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RESEARCH ARTICLE

Signal Peptide Variants That Impair Secretion of Pancreatic Secretory Trypsin Inhibitor (SPINK1) Cause Autosomal Dominant Hereditary Pancreatitis

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Variants of the *SPINK1* gene encoding pancreatic secretory trypsin inhibitor have been described in association with chronic pancreatitis (CP). These alterations are believed to cause a loss of function by either impairing the trypsin inhibitory activity or reducing expression. Here we report two novel *SPINK1* variants in exon 1 that affect the secretory signal peptide. The disease-associated c.41T>G (p.L14R) alteration was found in two European families with autosomal dominant hereditary pancreatitis, whereas the c.36G>C (p.L12F) variant was identified as a frequent alteration in subjects of African descent. The functional effects of both alterations and the previously reported c.41T>C (p.L14P) variant were characterized by activity assays and Western blots of wild-type and mutant SPINK1 expressed in human embryonic kidney 293T and Chinese hamster ovary cells. Alterations p.L14R and p.L14P destined the inhibitor for rapid intracellular degradation and thereby abolished SPINK1 secretion, whereas the p.L12F variant showed no detrimental effect. The results provide the first clear experimental demonstration that alterations that markedly reduce *SPINK1* expression are associated with classic hereditary pancreatitis. Therefore, these variants should be classified as severe and regarded as disease-causing rather than disease-modifiers. Hum Mutat 28(5), 469–476, 2007. Published 2007 Wiley-Liss, Inc.†

KEY WORDS: pancreatic secretory trypsin inhibitor; PSTI; chronic pancreatitis; signal-peptide alteration; hereditary pancreatitis; human cationic trypsin; SPINK1

INTRODUCTION

The pancreatic secretory trypsin inhibitor is a 6.5-kDa protein synthesized, stored, and secreted by the acinar cells of the exocrine pancreas. In humans, the inhibitor is encoded by the *SPINK1* (Serine Protease Inhibitor, Kazal type 1) gene on chromosome 5q32 (MIM# 167790). As a typical secretory protein, human SPINK1 contains a 23-amino-acid signal peptide (Fig. 1), which directs the protein to the endoplasmic reticulum, where, after translocation to the lumen and removal of the signal peptide, SPINK1 achieves its mature fold. It has long been assumed that the physiological role of SPINK1 is to protect the pancreas against premature trypsinogen activation by rapidly complexing free trypsin [Rinderknecht, 1986].

The strongest indication that SPINK1 plays an essential protective role in pancreatic physiology came from relatively recent genetic studies, describing an association between the *SPINK1* c.101A>G (p.N34S) variant [Chen et al., 2000] and chronic pancreatitis (CP) [Witt et al., 2000]. Data taken from eight larger studies in Europe and the United States indicate that 12.6% of patients with CP were heterozygous for N34S, and 3.6%

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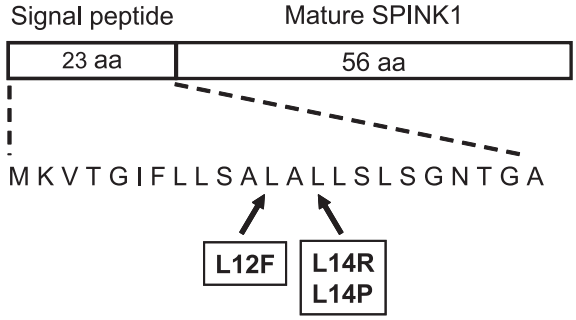

