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A Mutation Linked with Autism Reveals a Common Mechanism of Endoplasmic Reticulum Retention for the α,β-Hydrolase Fold Protein Family*

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A mutation linked to autistic spectrum disorders encodes an Arg to Cys replacement in the C-terminal portion of the extracellular domain of neuroligin-3. The solvent-exposed Cys causes virtually complete retention of the protein in the endoplasmic reticulum when the protein is expressed in transfected cells. An identical Cys substitution was reported for butyrylcholinesterase through genotyping patients with post-succinylcholine apnea. Neuroligin, butyrylcholinesterase, and acetylcholinesterase are members of the α,β-hydrolase fold family of proteins sharing sequence similarity and common tertiary structures. Although these proteins have distinct oligomeric assemblies and cellular dispositions, homologous Arg residues in neuroligin-3 (Arg-451), in butyrylcholinesterase (Arg-386), and in acetylcholinesterase (Arg-395) are conserved in all studied mammalian species. To examine whether an homologous Arg to Cys mutation affects related proteins similarly despite their differing capacities to oligomerize, we inserted homologous mutations in the acetylcholinesterase and butyrylcholinesterase cDNAs. Using confocal fluorescence microscopy and analysis of oligosaccharide processing, we find that the homologous Arg to Cys mutation also results in endoplasmic reticulum retention of the two cholinesterases. Small quantities of mutated acetylcholinesterase exported from the cell retain activity but show a greater K_m, a much smaller k_{cat}, and altered substrate inhibition. The nascent proteins associate with chaperones during processing, but the mutation presumably restricts processing through the endoplasmic reticulum and Golgi apparatus, because of local protein misfolding and inability to oligomerize. The mutation may alter the capacity of these proteins to dissociate from their chaperone prior to oligomerization and processing for export.

Several mutations that were found in human *neuroligin 3* and *4* genes appear to be associated with the autistic spectrum disorders (1, 2). One of the more interesting mutations is an Arg to Cys mutation found in neuroligin-3 (NL-3)[3] at position 451 near the C terminus of the extracellular domain (1). This mutation, identified in an affected twin set, has been shown to give rise to an altered cellular phenotype. The protein is largely retained intracellularly in the endoplasmic reticulum (ER), with little of the nascent protein reaching its cell membrane location (3, 4). In addition, the residual protein that reaches the cell surface has an altered affinity for its cognate partner, β-neurexin (3). When Arg-451 (rat numbering, Arg-471) is mutated to a Thr or a Glu, the mutant NL is exported normally but shows significantly lower affinity for β-neurexins (3). Mass spectrometry analysis of the secreted fraction of the R471C-NL3 mutant indicated that the mutated protein had an unaltered disulfide bonding pattern (3).

Arg-451 is highly conserved within the NL family and across mammalian species. This residue is also conserved in many other α,β-hydrolase fold proteins. Recently, a cysteine substitution for the conserved arginine in butyrylcholinesterase (BChE) was found as an infrequent mutation in patient populations with post-succinylcholine apnea (5–7). BChE appears as a soluble tetramer in the plasma but is synthesized in liver (8). The physiological function of BChE is not clear; nevertheless, the enzyme has proven critical in inactivating various administered drugs such as succinylcholine (9).

The cloning of the acetylcholinesterase (*AChE*) gene 2 decades ago revealed a new protein family structurally distinct from other hydrolases of the then known structures (10), and homology of AChE to a large domain in thyroglobulin suggested a new superfamily of proteins with diverse functions extending beyond hydrolytic reactions (10). Subsequently a variety of homologous hydrolases have been identified that fall in this family as do several proteins that lack hydrolase activity but function as adhesion proteins (11–13). Among these, the neuroligins are the only ones of mammalian origin that have been identified and characterized to date (14, 15). The commonality of tertiary structure has enabled one to classify these proteins as members of the α,β-hydrolase fold family (16).

The neuroligins are a family of multidomain transmembrane-spanning proteins expressed on the postsynaptic side of the synapse (14, 17, 18). The N-terminal extracellular region is homologous to AChE and serves as the extracellular recognition portion of the molecule. C-terminal to this recognition domain is a linking *O*-glycosylation-rich region followed by a transmembrane span and a cytoplasmic region with recognition capacity for PDZ proteins. The association of NL with β-neurexin and perhaps α-neurexin provides a potential trans-synaptic interaction. Accordingly, the neuroligins

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[3] The abbreviations used are: NL, neuroligin; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ER, endoplasmic reticulum; Endo H, endoglycosidase H; PNGase F, *N*-glycosidase F, HEK, human embryonic kidney; GPI, glycosylphosphatidylinositol; RT, reverse transcriptase.

presumably display a capacity to stimulate formation and maturation of new excitatory and inhibitory synapses in the central nervous system (19–23).

Although the molecular details of the neuroligin-neurexin association that underlie this function are not understood, a crystal structure of neurexin has been reported (24), and the structure of neuroligin has been inferred from its homology to AChE (25, 26). By constructing truncated, soluble forms of neuroligin, we characterized the disulfide bonding and glycosylation pattern of NL and demonstrated secondary structural features that are similar to AChE (26, 27). In contrast to NL, cholinesterases do not possess a transmembrane spanning region but rather associate with membranes through either an attached glycophospholipid or by attachment to structural subunits that associate with the plasma membrane or basement membrane (13, 28). Moreover, the alternative splicing mechanism that controls the nature of the membrane attachment of AChE also influences its degree of its oligomerization. Homomeric dimers and tetramers have been identified, as have heteromeric associations between catalytic and structural subunits (13, 29, 30).

Accordingly, given the distinct assembly modes of this family of proteins, we sought to determine whether a mutation at homologous residue positions in AChE and BChE would give rise to similar processing aberrations as those seen with the neuroligins. The data presented here suggest that the mutation results in a common folding and processing deficiency. This particular mutation may also uncover mechanistic details essential for catalytic activity, subunit assembly, and oligomerization of nascent proteins belonging to the α,β-hydrolase fold family.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—A cDNA encoding mouse GPI-AChE was subcloned into a FLAG-tagged vector (Sigma) for detection and purification. The natural leader peptide of AChE was replaced by the pre-protrypsin leader peptide. The N-terminal FLAG is followed by a linker peptide of 10 residues and by the AChE sequence beginning at Glu-1 (sequence of the mature mouse AChE protein). Soluble monomeric mouse AChE (AChE-548) was constructed by introducing a stop codon at Cys-549 of mouse AChE-GPI as described previously (31). Arg-395 → Cys (R395C) and Leu-386 → Cys (L386C) mutations were introduced using the QuikChange mutagenesis kit (Stratagene, San Diego, CA) and were subsequently subcloned into expression vectors. Mutations were verified by automated sequencing. Plasmids were purified using DEAE columns (Qiagen Inc., Valencia, CA). Rat NL1 and NL3 wild type constructs and the Arg to Cys mutants were described previously by Comoletti *et al.* (3). The pGS vector containing cDNA encoding human BChE was described previously (32). Mutagenesis introduced the Cys mutation at Arg-386. A FLAG tag was added to the C terminus of wild type BChE after Leu-574 (32).

Cell Culture and Transfections—HEK-293 cells were maintained at 37 °C and 10% CO_2 in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum. Plasmids (10 μg) carrying the neomycin resistance gene were transfected into HEK-293 cells with $(Ca)_3(PO_4)_2$ precipitation or FuGENE 6 (Roche Applied Science). Stably transfected cells were selected with G418 (Geneticin, Sigma) as described elsewhere (27). Although initial studies were conducted on protein generated after transient transfection, clonal cells were selected for studies of mRNA levels, enzyme activity at 31 and 37 °C, kinetic profiles, immunoblotting, and immunofluorescence. Wild type and mutant AChEs were also purified on affinity columns to apparent homogeneity for the kinetic and turnover studies. BChE was studied only after transient transfection.

