Pancreatology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Missense PNLIP mutations impeding pancreatic lipase secretion cause protein misfolding and endoplasmic reticulum stress

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ARTICLE INFO

Article history:
Received 26 May 2021
Received in revised form
28 July 2021
Accepted 30 July 2021
Available online xxx

Keywords:
Fat digestion
Genetic disease
Inflammation
Intracellular protein accumulation
Pancreatitis

ABSTRACT

Background/Objective: Mutation-induced misfolding of digestive enzymes has been shown to cause chronic pancreatitis. Recently, heterozygous pancreatic lipase (PNLIP) mutations leading to reduced secretion were identified. The aim of the present study was to investigate whether PNLIP mutants with a secretion defect result in endoplasmic reticulum (ER) stress in cell culture models.
Methods: We introduced the coding DNA for wild-type and A174P, G233E, C254R and V454F mutant PNLIP into two mammalian cell lines and carried out functional assays to assess PNLIP expression, secretion and ER stress.
Results: We found that wild-type PNLIP was readily secreted from the investigated cell lines. In contrast, none of the lipase mutants were detectable in the conditioned media. PNLIP variants accumulated in the cells as intracellular protein aggregates probably due to misfolding in the ER. Consistent with this notion, PNLIP mutants induced ER stress, as indicated by increased mRNA levels of spliced X-box Binding Protein 1 (XBP1) and the ER chaperone Immunoglobulin Binding Protein (BiP).
Conclusion: The results indicate that PNLIP mutations associated with a lipase secretion defect cause ER stress and thereby may increase the risk for chronic pancreatitis.

1. Introduction

Chronic pancreatitis is a progressive inflammatory disorder of the human pancreas characterized by permanent damage of the pancreatic parenchyma, fibrosis and infiltration of inflammatory cells [1,2]. Currently, there is no effective therapy or prevention for the disease, and treatments are limited to alleviating symptoms [3]. The most common etiologies for chronic pancreatitis are excessive alcohol consumption, and genetic predisposition, especially in pediatric populations [4].

Increased intrapancreatic trypsin activation can lead to autodigestion of the pancreas and the development of pancreatitis [5,6]. Common mutations in the gene PRSS1 encoding human cationic trypsin were first identified in the hereditary form of chronic pancreatitis [7–9]. Chymotrypsin C (CTRC), a minor chymotrypsin isoform in the pancreas, tightly regulates trypsin activation by limited proteolysis [10,11]. PRSS1 mutations that alter CTRC-mediated proteolytic cleavages and result in increased trypsin activation predispose to chronic pancreatitis [12]. Additionally, SPINK1, a small trypsin inhibitor secreted by the human pancreas, limits intrapancreatic trypsin activation by competitive inhibition. The importance of trypsin regulation is demonstrated by the fact that genetic alterations that reduce the levels of functional SPINK1 and CTRC are associated with chronic pancreatitis [13]. Recently, missense mutations in carboxypeptidase A1 (CPA1) were described as strong risk factors for the disease [14]. Functional experiments revealed that CPA1 mutations lead to pancreatitis through protein misfolding, intracellular retention and consequent endoplasmic reticulum (ER) stress rather than via enhanced trypsin activation [14–16]. Additional studies indicated that certain genetic alterations in PRSS1 and carboxyl ester lipase (CEL) might cause the disease through the same misfolding-dependent pathway [17–23]. Furthermore, missense mutations in secretory, cell membrane and ER resident proteins can lead to a number of other disorders via protein misfolding and consequent ER stress [24].

Pancreatic lipase (PNLIP) is one of the most abundant digestive

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https://doi.org/10.1016/j.pan.2021.07.008

enzymes secreted by the human exocrine pancreas [25,26]. PNLIP breaks down the majority of dietary triacylglycerols in the gastrointestinal tract, thereby significantly contributing to the absorption of lipids. PNLIP comprises a globular N-terminal domain and a β-sandwich C-terminal domain [27]. The N-terminal domain carries the catalytic site, while the C-terminal domain binds a cofactor called colipase also secreted by the pancreas. The activation of PNLIP requires the structural rearrangement of surface loops in the N-terminal domain rather than proteolytic cleavages. In contrast, colipase is produced as a precursor carrying an N-terminal propeptide, which is cleaved off by trypsin. The role of colipase is to stabilize the active state of PNLIP and restore its activity in the presence of inhibitory substances [28].

Recently, 19 rare heterozygous PNLIP mutations were identified in cases with chronic pancreatitis and controls [29]. Genetic analysis could not distinguish between disease-associated and harmless mutations. Experiments using purified enzymes indicated that wild-type PNLIP is highly resistant to proteolytic degradation. In contrast, a subset of mutations substantially decreased the proteolytic stability of lipase. Reduced proteolytic stability of PNLIP was associated with chronic pancreatitis, although the mechanism by which the mutations cause the disease remained elusive. The majority of PNLIP variants were readily secreted from transfected mammalian cells, with the exception of four mutants (A174P, G233E, C254R and V454F) for which secretion was barely detectable. In the present study, we investigated the expression and cellular effects of these non-secreted PNLIP variants in cell culture models. Our results demonstrated that PNLIP variants are misfolded and accumulate as intracellular protein aggregates. PNLIP secretion defect induced the development of ER stress, which may increase the risk for chronic pancreatitis.

2. Methods

2.1. Nomenclature

Nucleotide numbering of human PNLIP starts with the first nucleotide of the ATG translation initiation codon. Amino-acid residues are numbered starting with the initiator methionine of the primary translation product.

