6, Wt+Sy and Wt+Po). Wild type neuroserpin polymerized to a much lower degree under the same conditions (Fig. 6, left panels). The two bands in the lysate of cells expressing wild type neuroserpin on nondenaturing gels probably reflect different glycosylation states of the protein or possibly the formation of dimers (Fig. 6, black and white arrow and arrowhead). Only one band was detected in the culture medium of wild type neuroserpin, as expected for fully glycosylated monomeric neuroserpin (Fig. 6, black arrow). When the membranes where exposed for even longer times, a faint band of monomeric neuroserpin was also detected in the culture media of cells expressing the Syracuse and Portland mutants (data not shown).

Neuroserpin Is Partially Degraded by Proteasomes—Mutant proteins retained in the ER are frequently transported to the cytosol and degraded by proteasomes (27, 28). Some mutant proteins are also degraded in lysosomes (29, 30). We performed pulse-chase experiments in the presence of either the proteasome inhibitor lactacystin (31) or the lysosomal protease inhibitor leupeptin (32, 33). The chase period for these experiments was 6 h in order to allow sufficient protein to accumulate within the cells for reliable detection. Incubation of the cells with 200 μM leupeptin had no effect on the accumulation of wild type, Syracuse, or Portland neuroserpin (Fig. 7a, compare lanes marked −leup and +leup for cell lysates and media). In contrast, leupeptin was able to block lysosomal proteolytic maturation of cathepsin D under the same conditions (Fig. 7a, lower panel). Thus, neither wild type nor mutant neuroserpin are degraded by lysosomal proteases in our cell system. When the experiment was performed in the presence of 25 μM lactacystin, the intra- and extracellular amounts of wild type and both mutant neuroserpins increased (Fig. 7b). This was particularly marked for the Portland mutant, which accumulated significantly more than either wild type or Syracuse neuroserpin (graph in Fig. 7b). We conclude that a similar fraction of wild type and Syracuse neuroserpin misfolds in the ER of COS-7 cells and is transported to the cytosol, where it is degraded by proteasomes. Proteasome-mediated turnover is higher for the Portland mutant, which suggests a higher degree of misfolding.

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**Fig. 4. Intracellular neuroserpin is retained within the endoplasmic reticulum.** Endoglycosidase H (Endo-H) digestion of samples from a pulse-chase (3-h) experiment performed under the same conditions described in Fig. 3a. WT, wild type neuroserpin; Sy, Syracuse neuroserpin; Po, Portland neuroserpin; Lu, luciferase control. Arrow, fully glycosylated and secreted neuroserpin (55 kDa); arrowhead, intracellular neuroserpin intermediate (50 kDa); black and white arrow, deglycosylated intracellular neuroserpin (45 kDa).

**Fig. 5. Intracellular localization of wild type, Syracuse, and Portland neuroserpin in COS-7 transfected cells.** a, confocal microscopy of cells cultured for 24 h after transfection and stained for neuroserpin (labeled with Texas Red) and an ER-resident protein, calreticulin (labeled with fluorescein). b, cells in the same conditions stained for neuroserpin (labeled with Texas Red) and a Golgi-resident protein, GM130 (labeled with fluorescein). In the merged images, the yellow color corresponds to areas in which the red and green stainings overlap. The nucleus appears blue due to DNA staining with 4’,6-diamidino-2-phenylindole. Scale bar, 10 μm.
We have established a cellular model of the neurodegenerative disease FENIB that reproduces its main features, in particular the aggregation of mutants of neuroserpin within the cells as polymers. Our cell model shows more marked retention of the Portland mutant, which causes more severe disease in the patients, when compared with Syracuse neuroserpin. Despite its slower secretion, the intracellular half-life of Portland neuroserpin (3 h) was shorter than that of the Syracuse mutant (5.5 h). This can be explained by faster degradation of Portland neuroserpin within the Golgi at steady state, suggesting that the exit from the ER is the limiting step in the trafficking of the mutant proteins.