Cell Surface Activity and Preparation of Cell Extracts—Cells were rinsed and harvested by removal from the dish in phosphate-buffered saline, and activity was measured on the surface of intact cells. Proteins were extracted from cells in 20 mM sodium phosphate, pH 7.0, containing 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100 plus a mixture of protease inhibitors containing 5 μg/ml each of pepstatin A, leupeptin, and aprotinin, 10 μg/ml bacitracin, and 0.01 mM benzamidine (33). Cells were sonicated on ice for 10 min and then spun at 12,000 × *g* for 10 min. Supernatants containing all solubilized proteins were used for subsequent experiments.

Assay of AChE and BChE Catalytic Activity—To avoid serum AChE contamination, selected clones of transfected cells were maintained in serum-free Ultraculture medium supplemented with L-glutamine (Cambrex, Walkersville, MD). Media were collected at 48-h intervals, pooled for purification, and assayed for AChE or BChE activity using 0.5 mM acetylthiocholine iodide or 5 mM butyrylthiocholine iodide as substrates (34). Both media and cell-associated AChE activities were then normalized to the total cell protein content (35). To ascertain whether AChE expression could be enhanced at low temperature, 80% confluent cells were cultured for 48 h at 31 °C prior to harvesting.

Gel Electrophoresis and Immunoblotting—Western blots were performed as described by Towbin *et al.* (36). Briefly, crude cell extracts (20 μg of total protein) or batch anti-FLAG immunoprecipitated proteins from cell culture media were separated on 10% SDS-polyacrylamide gels (Invitrogen). After transfer to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), blocking of nonspecific binding was achieved by incubating the membrane with 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl, containing 5% nonfat dry milk, and 0.1% Tween 20 for 1 h. Primary antibodies were diluted in blocking buffer as follows: anti-AChE polyclonal (37), 1:4000; anti-FLAG M2 monoclonal antibody (Sigma), 1:1000; anti NL-1 monoclonal (Synaptic Systems, Goettingen, Germany), 1:5000; anti-PTP-1B monoclonal (Calbiochem), 1:10,000; anti-BChE monoclonal antibody, 1:1. Monoclonal antibody to human BChE was prepared by the Monoclonal Core Facility at the University of Nebraska Medical Center. The pure BChE protein used to form crystals (38) was injected into mice for production of antibodies. Human BChE protein was truncated at residue 529 thereby missing residues 530–574. It was also devoid of carbohydrate chains at Asn-17, -455, -481, and -486. Hybridoma clone C191 2.1.1 secreted the monoclonal antibody into culture medium. Antibody hybridization was detected with ECL (Pierce).

The proteasome inhibitor, lactacystin (10 μM, Sigma), was applied to the cells 24 h after transient transfection, and the altered cellular disposition of NL-3, AChE, and BChE was assessed after treatment for 24 h.

Total RNA Extraction, cDNA Preparation, and Real Time RT-PCR—Total RNA was extracted with TRIzol reagents (Invitrogen) from one 100-mm tissue culture dish of HEK-293 cells stably transfected with wild type and mutant AChE constructs. DNase I turbo treatment (Ambion, Austin, TX) was performed to avoid any genomic DNA contamination. Four μg of total RNA was reverse-transcribed into single-stranded cDNA using the Superscript First-strand System (Invitrogen). One μl of the resulting cDNA was subjected to real time RT-PCR using Taqman primer/probe technology (Applied Biosystems, Foster City, CA). The primer/probe sets for detecting AChE (exon 3 to exon 4) and the endogenous reference gene β-actin are, respectively, Mm00477275_m1 and Mm00607939_s1. PCRs were run according to the manufacturer's instructions. All assays were run in duplicate. The quantity of the target mRNA was calculated using the Sequence Detector software package version 1.7 (Applied Biosystems). AChE mRNA was normalized to β-actin mRNA allowing for measurement of the relative expres-

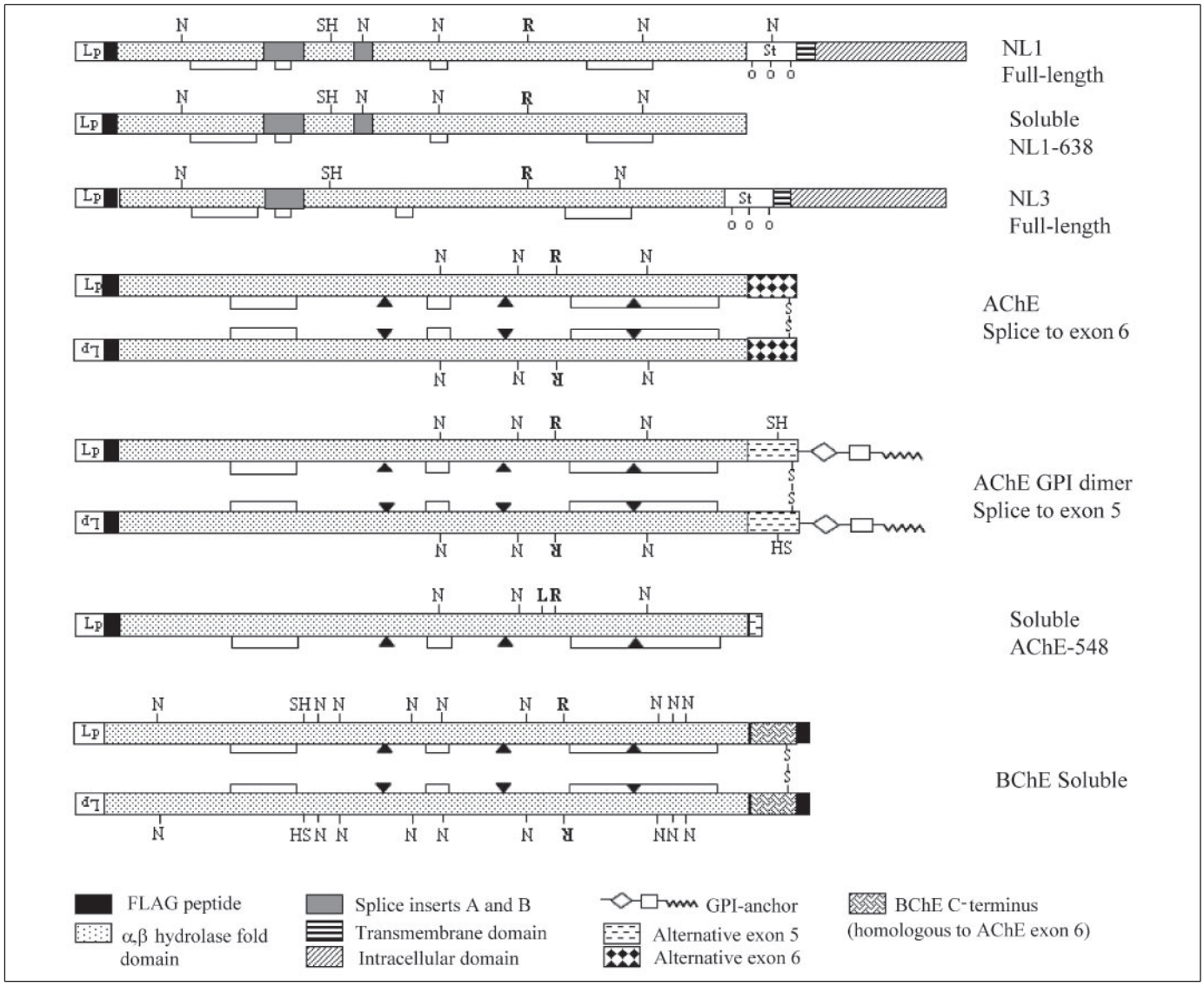


FIGURE 1. **Proteins of the α,β-hydrolase fold superfamily.** Schematic drawing of the domain organization and regions of homology between the rat neuroligins, mouse acetylcholinesterase, and human butyrylcholinesterase proteins as reported in UniProtKB/Swiss-Prot (us.expasy.org/sprot/). Leader peptides (*Lp*), disulfide bonds (*brackets*), unpaired cysteines (*SH*), and *N*-linked (*N*) and *O*-linked (*O*) glycosylation sites and the stalk region (*St*) for NLs are highlighted. The approximate position of the naturally occurring arginine (*R*) that is mutated in the three proteins and a reference leucine (*L*) residue in AChE are shown in boldface type. *Inverted triangles* show the catalytic triad (Ser-203, His-447, and Glu-334 in AChE and Ser-198, His-438, and Glu-325 in BChE). Size of domains is not drawn to scale.

sion level of AChE mRNA with the comparative C_T method (Applied Biosystems user's manual).