2.2. Expression plasmid and adenovirus construction

The expression plasmid pcDNA3.1(−) human PNLIP with a C-terminal 10 histidine tag was described previously [29,30]. Mutations were generated by overlap extension PCR mutagenesis and cloned into the expression plasmid as described earlier [29]. Adenovirus serotype 5 vectors carrying the coding DNA for PNLIP wild type and mutants with a C-terminal histidine tag were generated using the AdenoONE Cloning and Expression kit (Sirion Biotech). Briefly, PNLIP coding DNA from the pcDNA3.1(−) PNLIP plasmid was subcloned into the pO6A5-CMV shuttle vector using the *Nhe*I and *Kpn*I restriction sites. One Shot PIR1 chemically competent *Escherichia coli* cells (Invitrogen) were transformed with the pO6A5-CMV-PNLIP shuttle vector and incubated on an LB-agar-kanamycin plate for 16 h at 37 °C. A single colony was inoculated in 50 mL LB-kanamycin and grown overnight. The PNLIP shuttle vector was purified from the cells using NucleoBond Xtra Midi Plus kit (Macherey-Nagel). BA5-FRT electrocompetent *Escherichia coli* cells carrying the SIR-BAC-Ad5 vector (Sirion Biotech) were transformed with the PNLIP shuttle vector, plated onto an LB-agar-kanamycin-chloramphenicol plate and incubated for 16 h at 42 °C to allow homologous recombination of the PNLIP shuttle and BAC vectors. A single bacterial colony was grown in 300 mL LB supplemented with kanamycin and chloramphenicol at 37 °C. The PNLIP-BAC vector was purified from the cells using NucleoBond PC 100 kit (Macherey-Nagel), linearized with *Pac*I restriction enzyme and purified again with phenol-chloroform extraction. To ensure that only one copy of the pO6A5-PNLIP DNA was incorporated into the BAC vector, the characteristic banding pattern of linearized PNLIP-BAC DNA fragments was analyzed with agarose gel electrophoresis. Human embryonic kidney (HEK) 293AD cells were cultured into a well of a 6-well tissue culture plate in high-glucose DMEM supplemented with 10 % fetal bovine serum, 4 mM glutamine and penicillin-streptomycin. At 80–90 % confluence, the cells were transfected with 3 μg linearized PNLIP-BAC vector and 6 μL jetPEI transfection reagent (Polyplus-transfection) using the manufacturer's instructions. After 3 days of incubation, PNLIP-adenovirus particles were released from harvested cells by repeated freeze-thaw cycles. To obtain higher titer HEK 293AD cells were cultured in a T75 flask and re-infected with the virus particles and released again with freeze-thaw cycles after 3 days. The adenovirus was further amplified by re-infecting cells cultured in three T75 flasks. Finally, recombinant adenoviruses were purified with the AdenoONE Purification kit (Sirion Biotech) and stored in aliquots at −70 °C. The nucleotide sequences of PNLIP wild type and mutants in the adenoviral vectors were verified by capillary sequencing. The infectious titer expressed as infectious units per mL (IFU/mL) was determined using the AdEasy Viral Titer kit (Agilent). The typical yield of purified adenovirus samples was about 10^9–10^{10} IFU/mL. The relative amount of PNLIP wild type and mutant adenoviruses were verified by the quantitation of PNLIP mRNA levels in infected AR42J cells by qPCR using the Thermo Scientific TaqMan probes Hs00609591_m1 for human PNLIP and Rn01775763_g1 for internal GAPDH control (see protocol below). Adenovirus vector without PNLIP was also generated and used as a control.

2.3. Cell cultures and gene delivery

HEK 293T cells were grown in 2 mL growth media in 6-well tissue culture plates, as described previously [31]. Polyethylenimine (PEI) transfection reagent was prepared as described [32]. Transfections were carried out at 80–90 % confluence, by mixing 4 μg pcDNA3.1(−) PNLIP plasmid DNA and 20 μL PEI with 0.5 mL Opti-MEM I reduced serum medium. After 20 min incubation, 0.5 mL growth media were removed from the wells and replaced by the transfection mixtures. After 16 h incubation, the wells were rinsed with Opti-MEM and 2 mL fresh Opti-MEM supplemented with penicillin-streptomycin was added. The conditioned Opti-MEM media were harvested and the cells were lysed 48 h after medium change.

AR42J rat pancreatic acinar cells were maintained as described previously [30]. Prior to transduction, the cells were cultured in a 12-well tissue culture plate (4×10^5 cells per well) and grown for 48 h in the presence of 100 nM dexamethasone to induce differentiation. Transductions were carried out with 5×10^7 IFU/mL final adenovirus concentration in a total volume of 1 mL Opti-MEM medium supplemented with penicillin-streptomycin and 100 nM dexamethasone. The conditioned media were harvested and the cells were lysed after 48 h incubation.

2.4. Measurement of PNLIP protein secretion

Proteins in the conditioned media (200 μL from HEK 293T and 40 μL from AR42J cells) were precipitated with 10 % trichloroacetic acid and centrifuged for 10 min at 17,000 g. Protein pellets were resuspended in 15 μL Laemmli sample buffer supplemented with 100 mM dithiothreitol, heat denatured at 95 °C for 5 min and electrophoresed on 12 % SDS-polyacrylamide gels. The gels were

stained with Coomassie Brilliant Blue R-250.

2.5. Lipase activity assay

The activity of PNLIP in the conditioned media was measured using *p*-nitrophenyl palmitate (Sigma-Aldrich) substrate based on published protocols with some modifications [33,34]. The substrate was dissolved in isopropanol at a concentration of 3 mg/mL, and diluted to 0.3 mg/mL final concentration with 0.1 M Tris-HCl pH 8.0 buffer containing 1 mg/mL gum arabic and 0.4 mg/mL sodium deoxycholate. Substrate hydrolysis was initiated by mixing 180 μL emulsified substrate with 10 μL 6 μM recombinant human colipase and 10 μL conditioned medium. After 15 min incubation at 22 °C, the reaction was terminated by the addition of 10 μL Triton X-100 and the sample was clarified by 10 min centrifugation at 17,000 g. The released yellow *p*-nitrophenol was quantitated by measuring the absorbance of 100 μL supernatant with a plate reader at 405 nm. The activity value of fresh lipase-free Opti-MEM served as a blank, which was subtracted from the activity of the conditioned medium. OD_{405} values less than 0.5 were accepted, representing the initial velocity of enzymatic reaction.

2.6. Preparation of cell lysates

HEK 293T and AR42J cells were rinsed twice with PBS, scraped from the tissue culture plates in 1 mL PBS and centrifuged for 10 min at 850 g. The cell pellet was lysed with repeated freeze-thaw cycles in 200 μL Promega Reporter Lysis Buffer supplemented with cOmplete Mini Protease Inhibitor Cocktail (Roche). After 5 min centrifugation at 2,400 g, the supernatant was saved and its total protein content was determined with the BCA Protein Assay kit (Pierce).

2.7. Protein immunoblot

Conditioned media from transfected HEK 293T cells (5 μL) and transduced AR42J cells (3 μL), or 10 μg total protein from cell lysates were mixed with 10 μL 2x Laemmli buffer containing 100 mM dithiothreitol. After heat denaturation at 95 °C for 5 min the samples were electrophoresed on a 12 % polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% not-fat milk in PBS supplemented with 0.1 % Tween 20. To detect PNLIP, an anti-His tag antibody conjugated to horseradish peroxidase (HRP) (Qiagen, catalog number 34460) was added at a dilution of 1:2000 for 1 h. To detect Immunoglobulin Binding Protein (BiP, GRP78), the blocked membrane was incubated with rabbit anti-GRP78 antibody (Invitrogen, catalog number PA1-014A) at a dilution of 1:3000 for 1 h. HRP-conjugated anti-rabbit IgG (Bio-Rad, catalog number 1706515) was used as secondary antibody at a dilution of 1:15,000 for 1 h. To detect GAPDH protein the membrane was incubated with rabbit anti-GAPDH antibody (Sigma-Aldrich, catalog number G9545) at a dilution of 1:15,000 for 1 h followed by the addition of HRP-conjugated anti-rabbit IgG as secondary antibody at a dilution of 1:10,000 for 1 h. Protein bands were detected using the WesternBright ECL HRP substrate (Advansta).