Within the serpin family, the Z variant of α1-antitrypsin is the archetype of a disease causing polymorphic mutant serpin. The polymerization and retention of Z α1-antitrypsin within the ER of hepatocytes causes liver disease through a toxic gain-of-function mechanism (14, 18, 19). The trafficking of wild type and Z α1-antitrypsin has been extensively studied in cell culture and transgenic mouse models over the past few years (24, 36–40). In a hepatoma cell line stably expressing Z α1-antitrypsin, only 10–17% of the mutant protein is secreted into the culture medium, and the majority of the protein is degraded within 3 h, with less than 1% of the protein accumulating as insoluble aggregates inside the ER (37). In this cell model, most of the newly synthesized Z α1-antitrypsin was found as trimers inside the ER (41). In our cell system, Syracuse and Portland neuroserpin were more efficiently secreted and not degraded to the same extent; a significant amount of the mutant neuroserpin protein could still be detected as higher order polymers within the cells at a time after transfection when the wild type protein was no longer detectable (Fig. 6). A similar situation has been described for the Morioka mutant of antithrombin. This mutant protein is also secreted much more slowly than the wild type protein and not degraded after a 9-h chase but rather accumulates mainly as dimers within ER-derived structures (42).

We have shown here that Syracuse and Portland neuroserpin are secreted more slowly than the wild type protein, which is secreted with a half-time of 1 h, comparable with the secretion of other serpins (more than 90% of radiolabeled α1-antitrypsin is secreted after 30 min (37), and the half-time for wild type antithrombin is 1.5 h (42)). Portland neuroserpin was secreted more slowly (11 h) compared with 4.5 h for Syracuse, in keeping with the severity of the Portland mutant phenotype. Despite its slower secretion, the intracellular half-life of Portland neuroserpin (3 h) was shorter than that of the Syracuse mutant (5.5 h). This can be explained by faster degradation of Portland neuroserpin, since we detected a higher accumulation of Portland neuroserpin in cells treated with the proteasome inhibitor lactacystin. This faster degradation could be due to the structural characteristics of Portland neuroserpin, which may allow a more efficient recognition of the misfolded protein by the ER chaperone machinery, faster transport to the cytosol, or faster degradation by proteasomes. When the mutant neuroserpins were co-expressed with the wild type protein, the simultaneous presence of the wild type protein does not prevent the formation of mutant polymers, which suggests that wild type and mutant neuroserpin do not interact in vivo. This interpretation agrees with previous work from our laboratory, in which we demonstrated that mixing wild type α1-antitrypsin with the highly polymerogenic mutant Z α1-antitrypsin does not affect the polymerization rates of the wild type and mutant proteins (34), and with biochemical analysis that showed the intracellular inclusions from the brain of a patient with FENIB (Collins bodies) to be composed of only mutant neuroserpin (35).
half-times for neuroserpin secretion were reduced to 1.5 h for both combinations, and the intracellular turnover was also faster for Syracuse (2 h instead of 5.5 h), although it did not change for Portland neuroserpin (3 h). These effects can be explained in two different ways: the interaction between the wild type and mutant proteins partially prevent the polymerization of Syracuse and Portland neuroserpin, allowing a faster secretion of small chain polymers or mutant monomers; or alternatively, the signal that we detected both in steady state by Western blot and after the chase of radiolabeled proteins in the culture media is composed of only wild type monomers and mutant polymers, as suggested by our results with native PAGE analysis. We cannot discriminate between these two possibilities. However, the results shown in Fig. 6, the in vitro polymerization rates of wild type and Zα₁-antitrypsin (34), and the finding of only Portland neuroserpin in Collins bodies from heterozygous patients (35) all support the second explanation.

The mechanism of degradation of ER-retained Zα₁-antitrypsin is controversial, with evidence both in favor and against proteasomal degradation (41–45). In addition, a nonproteasomal degradation pathway and autophagy have been proposed as disposal pathways for Zα₁-antitrypsin (34), and the finding of only Portland neuroserpin in Collins bodies from heterozygous patients (35) all support the second explanation.