Analysis of Glycosylation—Cell lysates (10 μg) from HEK-293 cells expressing NL1 and AChE wild type and mutant proteins were incubated with 1500 units of endoglycosidase H (Endo H) or 250 units of *N*-glycosidase F (PNGase F) (New England Biolabs, Beverly, MA) at 37 °C for 3 h at pH 5.5 and 7.5, respectively.

FLAG-tagged Wild type and R395C-AChE-548 Expression and Purification—Serum-free culture medium was collected from AChE-548 wild type and R395C-expressing cells maintained in triple layer flasks at 37 °C and 5% CO_2. Enzymes were purified by passing the media over an M2 anti-FLAG-affinity column (Sigma). The column was then washed with 10 mM Hepes buffer, pH 7.4, with 450 mM NaCl and eluted using the FLAG peptide (Sigma) in 2.5-bed volumes of the Hepes buffer with NaCl containing 1 μg/ml leupeptin. Proteins were concentrated to ~4 μM using Centriprep 30 (Millipore, Bedford, MA) and stored at 4 °C. Purified proteins were used for AChE kinetics studies. Lower scale purification, from around 20 ml of media, was done to purify R395C-AChE and R386-BChE mutant proteins to be used for Endo H digestion. After purification, E386C BChE was also concentrated.

Immunohistochemical Localization and Confocal Microscopy—Parental HEK-293, NL1, and AChE-GPI stably expressing cells were plated on poly-D-lysine-coated glass coverslips and grown overnight in Dulbecco's modified Eagle's medium. Cells were fixed in 4% paraformaldehyde/phosphate-buffered saline for 20 min, washed, and labeled for immunofluorescence (3). Briefly, anti-FLAG M2 monoclonal antibody (Sigma) and anti-calnexin polyclonal antibody (Stressgen, Victoria, Canada) were mixed and diluted 1:500 and 1:200, respectively, with blocking buffer diluted 5-fold. Fluorescein isothiocyanate-conjugated anti-mouse antibody and Cy5-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) were diluted 1:100 in the same buffer. Image processing employed an MRC-1024 laser-scanning confocal system (Bio-Rad) coupled to a Zeiss Axiovert 35 M microscope.

Determination of Kinetic Parameters for the AChE Catalytic Activity—AChE activity was measured over acetylthiocholine concentrations of 0.010 to 100 mM, and individual kinetic constants were

TABLE 1
Expression of wild type and mutant mouse AChEs in HEK-293 cells

AChE gene product	AChE activity,[a] mean ± S.E.		% control[b]	
	Media	Lysates	Media	Lysates
	units/min/μg cell protein		*%*	
Monomeric, truncated				
Wild type AChE-548	1.30 ± 0.17	0.016 ± 0.003	100	100
R395C AChE-548	<0.003	0.002 ± 0.001	0.27	13.6
AChE gene product	**AChE activity,[a] mean ± S.E.**		**% control[b]**	
	Intact cell	**Lysates**	**Intact cells**	**Lysates**
GPI-linked dimer				
Wild type GPI dimer	0.019 ± 0.003	0.091 ± 0.017	100	100
R395C GPI dimer	0.008 ± 0.001	0.018 ± 0.006	42	20

[a] Activity is normalized to total cell protein content. Values represent the mean of at least four experiments.
[b] Percent values have been calculated considering the activities for both AChE-548 and AChE-GPI constructs equal 100%.

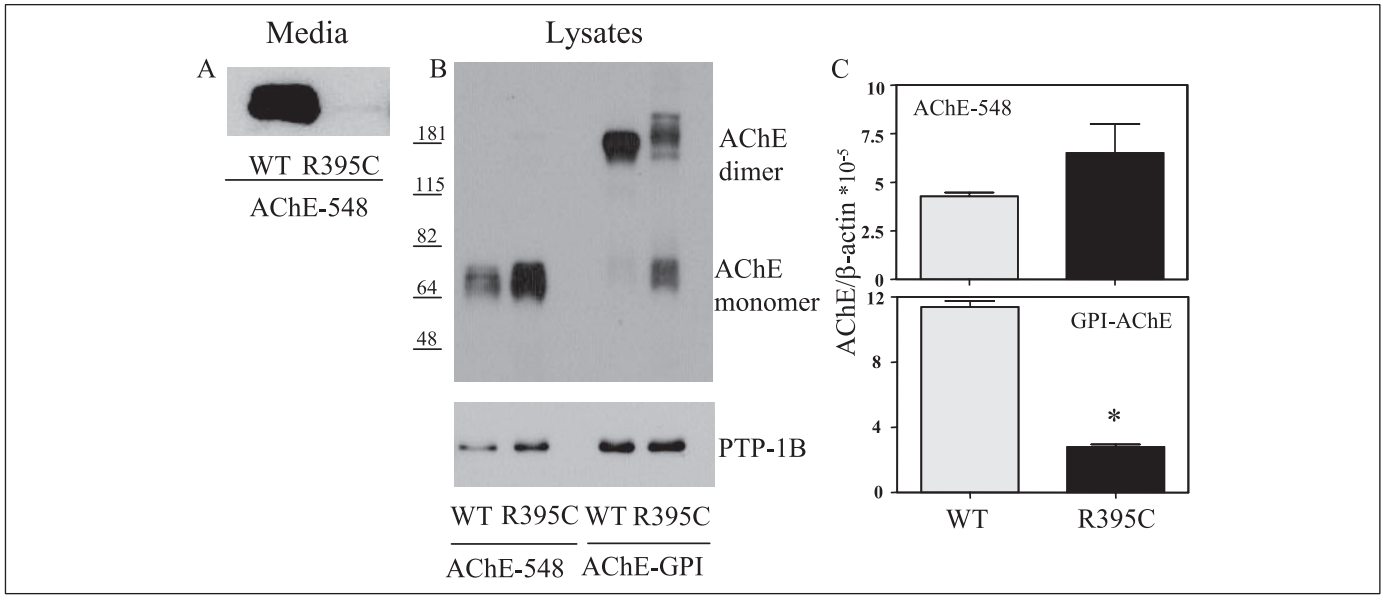


FIGURE 2. **Protein and mRNA expression levels of wild type and recombinant AChE.** *A*, immunoblot of secreted wild type (*WT*) and R395C AChE-548 immunoadsorbed with FLAG monoclonal antibody from culture medium of transfected clonal cells. An enhanced exposure reveals a minimal secreted fraction of the R395C AChE-548 protein compared with parental AChE-548. *B*, immunoblot of cellular lysates without β-mercaptoethanol showing monomers and dimers of AChE. Immunoblots were probed with rabbit polyclonal antibody against AChE. Adaptor protein PTP1B was used as loading control. *C*, relative quantification by real time RT-PCR analysis of AChE mRNA normalized to human β-actin. *, significantly different with $p < 0.05$.

assessed by nonlinear fitting of activities *versus* substrate concentration (39). The AChE catalytic turnover constant k_{cat} was then determined by normalizing V_m to the concentration of AChE active sites. The concentration of active sites was determined by titration of AChE activity using the covalent, "irreversible" organophosphate inhibitor, (S_p)-dimethylbutyl methylphosphonothiocholine (39).