2.8. RT-PCR and qPCR analysis

Total RNA from HEK 293T and AR42J cells was isolated using the NucleoSpin RNA Plus kit (Macherey-Nagel). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). X-box Binding Protein 1 (XBP1) mRNA splicing was studied with PCR as described previously [30]. BiP mRNA expression was measured by qPCR (Roche LightCycler 480 II) using TaqMan primers (Thermo Scientific, human Hs00607129_gH, rat Rn00565250_m1) and TaqMan Universal PCR Mastermix (Applied Biosystems). The measurement was carried out following a published protocol [31]: 10 min denaturation and enzyme activation at 95 °C followed by 40 two-step cycles of 15 s at 95 °C and 60 s at 60 °C. Gene expression was quantitated using the comparative CT method (ΔΔCT method). Threshold cycle (CT) values were determined with the LightCycler 480 Software 1.5.0 SP3. Expression levels of BiP was first normalized to the expression levels of GAPDH internal control (Thermo Scientific, human Hs02758991_g1, rat Rn01775763_g1) (ΔCT) and then to expression levels measured in cells infected with 5×10^7 IFU/mL control (vector only) adenovirus (ΔΔCT). Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta CT}$.

3. Results

3.1. PNLIP mutations

In the present study, we characterized four missense PNLIP mutations. Based on one of the available crystal structures, we generated a three-dimensional PNLIP model highlighting the affected amino acid residues (Fig. 1). Mutation sites Ala174, Gly233, and Cys254 are located in the PNLIP N-terminal catalytic domain. Ala174 is part of a structurally important alpha-helix, which is responsible for positioning the catalytic serine residue in the active site. Gly233 is part of the so called β9-loop, while Cys254 forms a disulfide bond with Cys278 and stabilizes another surface loop called the lid domain. These loops control access of the triacylglycerol substrate to the catalytic site. Mutation site Val454 is situated in a surface loop in the C-terminal domain in proximity of the so called β5′-loop. This loop together with the lid facilitate PNLIP binding to lipid droplets. Genetic studies indicated that PNLIP mutations A174P, G233E, C254R and V454F are all heterozygous and were present either in chronic pancreatitis patients or healthy individuals (Table 1).

3.2. Secretion and folding of PNLIP variants in HEK 293T cells

A recent publication described that PNLIP variants A174P, G233E, C254R and V454F are secreted poorly from mammalian cells [29]. To confirm and extend previous observations, we transfected HEK 293T cells with wild-type and mutant PNLIP plasmids and examined lipase levels in the conditioned media after 48 h with reducing SDS-PAGE followed by Coomassie blue staining or immunoblotting (Fig. 2A). In contrast to wild-type PNLIP which was readily secreted, none of the PNLIP mutants were detectable in the conditioned media with this method. Consistent with these findings, lipase activity measurements using the substrate *p*-nitrophenyl palmitate confirmed that only wild-type PNLIP was secreted effectively, as less than 10 % residual lipase activity was observed in the media of cells transfected with mutant PNLIP plasmids (Fig. 2B). As human procolipase can function as a cofactor of PNLIP [35], we co-transfected HEK 293T cells with both expression plasmids and followed mutant PNLIP secretion. The results indicated that the co-expression of procolipase with PNLIP did not improve the secretion of PNLIP variants (not shown).

Previous experiments suggested that the secretion defect of PNLIP variants in HEK 293T cells is due to either poor expression or protein misfolding and intracellular retention. In order to investigate the intracellular levels of PNLIP variants, cells were lysed 48 h after transfection, and PNLIP was detected with reducing SDS-PAGE followed by immunoblotting (Fig. 3). The results revealed that all PNLIP variants were readily expressed, and the levels of PNLIP variants in the cells were comparable with the amount of the wild-

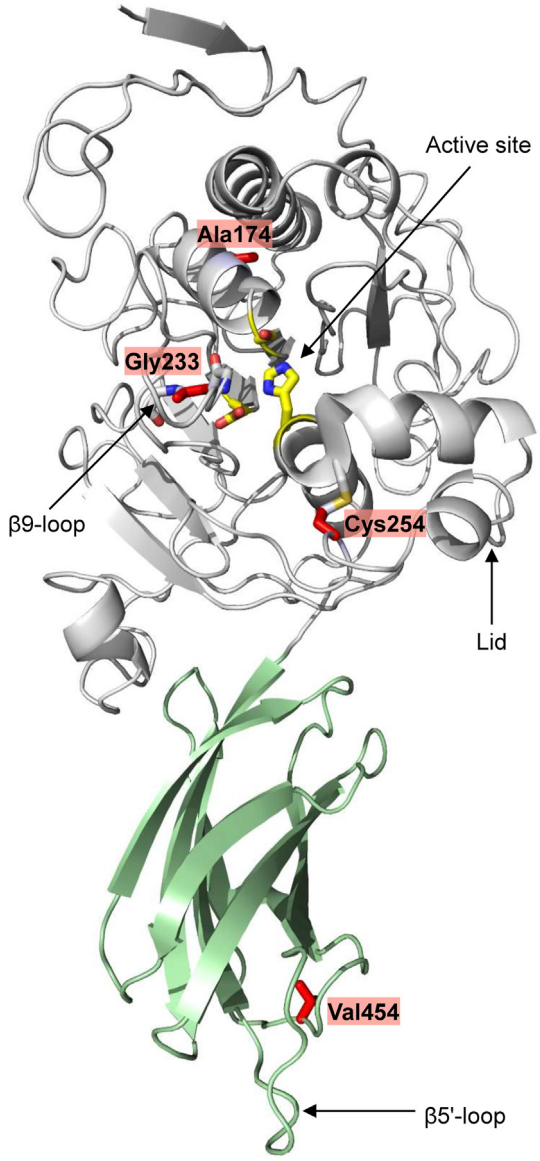

Fig. 1. Ribbon model of human pancreatic lipase (PNLIP) highlighting the studied mutation sites. Affected amino-acid residues are indicated in red, catalytic site residues are shown in yellow. The N-terminal and C-terminal domains of PNLIP are colored in grey and green, respectively. The image was rendered with PyMOL 2.4.1 (Schrödinger LLC) using the atomic coordinates of Protein Data Bank file 1LPB.