FIGURE 1. **Primary structure of human pancreatic secretory trypsin inhibitor (SPINK1). The amino acid sequence and alterations of the secretory signal peptide are indicated in the single-letter code.**

were homozygous [Witt et al., 2000; Pfützer et al., 2000; Plendl et al., 2001; Chen et al., 2001; Noone et al., 2001; Drenth et al., 2002; Threadgold et al., 2002; Truninger et al., 2002]. In contrast, homozygous individuals were never identified among these controls, whereas heterozygosity was detected at 1.9% on average. Thus, the large majority of carriers never develop pancreatitis. In addition to these studies, N34S was also recognized as a major genetic risk factor in tropical pancreatitis in the Indian subcontinent [Chandak et al., 2002; Hassan et al., 2002; Bhatia et al., 2002; Schneider et al., 2002] and as a minor susceptibility factor in alcoholic CP [Witt et al., 2001b]. The mode of inheritance of the N34S alteration in CP has been debated in the initial reports. The first study suggested that N34S may cause pancreatitis by an autosomal recessive mode with low penetrance (~25%) or in an oligogenic fashion [Witt et al., 2000]. Subsequently, based on negative genetic linkage studies, the lack of difference in age of onset between heterozygous and homozygous individuals and the observation that heterozygosity for N34S is common in the population, it was proposed that the N34S alteration is a "disease modifier," which does not cause pancreatitis, but modifies the effect of other disease-causing genes [Pfützer et al., 2000]. More recently, the N34S allele has been viewed as a relatively frequent genetic susceptibility factor to CP, which exerts its effect in the context of other genetic and environmental factors, resulting in the highly variable penetrance and expression of the disease. Because SPINK1 is supposed to protect the pancreas and the N34S variant seems to increase the risk of pancreatitis, the straightforward conclusion was drawn that the N34S alteration causes a loss of inhibitor function [Witt et al., 2000]. However, such a functional defect has not so far been demonstrated, as a recent study indicated that recombinant N34S SPINK1 retains full inhibitory activity [Kuwata et al., 2002].

In addition to the N34S alteration, several other *SPINK1* variations have been described in pancreatitis patients as well as healthy controls (see www.uni-leipzig.de/pancreasmutation). The large majority of alterations found in pancreatitis patients represent rare variants; therefore, disease-association and mode of inheritance has not been conclusively determined. Nevertheless, in some cases, the nature of the alteration indicates an obligatory loss of SPINK1 expression, either due to splicing problems (c.87+1G>A; c.194+2T>C), shift of reading frame with premature termination (c.98dupA; c.27delC), loss of the initiator methionine codon (c.2T>C; p.M1?), or large genomic deletions in the *SPINK1* gene [Witt et al., 2000; Gaia et al., 2002; Le Maréchal et al., 2004; Masson et al., 2006].

Here we report that *SPINK1* alterations in the signal peptide are associated with a severe pancreatitis phenotype and these alterations result in decreased secretion of the mature SPINK1 peptide due to intracellular degradation.

MATERIALS AND METHODS

Plasmid construction, transfection, and Western blot analysis were executed independently in the Brest and Boston laboratories using somewhat different methods. Unless indicated otherwise, the Materials and Methods section refers to the experiments performed in Boston.

Study Subjects

The study was approved by the local ethical review committees. All patients gave their informed consent for genetic analysis. The study included 574 patients with hereditary or idiopathic CP originating from France (n = 320) and Germany (n = 254). All 254 CP patients originating from Germany (144 female, 110 male; mean age ± standard deviation [SD], 11.9 ± 6.9 years) were of German ethnicity. Of these subjects, 65 patients (42 female, 23 male; mean age ± SD, 12.1 ± 7.9 years) were classified as having hereditary pancreatitis and 189 patients (102 female, 87 male; mean age ± SD, 11.8 ± 6.5 years) were classified as having idiopathic CP. The clinical diagnosis of CP was based on two or more of the following criteria: presence of a typical history of recurrent pancreatitis, radiological findings such as pancreatic calcifications and/or pancreatic ductal irregularities revealed by endoscopic retrograde pancreaticography or magnetic resonance imaging of the pancreas, and/or pathological sonographic findings. Hereditary CP was diagnosed when two first-degree relatives, or three or more second-degree relatives, suffered from recurrent acute or CP without any apparent precipitating factor. Patients were classified as having idiopathic CP when precipitating factors such as alcohol abuse, trauma, medication, infection, metabolic disorders, and/or a positive family history were absent. Altogether 589 control subjects were included. The French controls consisted of 98 unrelated healthy bone marrow donors from Brest recruited between 1998 and 2000. The 340 German control subjects (181 female, 159 male; mean age ± SD, 56.9 ± 29.2 years) consisted of 162 medical students and staff recruited in Berlin between 1996 and 1998 and 178 healthy individuals recruited in Berlin for the Berlin Aging Study (BASE) between 1993 and 1995. To determine the frequency of *SPINK1* variants in non-Caucasians, we studied 151 individuals of African descent originating from Cameroon who were recruited for genetic population studies between 1986 and 1990 (132 female, 19 male; mean age ± SD, 33.8 ± 6.6 years).