RESULTS

Expression of Cysteine-substituted AChE—The *AChE* gene was mutated so that the homologous Arg mutation in NL (Arg-451 → Cys) was also converted to a Cys in AChE (R395C) (Fig. 1). To analyze the effect of oligomeric assembly on the processing and export of AChE and its related proteins, two forms of mutated AChE were generated. The first is a truncated form of AChE terminating at residue 548 (AChE-548) that is expressed as a soluble monomer. This form lacks sequence necessary to form disulfide-linked dimers or larger oligomers of AChE (40). Similar monomeric species of AChE have been identified *in situ*. The second form contained an amino acid sequence capable of forming a disulfide-linked dimer and the signal capable of adding a glycophospholipid linkage at its C terminus after cellular processing, AChE-GPI (42). This form appears largely in hematopoietic cells as a dimer with the glycophospholipid tethering it to the outer leaflet of the plasma membrane (42, 43).

In contrast to the wild type form of AChE-548, activity is not detected in the culture medium when the R395C AChE-548 mutant is transfected into HEK-293 cells (Table 1). Measurement of cell-associated activity in the cell lysates of AChE-548 and R395C AChE-548 reveals intracellular activity in both the wild type and in the Cys mutant, although the R395C mutant has far lower activity than the wild type enzyme (Table 1).

Expression of the cDNA encoding AChE with the requisite signal sequence for addition of a glycophospholipid anchor at its C terminus, AChE-GPI, yields cells expressing AChE on the cell surface. Activity measurements on the intact cells show substantially reduced activity for the R395C mutant of the GPI form of AChE (Table 1). This reduction is also evident when the cells are extracted with Triton X-100 to reveal total cellular activity, suggesting that transport of AChE-GPI to its cell surface location is compromised with the R395C mutation (Table 1).

Electrophoretic Analysis of Expressed AChE—Western blots to detect AChE protein production confirmed the activity measurements showing that virtually no R395C AChE-548 was exported into the media, whereas expression of the parent AChE-548 showed robust secretion into the media (Fig. 2*A*). Western blots using cell lysates in nonreducing

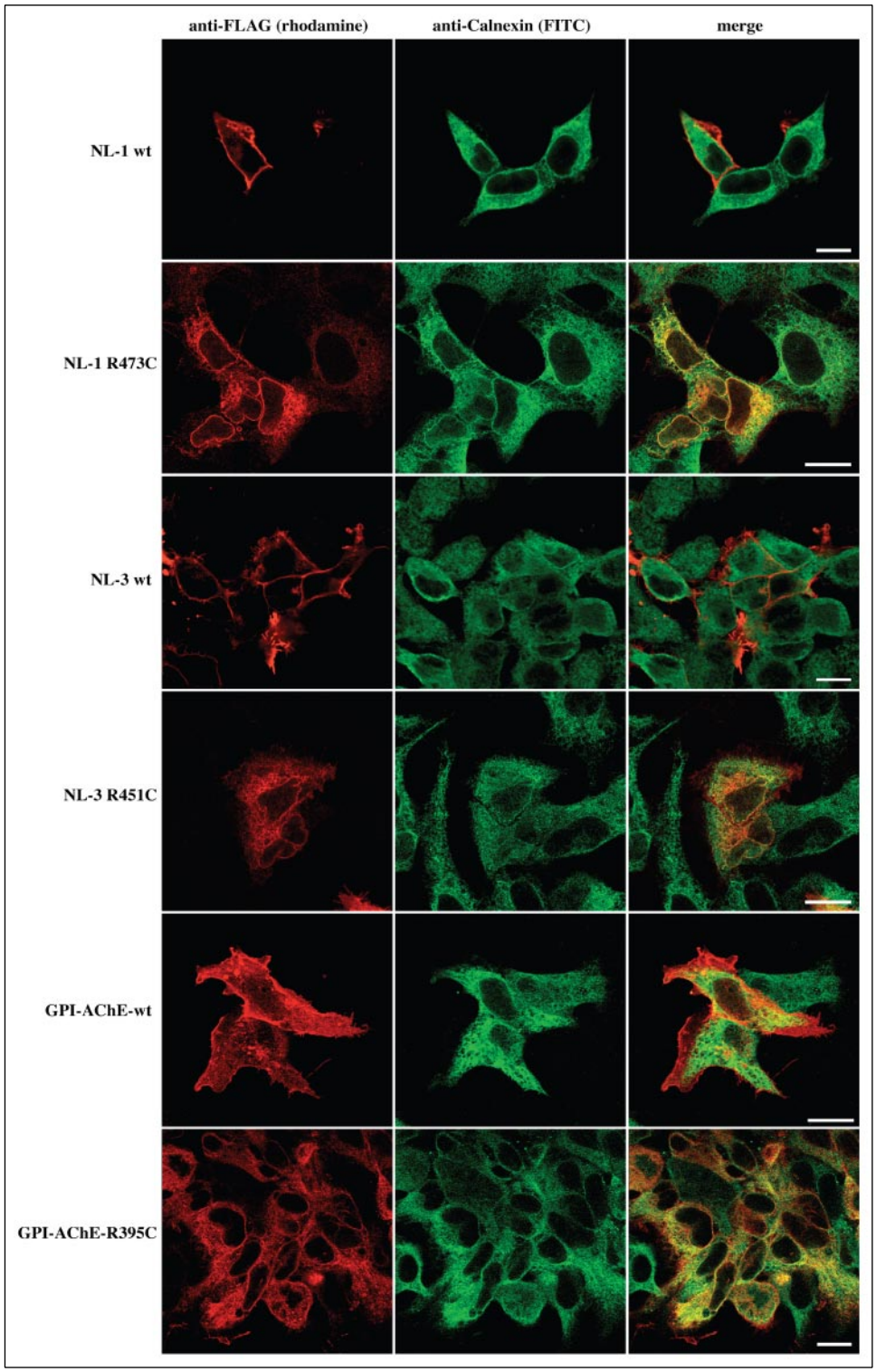


FIGURE 3. **Co-localization of NL and AChE wild type and mutant proteins with calnexin.** Representative immunofluorescence images of HEK-293 cells expressing FLAG-tagged wild type (*WT*) NL1 and -3, AChE-GPI wild type, and their Arg to Cys mutations. Images show the FLAG staining (*left column, red*) and the ER marker calnexin staining (*middle column, green*) and the merged view (*yellow*) of the two fluorescence signals in the presence of saponin. Mutated proteins co-localize with calnexin indicating that the protein is retained in the ER. *Scale bar*, 10 μm.

conditions (Fig. 2*B*) show slightly higher cellular levels of R395C AChE-548 compared with the wild type enzyme indicating that the mutated R395C protein is synthesized but compromised in its capacity to be exported from the cell. The lower catalytic activity found for the cell lysates may reflect either inactivation of a metastable enzyme conformation or altered catalytic properties of the mutated AChE.

The glycophospholipid forms of AChE, whether expressed in transfected cells or natively expressed on the erythrocyte surface, exist as disulfide-linked dimers with the C-terminal cysteine forming the inter-subunit linkage (41). To examine whether introduction of a second cysteine near the C terminus might interfere with oligomerization, we examined the electrophoretic migration of both R395C AChE-GPI and the wild type form of AChE-GPI in nonreducing conditions. As is evident in Fig. 2*B*, expression of the R395C AChE-GPI mutant yields an appreciable fraction of monomer, in contrast to the wild type AChE-GPI that is mainly expressed as a dimer. Other satellite bands are present in the lane of the R395C GPI-AChE mutant. This heterogeneity of molecular forms may reflect accumulation of ubiquitinated species of

the mutant protein and/or different intermediates of glycosylation processing.