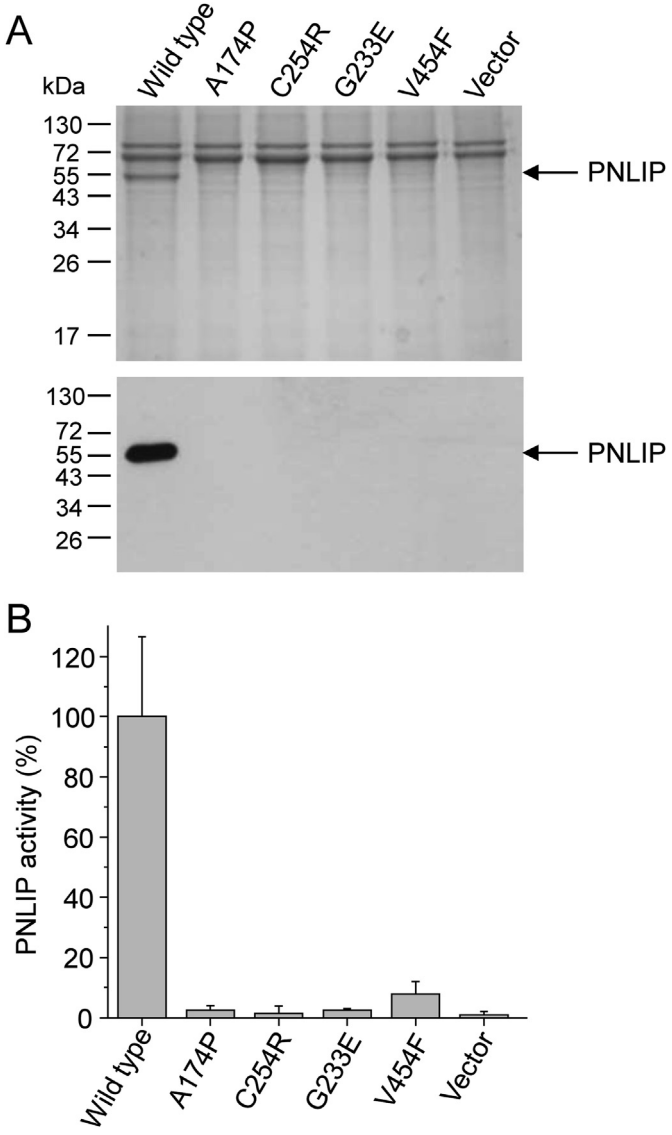

Fig. 2. Secretion of wild-type PNLIP and variants in HEK 293T cells. (A) PNLIP secretion in the conditioned medium was analyzed 48 h after transfection by SDS-PAGE and Coomassie Blue staining (upper panel) and immunoblotting (lower panel), as described in *Methods*. Representative pictures of four experiments are shown. (B) PNLIP activity in the conditioned media was measured using the *p*-nitrophenyl palmitate substrate, as described in *Methods*. Data points represent the average of four experiments ± SD.

Table 1
Missense PNLIP mutations exhibiting reduced secretion identified in patients with chronic pancreatitis (CP carriers) and healthy controls (non-CP carriers).

Exon	Nucleotide change	Amino acid change	CP carriers reported	Non-CP carriers reported	Carrier frequency in gnomAD
6	c.520G > C	p.A174P	0	1	Not reported
8	c.698G > A	p.G233E	1	0	0.0008 %
8	c.760T > C	p.C254R	1	0	0.0035 %
13	c.1360G > T	p.V454F	0	1	0.0040 %

CP, Chronic pancreatitis.
Data were obtained from the publication Lasher et al. [29] and the web site https://gnomad.broadinstitute.org.

type protein. Misfolded proteins may form insoluble intracellular aggregates. Therefore, the level of intracellular PNLIP in the soluble and insoluble fractions were determined by ultracentrifugation of the cell lysates followed by SDS-PAGE and immunoblotting (Fig. 4). As expected, the wild-type PNLIP protein was mostly localized in the soluble fraction, whereas PNLIP mutants were primarily detected in the insoluble pellet.

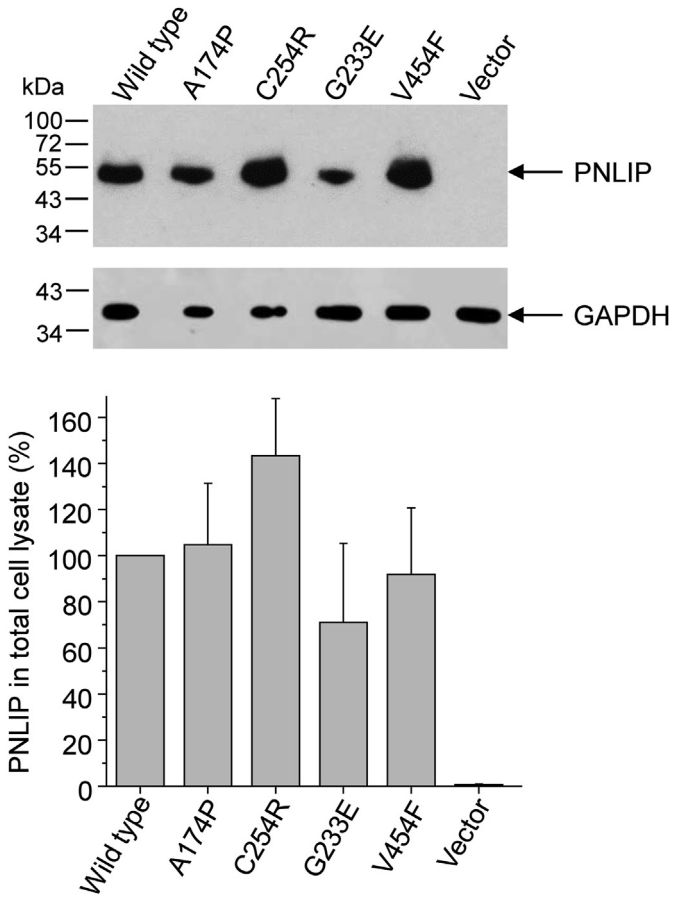

Fig. 3. Expression levels of wild-type PNLIP and variants in HEK 293T cell lysates. Cells were lysed 48 h after transfection. PNLIP in total cell lysates was analyzed with SDS-PAGE followed by immunoblotting as described in *Methods*. As a control, GAPDH was also assessed. Representative pictures of three experiments are shown (upper panel). The relative abundance of PNLIP protein in total cell lysates was quantitated with densitometry (lower panel). The intensity of PNLIP bands was normalized to that of the GAPDH bands and the abundance of PNLIP mutants was expressed as percent of wild-type PNLIP. Data points represent the average of three experiments ± SD.