Numbering of genetic variants is based on the cDNA sequence, indicated by a "c." before the number [den Dunnen and Paalman, 2003]. Position+1 corresponds to the A of the ATG translation initiation codon located at nucleotide 121 in the NM_003122.2 *SPINK1* mRNA reference sequence.

Plasmid Construction

The *SPINK1* cDNA was amplified from IMAGE clone #1679809 (GenBank AA844948) using the oligonucleotide primers SPINK1_XhoI_sense 5′-CGG TGC TCG AGT CAA CTG ACC TCT GGA CGC AGA ACT TC-3′ and SPINK1_-BamHI_antisense 5′-ATA CAG GAT CCC AAC AAT AAG GCC AGT CAG GCC TCG CGG-3′. The PCR product was digested with *Xho*I and *Bam*HI and ligated into the expression vector pcDNA3.1(–) (Invitrogen) digested with the same enzymes. Signal peptide mutants and the Glu-Glu-tagged SPINK1

constructs were generated by overlap extension PCR mutagenesis and ligated into pcDNA3.1(–). The Glu-Glu tag sequence (EYMPME) is derived from the polyoma virus medium T antigen [Grussenmeyer et al., 1985].

Cell Culture and Transfection

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin/streptomycin solution at 37°C in a humidified atmosphere containing 5% CO_2. A day before transfection, approximately 10^6 cells were plated in six-well tissue culture plates in culture medium without antibiotics. Transfections were performed using 4 μg pcDNA3.1(−)_SPINK1 plasmid, 2 μg pSV-β-galactosidase control plasmid (Promega, Madison, WI), and 10 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 2 mL OptiMEM medium supplemented with 2 mM glutamine. After 5 hr incubation at 37°C, 2 mL of feed medium (DMEM with 20% fetal bovine serum, 4 mM glutamine) was added to each well and cells were incubated for an additional 24 hr. Cells were then washed with 1 mL OptiMEM supplemented with 2 mM glutamine, and the transfection medium was replaced with 2 mL OptiMEM supplemented with 2 mM glutamine. Time-courses of expression were measured starting from this medium change and were followed for 48 hr. At the indicated times, 200 μL conditioned medium (i.e., 10% of total volume) was removed from each well for activity assays and replaced with 200 μL fresh medium. At the conclusion of the transfection experiments, conditioned media and cells were harvested for Western blot analysis and β-galactosidase assays.

Trypsin Inhibitory Activity Determinations

Conditioned media from transfected cells were centrifuged for 5 min and 40 μL of the supernatant was mixed with 4 mg/mL bovine serum albumin and 80 nM recombinant human cationic trypsin (final concentrations) in a final volume of 50 μL and incubated for 1 min at room temperature. The chromogenic substrate CBZ-Gly-Pro-Arg-p-nitroanilide (150 μL) was then added to 0.14 mM concentration and 1 min rates of p-nitroaniline release were measured at 405 nm. Residual trypsin activity was expressed as percentage of the uninhibited maximal activity determined in the presence of 40 μL fresh medium devoid of SPINK1. Residual trypsin activities were converted to inhibitor concentrations using a calibration curve obtained by assaying serial dilutions of purified recombinant SPINK1.