To evaluate if the higher protein expression seen in the cells stably transfected with R395C AChE compared with the wild type construct was resulting from different expression levels, mRNA levels were measured by real time reverse transcriptase (RT) (Fig. 2*C*). No significant differences in the amount of AChE mRNA was found comparing R395C AChE-548 and wild type AChE-548, indicating that the higher protein amount found in the cells expressing the mutant protein is the result of a cellular retention mechanism. On the other hand R395C AChE-GPI shows reduced mRNA expression compared with WT AChE-GPI (Fig. 2*C*).

Co-localization of NL and AChE with the ER Protein, Calnexin—Previous studies have shown that the R471C-NL3 mutation is retained in the endoplasmic reticulum with minimal mutant protein being exported (3, 4). On the other hand, mutation to residues other than cysteine at 471 did not result in cellular retention (3). To ascertain whether this behavior is common to the α,β-hydrolase fold family, we examined the immunochemical localization of wild type and R395C AChE-GPI compared with the ER residing protein, calnexin (Fig. 3).

We observed in the immunohistochemical analysis that wild type AChE-GPI and wild type NLs 1 and 3 are localized to the cell surface as expected. Minimal co-localization with the chaperone protein calnexin, which is present endogenously, is evident. On the other hand, the R473C NL1, R471C NL3, and the R395C AChE-GPI mutants are retained in the endoplasmic reticulum and show clear co-localization with calnexin. Similar retention is seen with R395C AChE-548; however, it is more difficult to quantitate retention of this form of AChE because the wild type and mutant are located in the ER during biosynthesis (data not shown).

Analysis of Glycosylation in NL and AChE—To further investigate the stage of processing of NL and AChE that the common mutation influences, we examined the sensitivity of NL and AChE to glycosidase treatment. We found that the predominant portion of wild type NL1, when extracted from the cell lysate, is resistant to Endo H treatment. Extracellular NL migrates to the higher molecular mass position found for the fully processed protein (Fig. 4*A*). This is to be expected because a recent characterization of the oligosaccharide content by mass spectrometry shows primarily bi- and tri-antennary chains containing several terminal sialic acids (26). By contrast, the sensitivity of R473C NL1 to Endo H digestion is virtually complete, indicating the predominance of high mannose chains and the incomplete oligosaccharide processing of the mutant protein. Hence, with the mutation the majority of NL retained in the cell fails to reach the trans-Golgi stages of oligosaccharide processing.

Analysis of the Endo H treatment of the cell lysates of the wild type form of AChE-GPI reveals that only a portion of the glycoprotein is digested (Fig. 4*B*). The total cell extract contains various biosynthetic fractions of the enzyme that transit through the organelles in addition to the processed protein on the cell surface. These intracellular fractions presumably have not yet acquired the fully processed oligosaccharides, whereas the fraction that is exposed at the cell surface contains oligosaccharides that should be processed completely. By contrast, R395C AChE-GPI is completely sensitive to the Endo H treatment. The R395C AChE-GPI mutant also migrates faster than wild type AChE-GPI because the oligosaccharides are largely unprocessed (Fig. 4*B*). PNGase F treatment of wild type and mutant GPI-AChE reveals that both forms

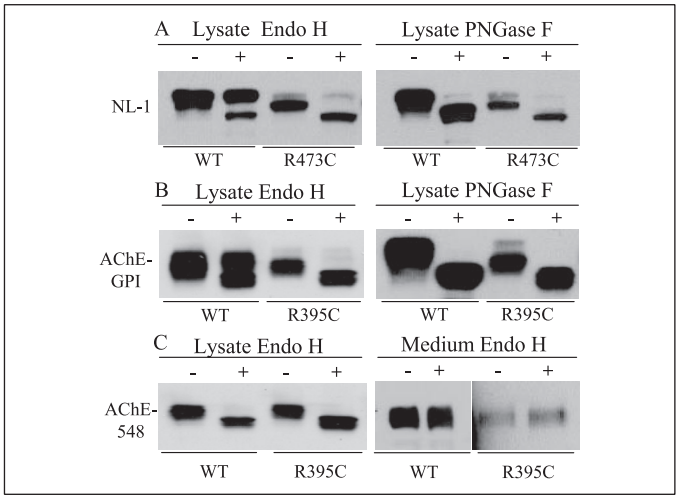


FIGURE 4. **Endo H and PNGase F sensitivity of NL1 and AChE wild type and mutant proteins.** Lysates of HEK-293 cells, transiently transfected with wild type (*WT*) or mutated NL1 (*A*), AChE-GPI (*B*), and AChE-548 (*C*) were incubated with either Endo H or PNGase F (+) or mock-digested (−) for 3 h at 37 °C. Wild type and R395C-AChE-548 proteins immunoadsorbed from conditioned medium were also incubated with Endo H (*C*). After digestion, samples were subjected to 10% SDS-PAGE and blotted with rabbit anti-AChE antibody for AChE detection.

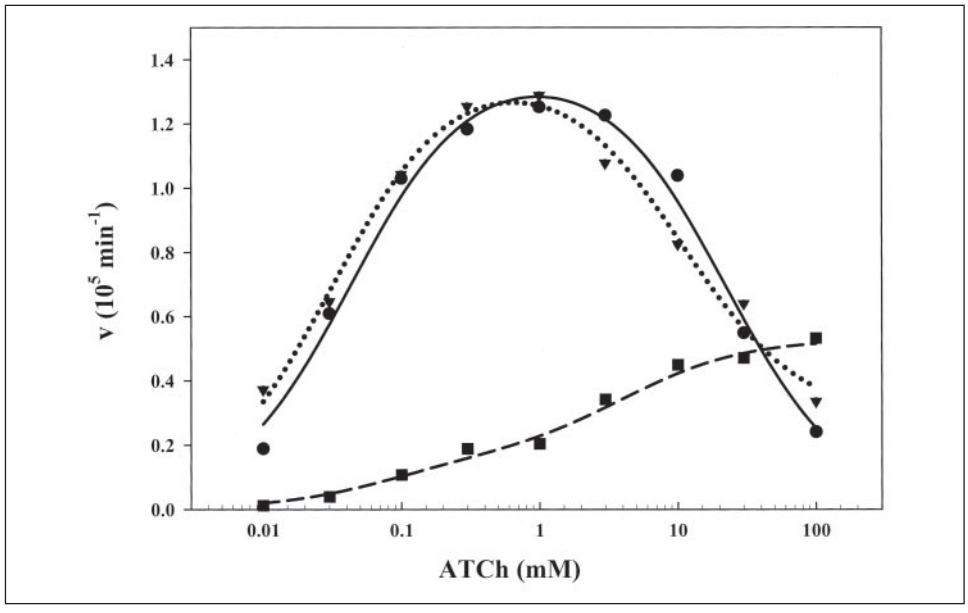
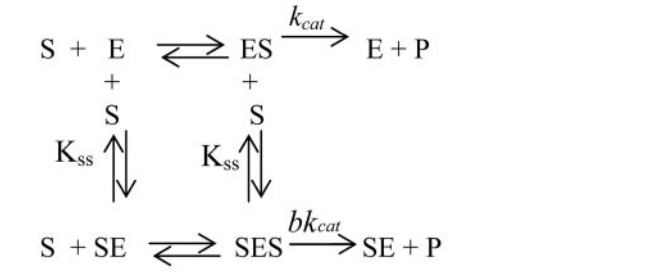


FIGURE 5. **Catalytic parameters of purified wild type and mutants of AChE-548.** Curves were generated over a range of acetylthiocholine concentrations using the Ellman assay, and catalytic constants are calculated by nonlinear regression of AChE activity data as described previously (39). For the kinetic Scheme 1 and the corresponding equation (see Ref. 39), the following catalytic constants were determined. For the wild type AChE (—), $K_m = 0.043 \pm 0.009$ mM; $K_{ss} = 22 \pm 9$ mM; $k_{cat} = 1.4 \times 10^5$ min^{-1}; and $b = 0$. For R395C-AChE (- - -), $K_m = 0.082 \pm 0.055$ mM; $K_{ss} = 4.2 \pm 1.8$ mM; $k_{cat} = 0.18 \times 10^5$ min^{-1}; and $b = 2.94 \pm 0.77$. For L386C-AChE (···), $K_m = 0.032 \pm 0.004$ mM; $K_{ss} = 11 \pm 3$ mM; $k_{cat} = 1.3 \times 10^5$ min^{-1}; and $b = 0.20 \pm 0.04$.

are equally sensitive (Fig. 4*B*). This is to be expected because PNGase F does not have altered specificity for differentially processed *N*-linked glycosylated sugars.