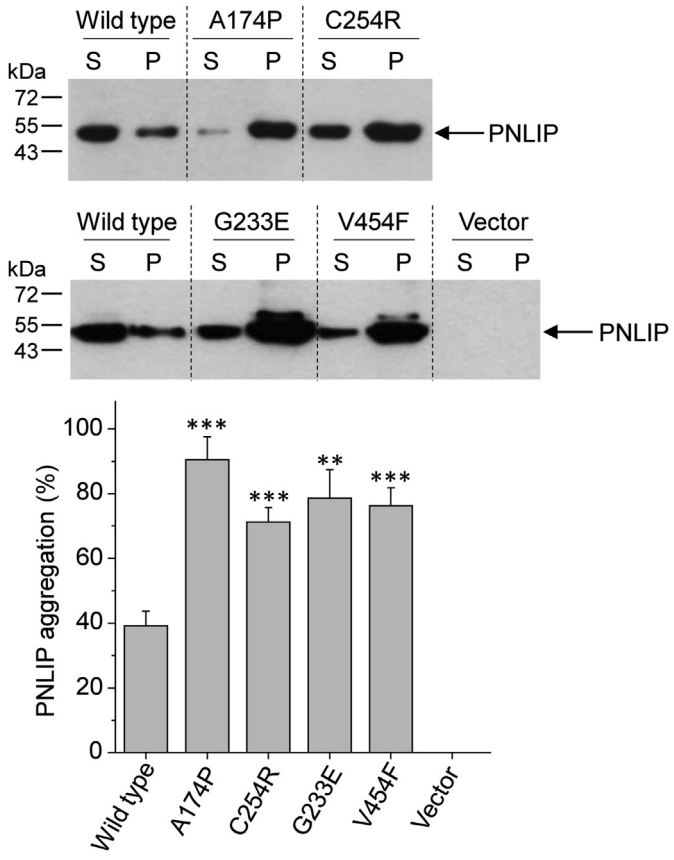

Fig. 4. Intracellular accumulation of PNLIP variants in HEK 293T cells. Cell lysates (0.1 mL) were ultracentrifuged at 90,000 g for 12 min. The supernatants were transferred into new microtubes and the protein pellets were reconstituted in 0.1 mL Laemmli buffer containing 0.1 M dithiothreitol. The levels of PNLIP in the soluble (S) and pellet (P) fractions (5 μL each) were determined by SDS-PAGE followed by immunoblotting (upper panel). Intensity of PNLIP bands were quantitated by densitometry. PNLIP aggregation was calculated from intensity values using the formula P/(S + P)*100 (lower panel). Data points represent the average of three experiments ±SD. **P ≤ 0.01; ***P ≤ 0.001.

3.3. ER stress response in HEK 293T cells

The results described above indicated that PNLIP variants accumulated in HEK 293T cells as intracellular protein aggregates. These observations suggested that PNLIP variants exhibiting a severe secretion defect may induce ER stress. To test this notion, the cells were lysed 48 h after transfection, total RNA was isolated and reverse transcribed. The levels of ER stress markers were determined by analyzing splicing of the XBP1 transcription factor mRNA and by measuring upregulation of the mRNA for the ER chaperone BiP (Fig. 5). As a response to the accumulation of misfolded proteins in the ER, the signal transducer Inositol Requiring Enzyme 1α (IRE1α) processes XBP1 mRNA to a shorter form which serves as a template for XBP1 protein synthesis [36]. XBP1 mRNA splicing was studied with RT-PCR followed by agarose gel electrophoresis (Fig. 5A). We found that XBP1 mRNA splicing was significantly increased in cells transfected with PNLIP variants in contrast to cells expressing wild-type PNLIP.

The expression of the ER chaperone BiP is induced if the load of misfolded proteins in the ER is increased [36]. The level of BiP mRNA in cells transfected with wild-type and mutant PNLIP were assessed using RT-qPCR and TaqMan probes (Fig. 5B). In comparison with cells expressing wild-type PNLIP, a 2.2−3.0-fold increase in BiP mRNA levels was observed in cells expressing PNLIP mutants. The results indicated that the misfolding of PNLIP variants induced ER stress in the HEK 293T cell line.

3.4. Secretion and folding of PNLIP variants in AR42J rat pancreatic acinar cells

AR42J is a more relevant cell line to investigate the secretion and cellular effects of pancreatic secretory protein variants [14,17,30,31]. The only disadvantage of this cell line is the ineffective transfectability. To introduce the coding DNA of PNLIP into AR42J cells, we developed recombinant adenoviral vectors carrying wild-type and mutant PNLIP. The secretion of wild-type PNLIP and variants was studied 48 h after transduction with reducing SDS-PAGE followed by Coomassie staining or immunoblotting (Fig. 6A). Consistent with the results using HEK 293T cells, wild-type PNLIP was readily secreted by AR42J cells. The investigated PNLIP variants resulted in a severe secretion defect, as lipase protein was not detectable in the conditioned media in those cases. We were unable to measure PNLIP activity in the conditioned media of the pancreatic cell line probably due to a highly active endogenous secretory hydrolase enzyme, which quickly degraded the substrate *p*-nitrophenyl palmitate and masked PNLIP activity.