An intriguing result is the finding of high molecular mass polymers of mutant neuroserpin in the culture media of transfected cells (Fig. 6, M lanes). Wild type neuroserpin was clearly detected in the culture medium as a monomeric band, but both combinations, and the intracellular turnover was also faster for Syracuse (2 h instead of 5.5 h), although it did not change for Portland neuroserpin (3 h). These effects can be explained in two different ways: the interaction between the wild type and mutant proteins partially prevent the polymerization of Syracuse and Portland neuroserpin, allowing a faster secretion of small chain polymers or mutant monomers; or alternatively, the signal that we detected both in steady state by Western blot and after the chase of radiolabeled proteins in the culture media is composed of only wild type monomers and mutant polymers, as suggested by our results with native PAGE analysis. We cannot discriminate between these two possibilities. However, the results shown in Fig. 6, the in vitro polymerization rates of wild type and Zα₁-antitrypsin (34), and the finding of only Portland neuroserpin in Collins bodies from heterozygous patients (35) all support the second explanation.

The mechanism of degradation of ER-retained Zα₁-antitrypsin is controversial, with evidence both in favor and against proteasomal degradation (41–45). In addition, a nonproteasomal degradation pathway and autophagy have been proposed as disposal pathways for Zα₁-antitrypsin (29, 41, 45). All of these may contribute to degradation, but to different extents in different cell lines (46). Recently, Sifers and colleagues have proposed an interesting model to explain the fate of mutant Zα₁-antitrypsin that polymerizes within the ER, based on the activity of the enzyme ER mannosidase I, which generates a degradation signal by removing a single mannose residue from the Man₉GlcNAc₂ N-glycan chain. In this model, at physiological levels of ER mannosidase I, polymerization of Zα₁-antitrypsin occurs before the mannosidase I can target the mutant monomeric protein for proteasome degradation, and polymers are directed to an alternative nonproteasomal degradation pathway. When ER mannosidase I is overexpressed in the same cells, it efficiently targets Zα₁-antitrypsin monomers for proteasomal degradation before they can polymerize (47). It is possible that the partial degradation of neuroserpin that we observe in our system (Fig. 7b) corresponds to proteasomal degradation of monomers shortly after their synthesis, whereas the polymerized mutant protein is not degraded in COS-7 cells but rather accumulates stably in the ER.
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pathway. Likewise, it is also possible that a fraction of wild type neuroserpin polymerizes in the ER at the high concentrations that are achieved during transient transfection and that the polymers are then secreted. A third alternative is that the overexpression of mutant neuroserpin in COS-7 cells may saturate the ER quality control system and therefore result in the secretion of polymers. Future research on the cellular trafficking of mutant neuroserpin polymers will resolve this question and provide insight into the mechanisms involved in the ER quality control of polymeric mutant proteins.

One important phenotypic difference between our cell culture model for neuroserpin expression and secretion and the FENIB patients is the apparent lack of toxic effects of the retained mutant neuroserpin polymers on the cells. Cells expressing wild type or mutant neuroserpin did not show any features of nuclear degeneration as detected by others when expressing the exon 1 of the Huntington’s disease gene in COS-7 cells (30). Moreover, we detected dividing cells containing abundant protein accumulation (results not shown). This could be due to a differential toxicity of neuroserpin aggregation in neurons and COS-7 cells and/or to the transient character of the wild type and mutant neuroserpin overexpression in our cell model. Our next goal will be to establish neuronal cell lines that stably express wild type and mutant neuroserpin in order to better characterize the cellular mechanisms that cause neuronal dysfunction and death in FENIB.

In summary, we have shown here that mutants of neuroserpin that cause FENIB are retained as polymers within the ER, and provide insight into the mechanisms involved in the ER quality control of polymeric mutant proteins.

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