Although secretion of the mutant R395C AChE-548 is severely compromised, a small fraction of AChE becomes fully processed (thus becoming Endo H-resistant) and secreted as soluble entity (Fig. 4*C*). This fraction was affinity-purified and was used for subsequent monitoring of the enzymatic activity. The wild type form of AChE-548 found in the cell lysates is completely sensitive to Endo H, and therefore the oligosaccharide pattern of the R395C mutant and wild type AChE-548 forms cannot be distinguished in the cell lysates (Fig. 4*C*).

Catalytic Properties of the AChE Mutants—To understand whether the mutated Cys affects the enzymatic function of the cholinesterases, we compared the catalytic constants of the wild type and R395C AChE-548-purified enzymes. A second Cys mutation near the C terminus (L386C) was employed as a reference to assess the importance of positioning of the inserted Cys. By simultaneously examining the substrate dependence and titrating the number of active sites with an irreversible organophosphate, we deconstructed the steady-state kinetics into the catalytic parameters for substrate hydrolysis and inhibition. Although the R395C AChE-548 retained activity, it exhibited marked differences in catalytic parameters when compared with the wild type form (Fig. 5 and Scheme 1). In particular, hydrolysis was compromised at low substrate concentration but showed substrate activation ($b > 1$), rather than the characteristic substrate inhibition ($b < 1$) of AChE at high substrate concentration. A comparison of catalytic through-puts (k_{cat}/K_m) reveals a 13-fold difference between the R395C and wild type AChE-548 resulting from a greater K_m and smaller k_{cat}. By contrast, the reference mutation L386C AChE-548 shows virtually identical catalytic parameters with the classical substrate inhibition characteristic of the mammalian AChEs (39).



SCHEME 1

Temperature Dependence of AChE Expression—Because the retention of AChE in the ER and the altered kinetic parameters of secreted AChE may reflect incomplete or abnormal folding of the enzyme, we examined expression at lower temperatures to minimize misfolding (44). Although the lower temperature diminishes expression of the wild type AChE-548, relative activities are enhanced for R395C AChE-548 in both the cell lysate and medium (Fig. 6, *A* and *C*). Protein levels appear to be slightly reduced in the wild type and mutant lysates (Fig. 6*B*), but only expression at 31 °C shows appreciable mutant protein in the media (Fig. 6*D*). Insufficient quantities of mutant enzyme precluded a complete kinetic analysis of AChE produced at 31 °C. However, an examination of activity at low and high substrate concentration suggests that expression at low temperature yields the same concentration profile as the respective enzyme produces at 37 °C. Hence expression at 31 °C

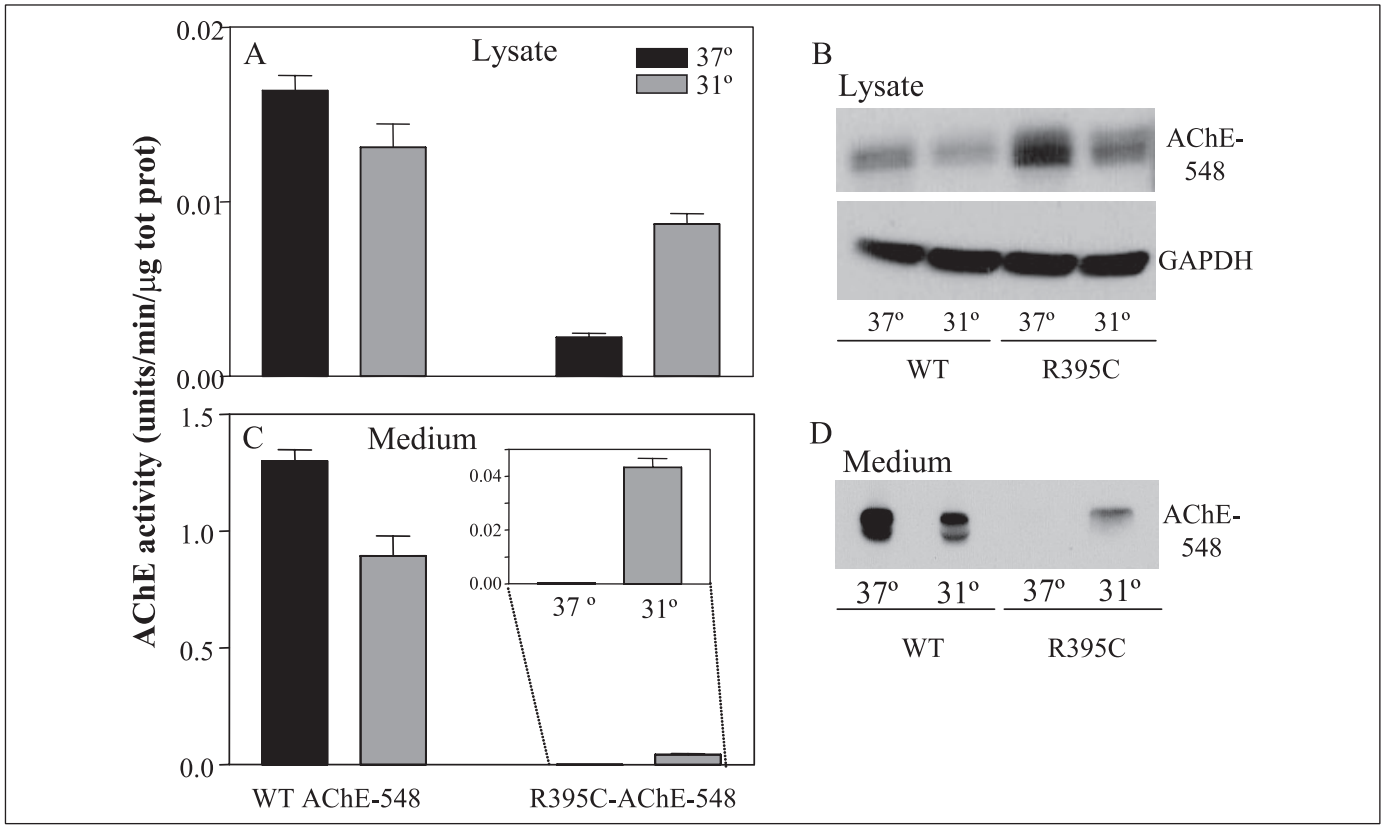


FIGURE 6. **Temperature dependence of AChE expression.** AChE activity levels measured by Ellman assay and immunoblot analysis of cellular lysates (*A* and *B*) and media (*C* and *D*) from HEK-293 stable cell lines expressing soluble AChE-548 wild type (*WT*) and the R395C mutation. Following a medium change, cells were cultured in Ultraculture medium at 37 and 31 °C for 48 h. Immunoblots were probed with rabbit polyclonal antibody against AChE and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as loading control.

does not affect the substrate dependence but rather alters the expression levels of fully active enzyme.

Expression of Wild Type and R386C BChE—BChE is known to have a single splice option giving rise to a soluble tetramer of identical catalytic subunits that assembles as dimer of two disulfide-linked dimers (32, 45). This form closely resembles the dominant splice option of AChE expressed in brain and muscle. As seen with the two forms of AChE, the Cys substitution for Arg in BChE impaired secretion of the protein. Also, a diminished amount of enzyme activity in the cell lysates was evident (Fig. 7*A*). Similar reductions in cellular and exported BChE are evident in comparing immunoreactive protein (Fig. 7*B*). Endo H treatment of the cell lysates shows a similar result to that observed for the soluble AChE-548 form for the wild type and mutant BChE proteins, both resulting in sensitivity to the enzymatic digestion. Endo H digestion of the purified proteins from the media shows a shift after Endo H treatment of a far smaller magnitude than in the lysate. This suggests that a small fraction of the nine *N*-linked oligosaccharides may still retain high mannose composition.