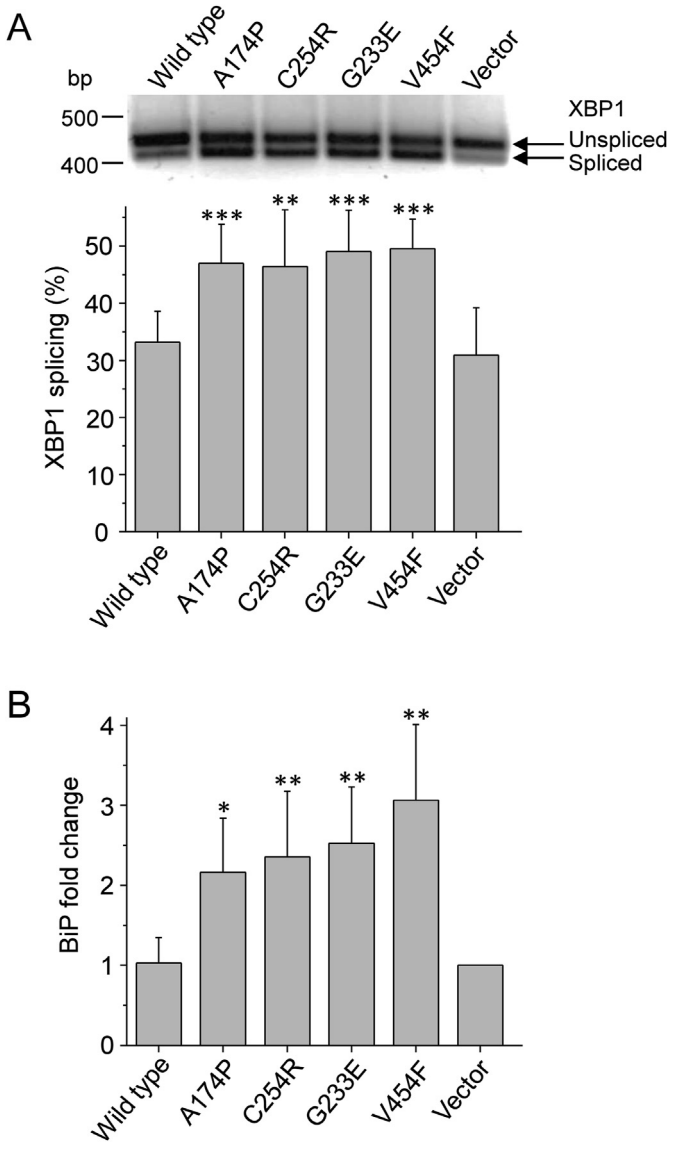

Fig. 5. Effect of PNLIP variants on endoplasmic reticulum (ER) stress markers in HEK 293T cells. Transfected cells were lysed 48 h after transfection, and total RNA was isolated and reverse transcribed. (A) XBP1 mRNA splicing was examined with PCR amplification and agarose gel electrophoresis (upper panel). The intensity of spliced and unspliced DNA bands were quantitated by densitometry. The extent of XBP1 mRNA splicing was calculated using the following formula spliced/(unspliced + spliced)*100 (lower panel). Data points represent the average of nine experiments ± SD. (B) The levels of BiP mRNA expression was determined with quantitative real-time PCR using TaqMan probes as described in *Methods*. BiP expression was normalized to GAPDH mRNA levels and then expressed as fold changes relative to levels measured in cells transfected with pcDNA3.1(−) plasmid DNA. Data points represent the average of six experiments ±SD. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

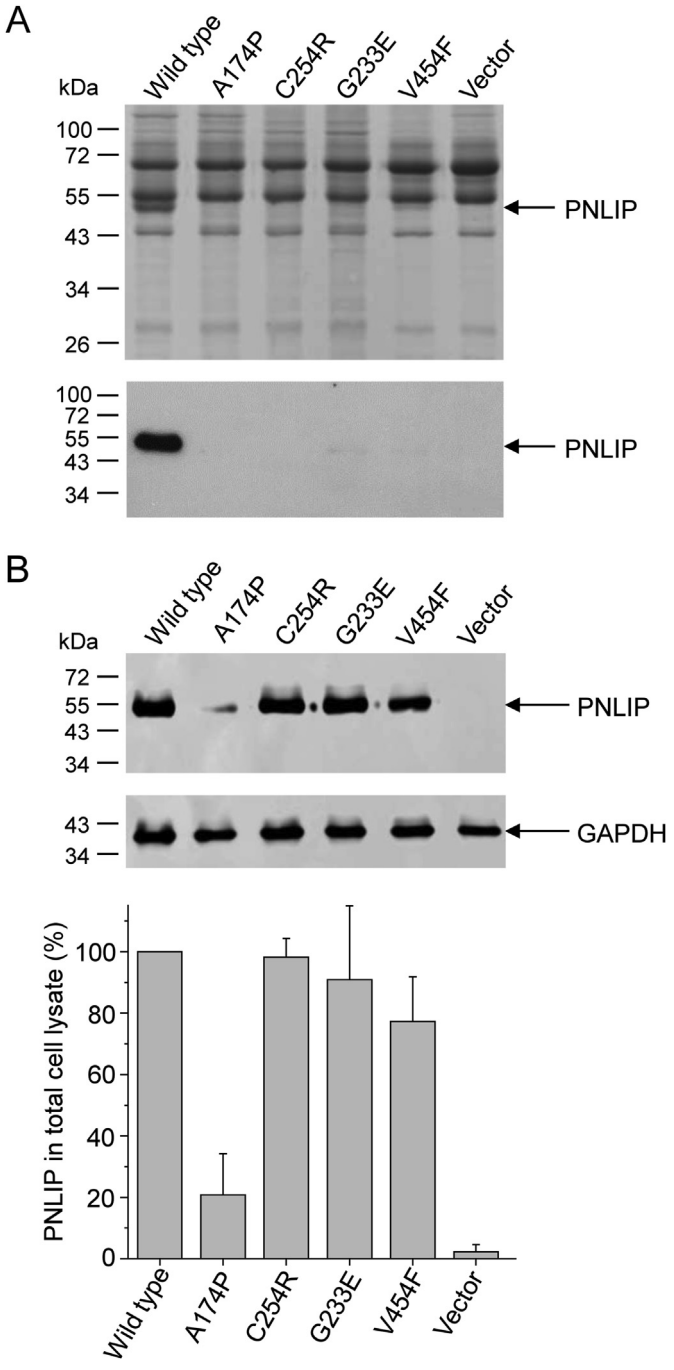

Fig. 6. Expression of wild-type PNLIP and variants in AR42J cells. Cells were transduced by adenoviral vectors carrying the coding DNA for PNLIP, as described in *Methods*. Conditioned media were harvested and cells were lysed 48 h after transduction. (A) Secretion of PNLIP variants in the conditioned medium was assessed by SDS-PAGE and Coomassie Blue staining (upper panel) and immunoblotting (lower panel). Representative pictures of three experiments are shown. (B) Total cell lysates of the transduced cells were examined by immunoblotting. As a control, GAPDH in the cell lysates was also assessed. Representative pictures of three experiments are shown (upper panel). The relative abundance of PNLIP protein in total cell lysates was quantitated with densitometry (lower panel). The intensity of PNLIP bands was normalized to that of GAPDH bands and the abundance of PNLIP mutants was expressed as percent of wild-type PNLIP.

To study the intracellular level of PNLIP variants, AR42J cells were lysed 48 h after transduction and PNLIP was detected with reducing SDS-PAGE followed by immunoblotting (Fig. 6B). We found that the intracellular protein levels of PNLIP variants G233E, C254R and V454F was comparable to that of the wild type. In contrast, the protein levels of variant A174P was substantially reduced possibly due to intracellular degradation.

3.5. ER stress response in AR42J cells

The secretion defect and intracellular retention of PNLIP variants in AR42J cells suggested that ER stress might also develop in this

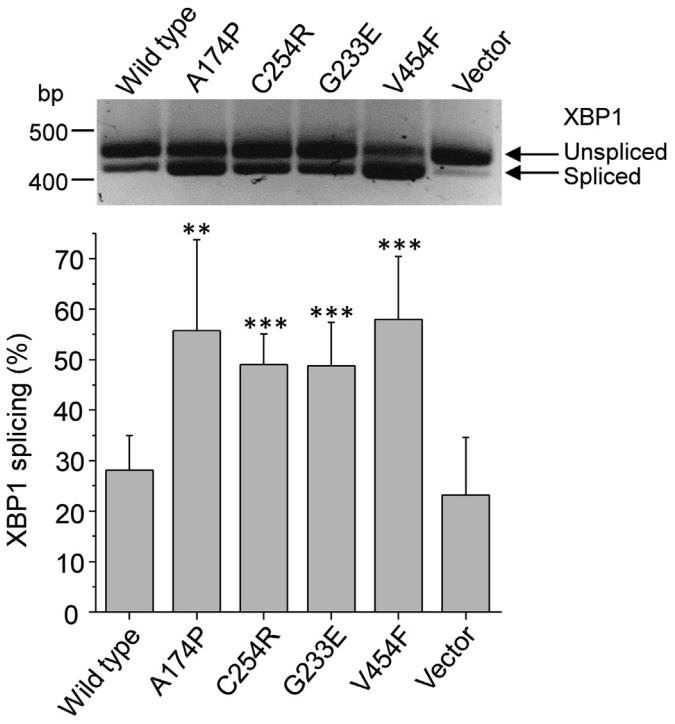

Fig. 7. Effect of PNLIP variants on endoplasmic reticulum (ER) stress marker XBP1 mRNA splicing in AR42J cells. Cells were lysed 48 h after transduction and total RNA was isolated and reverse transcribed. XBP1 mRNA splicing was examined with PCR amplification and agarose gel electrophoresis (upper panel). The intensity of spliced and unspliced DNA bands were quantitated by densitometry. The extent of XBP1 mRNA splicing was calculated using the following formula spliced/(unspliced + spliced)*100 (lower panel). The mean and SD was calculated from six independent experiments. **P ≤ 0.01; ***P ≤ 0.001.

cell line. Therefore, the levels of ER stress markers spliced XBP1 mRNA (Fig. 7) and BiP mRNA (Fig. 8) were assessed 48 h after transduction. Misfolded PNLIP variants in the pancreatic acinar cell line induced XBP1 mRNA splicing about 2-fold (Fig. 7). Additionally, elevated levels of BiP mRNA expression was observed in cells producing PNLIP variants (Fig. 8A). The amount of BiP mRNA was about 4.0–7.6-fold higher in PNLIP A174P, G233E and C254R expressing cells than in cells producing wild-type PNLIP. Interestingly, BiP mRNA levels in acinar cells expressing PNLIP mutant V454F were about 15-fold greater than in wild-type PNLIP producing cells. To validate these results, the levels of BiP protein in AR42J cells expressing wild-type PNLIP and variants were also assessed (Fig. 8B). Surprisingly, BiP protein levels were comparable in cells expressing PNLIP variants A174P, C254R, G233E and wild-type PNLIP, and only a two-fold increase was observed in cells expressing PNLIP variant V454F.

4. Discussion

Recently, a large collaborative study described 19 rare heterozygous PNLIP mutations in cases with non-alcoholic chronic pancreatitis and controls [29]. Functional characterization revealed that the majority of PNLIP variants were readily secreted in transfected cells, with the exception of PNLIP mutants A174P, G233E, C254R and V454F, which exhibited substantially reduced secretion