Degradation via the Proteasome Pathway of NL, BChE—To examine the influence of the degradation pathway of the Arg to Cys mutant proteins retained in the ER, lactacystin was used to block degradation via the proteasome pathway. Lactacystin causes an increase in cellular levels for both the wild type and Arg to Cys mutant in the NL3 and BChE proteins but has a more pronounced effect upon R451C-NL3 and R386C-BChE (Fig. 8, *A* and *C*). This suggests that a greater fraction of the mutant proteins is degraded intracellularly via the proteasome pathway. Similarly, lactacystin increases cellular levels of AChE-548; curiously, the differential effect on the mutation was not evident. This may be due to the truncated monomeric subunit being more susceptible to proteasome degradation, because it lacks a capacity for protection by oligomerization. Cells exposed to 0.2% Me_2SO, the solvent required for lactacystin, show unaltered expression, as shown for BChE (Fig. 8*C*).

DISCUSSION

We demonstrate here that single point mutation of a conserved Arg found in the neuroligin gene of an autistic twin set (1) and in patients with post-succinylcholine apnea (5–7) causes a similar defect in protein expression for both NL3 and BChE. Demonstration of processing deficiencies in AChE and BChE, as α,β-hydrolase fold family members related to the neuroligins, carries the advantage that catalytic parameters of the folded protein can be monitored during the intracellular processing and extracellular secretion steps of protein biosynthesis.

Velan *et al.* (46) have examined AChE biosynthesis through methionine pulse-chase labeling and immunoprecipitation. The initial monomeric species containing exon 6 formed intracellularly was not exported from the cell; rather dimerization and formation of the disulfide linkage are required for progression through the trans-Golgi, with concomitant oligosaccharide processing and subsequent secretion from the cell. Of particular interest is their observation that mutation of the C-terminal cysteine responsible for the *inter*-subunit disulfide bond (Cys-580) leads to secretion of the monomeric form of AChE and that secreted AChE also contains Endo H-resistant or terminally processed oligosaccharides (47). Accordingly, Cys-580 in mammalian AChE was proposed to be part of the oligomerization signal. Similarly, our results indicate that the introduction of a cysteine at an exposed but abnormal position near the C terminus may be the aberrant signal for association of a chaperone protein rendering the α,β-hydrolase fold protein unable to proceed efficiently through processing and secretion. Consequently, the protein accumulates in the endoplasmic reticulum and is more susceptible to proteosomal degradation.

Extensive cysteine substitution mutagenesis has been conducted on mammalian cholinesterase with the intent of attaching labels to the free cysteine side chain (48, 49). These studies have shown that cysteines introduced in the N-terminal two-thirds of AChE, at least up through residue 386, do not appear to alter secretion. Moreover, *Torpedo* AChE contains a free cysteine at position 231. Thus, it would appear that only Cys residues residing near the C terminus give rise to the assembly and secretion deficiency. This was also shown for NL1 where the unpaired cysteine at the homologous position (Cys-286) did not interfere with

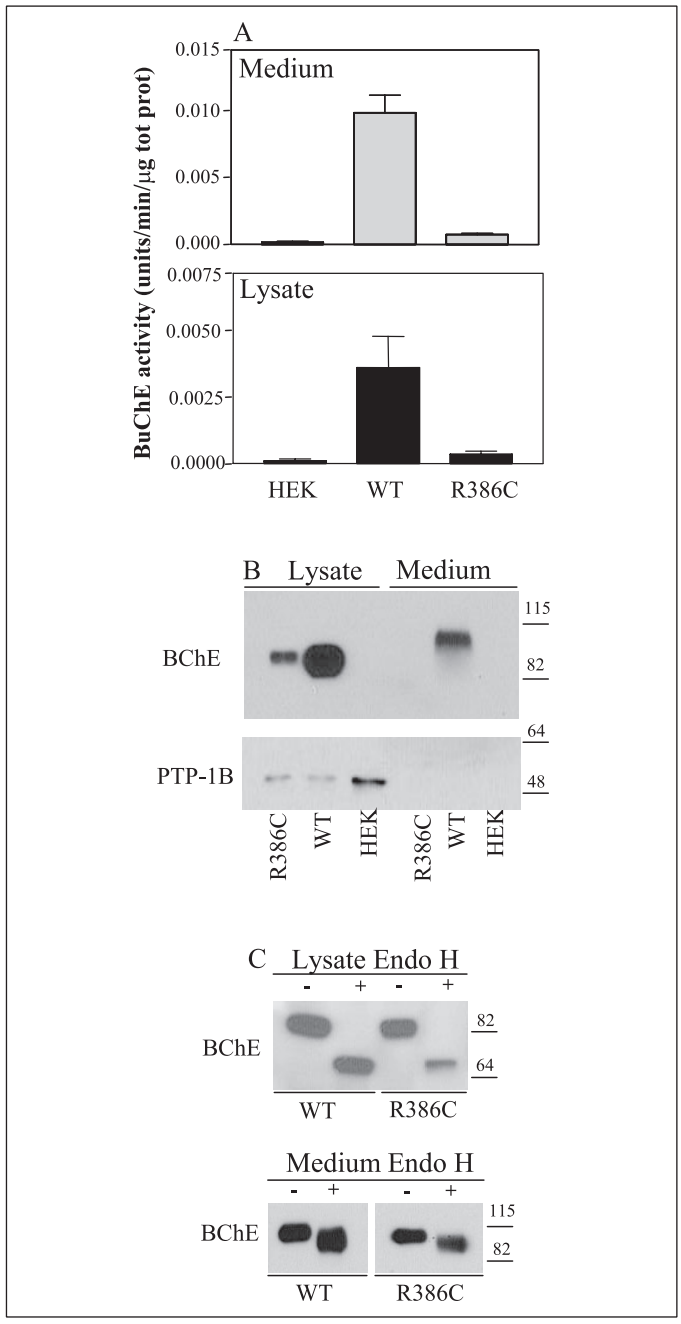


FIGURE 7. **Expression analysis of cellular and secreted recombinant BChE.** *A*, BChE activity is measured in lysate and medium from HEK-293 cells transiently transfected with BChE wild type (*WT*) and R386C expression plasmids. *B*, BChE expression levels by Western blot analysis of lysate and medium probing with mouse anti-BChE antibody and an antibody to the cellular protein PTP-1B as a loading control. *C*, immunoblot after Endo H treatment of lysate and medium for BChE wild type and R386C immunoprecipitated from growth media. Media samples were immunoadsorbed with a FLAG antibody prior to elution for gel electrophoresis. The R386C required additional concentration (5-fold) for detection.

expression nor rescue the deficiency in expression found in the neuroligin mutant (3).

There are some distinguishing structural features between members of the α,β-hydrolase fold family. The cholinesterases contain a four-helix bundle near the C terminus that has the requisite proximity to be essential for dimerization and the proper placement of the disulfide-linking cysteines. Although NL by sequence homology is believed to have the same helices (25), it does not contain a cysteine C-terminal to the four-helix bundle found in the cholinesterase sequence. Yet, the neuroligin extracellular domain alone, examined by analytical ultracentrifugation, dimerizes (27). By contrast, AChE devoid of the cysteine in the C-terminal region, as in AChE-548 or in a naturally occurring species containing a retained intron between exons 4 and 5, is normally secreted and behaves as a monomer in dilute solution. Formation of tetrameric forms of AChE and BChE occurs noncovalently between disulfide-linked dimers (9).

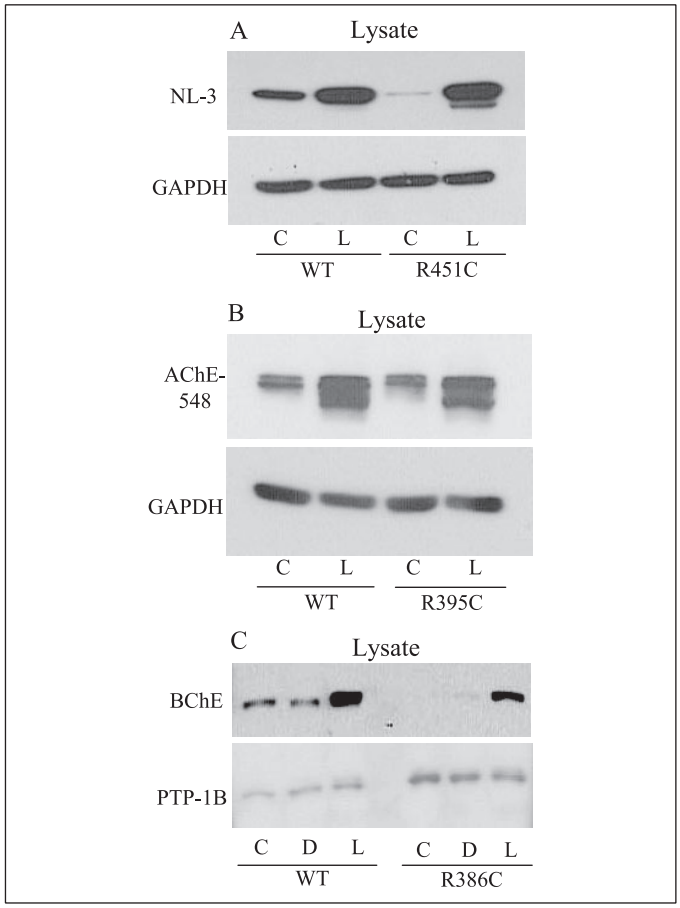


FIGURE 8. **Western blot of lysates expressing wild type or Arg to Cys mutant proteins after treatment with proteasome inhibitor, lactacystin (10 μM).** NL3 (*A*), AChE-548 (*B*), and BChE (*C*). Me_2SO (0.2%) was used to dissolve lactacystin. *C*, Me_2SO-treated cells are included as a control. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and PTP-1B were used as loading controls. Lanes are labeled as follows: *C*, control; *L*, lactacystin; *D*, Me_2SO (*DMSO*) treatment.

Accordingly, the cysteine mutations originally identified in human NL and BChE affect processing and cellular export irrespective of the oligomeric assembly of members of the α,β-hydrolase fold family. A possible mechanism for the diminished secretion of the Arg to Cys mutant involves the attachment of a chaperone protein to the introduced cysteine forming a complex that is not immediately dissociable when the partnering subunit comes into proximity. This complex can accumulate and become routed to the proteasome for degradation. Analysis of the chaperone shuttling mechanism through immunoprecipitation may shed further light on the aberrant mechanism seen in the naturally occurring Cys mutations. However, abnormal processing and intracellular retention and degradation are not solely responsible for diminished NL adhesion activity or cholinesterase catalytic activity. Rather Cys mutants, when secreted, have compromised intrinsic activity despite the normal disulfide bond interactions (26) (Fig. 5 and Scheme 1).

Irrespective of the fractional contributions of the above two proposed mechanisms for diminished activity, losses of expression are not complete, and it may be possible through the use of particular stabilizing agents to alter the folding and/or enhance expression of the mutant protein (50).

Interestingly, the truncated monomeric form of AChE-548 present within the cells shows complete sensitivity to Endo H. Hence, no difference can be detected between the mutant and native AChE by this approach. This may, in part, be due to the difference in processing rate between the ER and the Golgi apparatus. Once the proteins complete the primary processing in the ER and enter the Golgi, final maturation and secretion could happen at a sufficient rate that no intermediate stages are detectable. A similar finding was reported previously by Kerem *et al.* (47), who observed that removal of a cysteine near the C terminus of human AChE not only resulted in the secretion of monomers but that the monomers were Endo H-resistant. Hence, the absence of a disulfide bond, as is the case for AChE-548, results in secretion and carbohydrate processing by a default or other pathway. The changes in catalytic parameters for the enzyme with the introduced Cys are surprising because Arg-395, where the sulfhydryl is introduced (Fig. 9), is ~22 Å removed from the active center serine and about 17 Å from the other two catalytic triad residues (Glu-334 and His-447). Arg-395 is, however, in the immediate vicinity of Trp-442 that is linked to Met-443

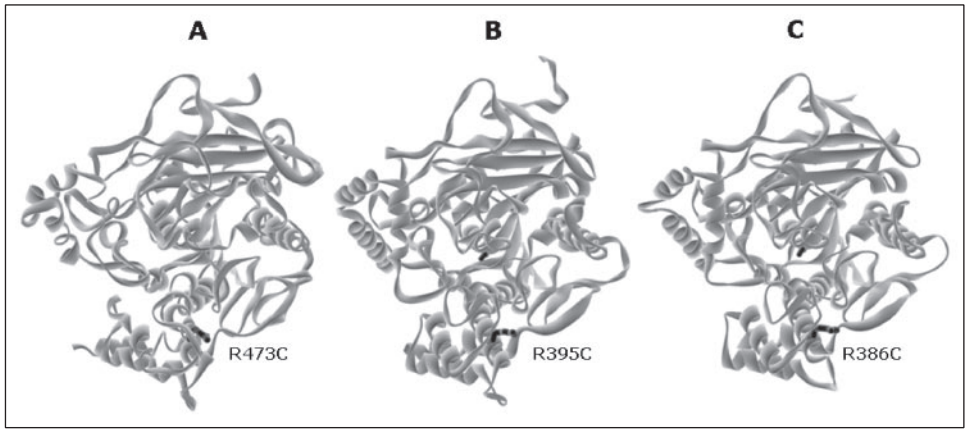


FIGURE 9. **Position of Arg to Cys mutation in three-dimensional structures of α,β-hydrolase fold proteins.** *A*, homology model of NL-1 (27). *B*, crystal structure of mouse AChE (53). *C*, crystal structure of human BChE (39). The mutated Arg residue in the three proteins appears in structures as a *black stick*. The centrally located catalytic serine, located at the base of the active center gorge of AChE and BChE some 18–20Å from the surface, is similarly indicated.

and on the same loop as His-447. As oxidation of Met-443 is known to significantly compromise AChE activity (37), small perturbations in Trp-442 position because of the absence of the positive charge in R395C could indirectly affect the alignment of the catalytic triad resulting in reduction of k_{cat} for R395C. On the other hand, the molecular basis for the shift from substrate inhibition to substrate activation for the AChE is not understood, being generated with several independent side chain modifications in the AChE active center gorge (39, 51).

A molten globule state of AChE has also been described (52). Although it could be considered as a precursor to the fully folded native form of the enzyme, it is devoid of activity rather than having markedly reduced activity and altered catalytic parameters as seen for the R395C mutant AChE.

The arginine position associated with a natural mutation in neuroligin implicated in autism is conserved in many proteins in the α,β-hydrolase fold family. When mutated to Cys, we demonstrate that it gives rise to retention of homologous proteins in the cell. The majority of protein is incompletely processed and is likely shuttled to the proteasome for degradation. This is evident irrespective of whether the protein is a dimer as in neuroligin or a monomer, dimer, or tetramer as in the case of AChE or BChE. Chaperone association may be an initial step of oligomerization, and introduction of an abnormal cysteine may interfere with chaperone association and subsequent oligomerization of the α,β-hydrolase fold family proteins. Small quantities of folded proteins containing the mutation are exported from the cell, but their conformation is altered and catalytic or adhesive activities are compromised. It will be of interest to ascertain whether altering intracellular redox parameters can enhance export and proper folding of the mutated proteins.

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