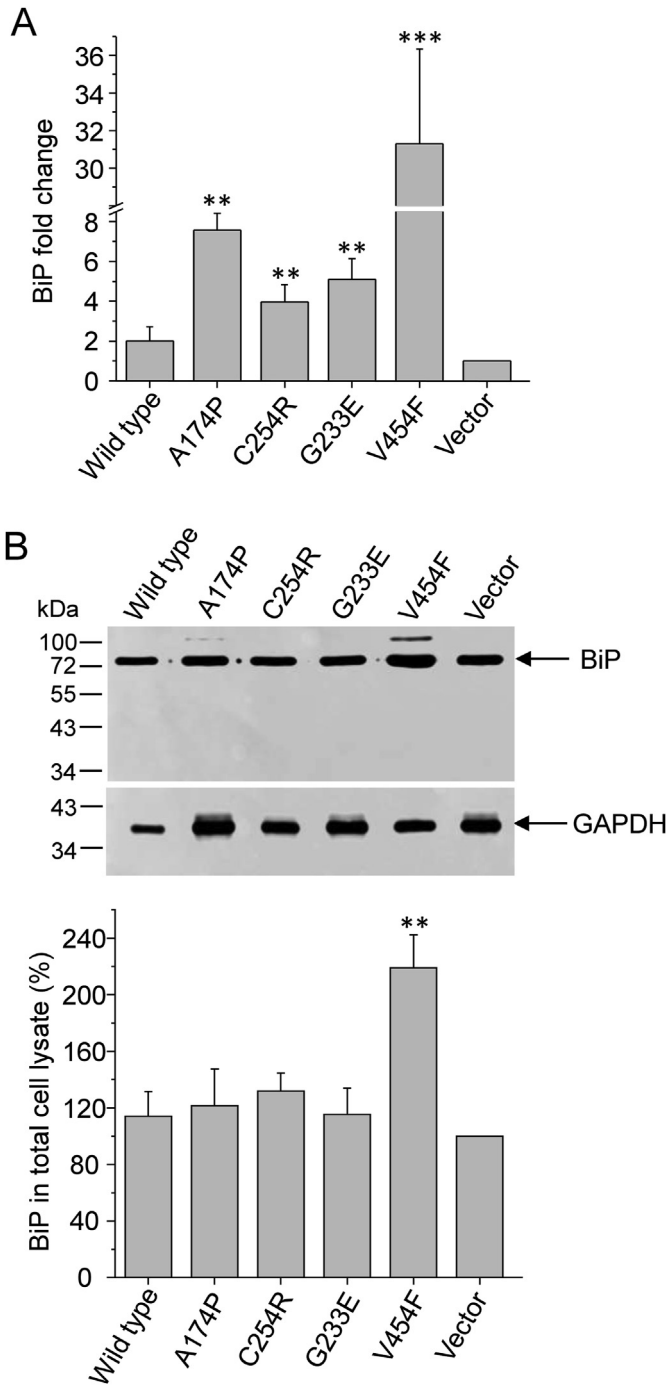

Fig. 8. Effect of PNLIP variants on the expression of endoplasmic reticulum stress marker BiP in AR42J cells. (A) The level of BiP mRNA expression was determined with quantitative real-time PCR using TaqMan probes, as described in *Methods*. Expression was normalized to GAPDH mRNA levels and then expressed as fold changes relative to levels measured in cells transfected with the empty adenovirus vector. Data points represent the average of three experiments ± SD. (B) The level of BiP protein in total AR42J cell lysates was determined by SDS-PAGE and immunoblotting. As a control, GAPDH protein in the cell lysates was also assessed. Representative pictures of three experiments are shown (upper panel). The relative abundance of BiP protein in cell lysates was quantitated with densitometry (lower panel). The intensity of BiP bands was normalized to that of GAPDH bands and then to the level of BiP in cells transduced by empty adenovirus vector. Data points represent the average of three experiments ±SD. **P ≤ 0.01; ***P ≤ 0.001.

[29]. In the present study, structural modeling indicated that PNLIP mutations A174P, G233E and C254R are located in the core of the N-terminal lipase domain in the vicinity of the active site, whereas mutation V454F is situated in the C-terminal domain close to a region responsible for substrate binding. Amino-acid sequence alignment indicated that the affected mutation sites are highly conserved among other human and mammalian pancreatic lipases (not shown). Thus, these residues may play a critical role in stabilizing the structure of lipase. On the basis of these findings, we carried out functional assays using HEK 293T and AR42J cells to study the cellular effects of these four PNLIP variants. Although PNLIP variants were expressed, they were retained and accumulated in the cells as misfolded protein aggregates. Co-expression of PNLIP variants and procolipase did not improve lipase secretion. The intracellular accumulation of misfolded proteins increased the level of ER stress markers such as XBP1 mRNA splicing and BiP mRNA expression in both cell lines. The greatest impact was observed with PNLIP variant V454F which caused more than an order-of-magnitude upregulation of BiP mRNA levels in AR42J cells. Nevertheless, the elevated BiP mRNA levels only slightly increased BiP protein production.

PNLIP missense mutations are rarely described in disease etiology [37,38]. A recent publication reported two brothers from a consanguineous marriage who had congenital lipase deficiency with steatorrhea as the main symptom [37]. The index patients also had exocrine insufficiency indicated by low fecal elastase level which raised the possibility of underlying chronic pancreatitis. Sequencing of the PNLIP gene identified a homozygous missense mutation in both patients [37]. The mutation resulted in Thr221 to Met change in the core of the N-terminal lipase domain. Although the T221M mutation site is located in the vicinity of the active site, functional characterization revealed that reduced PNLIP secretion is due to protein misfolding, intracellular retention and accumulation rather than impaired lipase activity [30]. The misfolded T221M PNLIP protein also caused ER stress demonstrated by increased XBP1 mRNA splicing and BiP expression. Therefore, it is possible that the homozygous form of this PNLIP mutation play a role in chronic pancreatitis development as robust misfolding of pancreatic secretory proteins and the activation of ER stress pathways are risk factors of the disease [30,39]. The parents of the patients who were heterozygous carriers of the mutation did not have any symptoms. It is likely that the T221M mutation in heterozygous form does not cause pancreatitis, although no clinical testing has been done on the parents [37]. More recently, another case of congenital pancreatic lipase deficiency has been described in identical twin boys [38]. Sequencing of the PNLIP gene revealed two compound heterozygous mutations (W102X and R188C) affecting both PNLIP alleles of the patients. Nonsense mutation W102X was predicted to cause loss of function via early termination of PNLIP protein synthesis, whereas missense mutation R188C likely results in protein misfolding through the disruption of normal disulfide bond formation. Although functional characterization has not been performed yet, the combined effect of these mutations may be similar to that of the homozygous T221M mutation.

Protein misfolding and ER stress is a common pathological pathway in chronic pancreatitis onset and progression [39]. CPA1 is one of the major digestive proteases secreted by the human pancreas [25,26]. Heterozygous CPA1 mutations characterized by reduced secretion and the development of strong ER stress were identified in cases with chronic pancreatitis [14]. CPA1 mutation N256K which triggers protein misfolding and secretion defect was over-represented in disease cases. Knock-in mouse model of CPA1 N256K indicated that the mutation induces misfolding and the upregulation of ER stress markers causing spontaneous and progressive chronic pancreatitis [40]. Cationic trypsinogen, encoded by the PRSS1 gene, is also secreted in high quantities by the human exocrine pancreas. Studies demonstrated that infrequent heterozygous mutations in PRSS1, such as R116C and C139S, associated with chronic pancreatitis resulted in a substantially reduced trypsinogen secretion due to protein misfolding and intracellular retention [17,39]. Functional characterization of transfected cells indicated that the misfolded PRSS1 protein caused pancreatitis through increased level of ER stress. Although PNLIP is one of the most abundant enzyme secreted by the human exocrine pancreas, its expression is probably lower than that of CPA1 and PRSS1 based on protein abundance analysis of human pancreatic juice [25] and mRNA expression data available on the Genotype-Tissue Expression (GTEx) Portal (https://www.gtexportal.org/home) [41]. Our results suggest that due to the lower expression, the heterozygous PNLIP mutations studied here probably induce milder ER stress and cellular injury than the misfolding CPA1 and PRSS1 mutations associated with chronic pancreatitis. In contrast, the previously characterized homozygous T221M misfolding PNLIP mutation might have a much greater damaging impact on acinar cells.

In summary, we characterized the cellular effects of four potentially pathogenic PNLIP mutations in cell culture models. We found that mutations A174P, G233E, C254R and V454F caused PNLIP misfolding, intracellular accumulation as protein aggregates and ER stress. On the basis of our results we anticipate that the investigated PNLIP mutations are risk factors of chronic pancreatitis but in the heterozygous form they require additional risk factors to cause the disease.

Acknowledgements

This work was supported by the Development and Innovation Office of Hungary grant FK127942, the János Bolyai Research Scholarship of the Hungarian Academy of Sciences grant (BO/00514/19/5) and the ÚNKP-20-5 New National Excellence Program of the Ministry for Innovation and Technology of Hungary (to AS). Miklós Sahin-Tóth and Zoltán Balajthy are gratefully acknowledged for critical reading of the manuscript and helpful suggestions. The authors declare that they have no conflicts of interest with the contents of this article.

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