β-Galactosidase Activity Assays to Determine Transfection Efficiency

Cells were cotransfected with 2 μg pSV-β-galactosidase plasmid. At 48 hr after transfection, the growth medium was removed and the cells were washed twice with phosphate buffered saline (PBS). Reporter Lysis Buffer (Promega; 200 μL) was added to each well and the plate was incubated for 15 min at room temperature. Cells were then scraped from the wells, and the lysates were briefly vortexed and centrifuged for 5 min at 4°C. Supernatants were assayed for β-galactosidase activity by measuring rates of o-nitrophenyl β-D-galactopyranoside hydrolysis using the β-galactosidase Enzyme Assay System (Promega). The resulting β-galactosidase activity rates were used to normalize SPINK1 expression to variations in transfection efficiency. SPINK1 concentrations were divided by the corresponding β-galactosidase activity values and multiplied by the average of β-galactosidase activities from all transfections in the experiment.

Total RNA Isolation and RT-PCR

RNA was isolated from cells transfected with wild-type and mutant *SPINK1* constructs using the RNAqueous kit (Ambion, Austin, TX) and 0.75 μg RNA was reverse-transcribed with the SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA). *SPINK1* cDNA was measured by semiquantitative PCR using the gene-specific primer pair described in "Plasmid Construction." As a reference control, the cDNA of the glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) gene was also amplified using the pseudogene-free amplification conditions described by Harper et al. [2003].

Western Blotting

Samples of conditioned medium or cell extracts were run on a 21% Tris-glycine gel under reducing conditions and electrophoretically transferred onto an Immobilon-P membrane (Millipore, Billerica, MA) at 300 mA for 3 h. To detect the Glu-Glu-tag, the membrane was blocked with 5% milk powder solution overnight and incubated with an horseradish peroxidase (HRP)-conjugated goat polyclonal antibody raised against the Glu-Glu tag (Abcam, Cambridge, MA) at a concentration of 0.1 μg/mL for 1 hr at room temperature. When untagged proteins were analyzed, the membrane was blocked in 5% milk/3% bovine serum albumin (BSA) overnight and hybridized with anti-human SPINK1 rabbit serum at a dilution of 1:5,000 for 1 hr at room temperature. The secondary antibody (ImmunoPure peroxidase-conjugated goat anti-rabbit immunoglobulin G [IgG]; Pierce, Rockford, IL) was applied at a dilution of 1:50,000 for 1 hr. HRP was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Western blot analysis of conditioned media of Chinese hamster ovary (CHO) cells was performed using the PSKAN2 monoclonal mouse IgG antibody (Mobitec; Göttingen, Germany) at a 1:250 dilution, followed by peroxidase-conjugated anti-mouse IgG (Amersham, Orsay, France) at a 1:10,000 dilution.

RESULTS

SPINK1 Signal Peptide Variants in Patients With Chronic Pancreatitis

Screening a previously described cohort of patients with familial or hereditary idiopathic pancreatitis from France for alterations in the *SPINK1* gene by DHPLC [Le Maréchal et al., 2004] identified a heterozygous T to G transversion at position c.41 (Supplementary Fig. S1A; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat) in a family of Bulgarian origin (Fig. 2). This c.41T>G alteration resulted in the replacement of the leucine at amino acid position 14 with an arginine (p.L14R; Fig 1). The 20-year-old index patient was diagnosed at 10 years of age. His father died from an acute attack of pancreatitis, and because the mother tested negative, he was an obligate carrier of the alteration. The father's mother was diagnosed with CP at the age of 59 years and was also heterozygous for L14R. Thus, the alteration segregated with pancreatitis over three generations, with complete penetrance. We also analyzed 254 German patients with CP by direct DNA sequencing [Witt et al., 2000] and detected the L14R alteration in a 9-year-old male patient from the county of Niedersachsen (Lower Saxony, Germany) (Fig. 2). The alteration was present in the unaffected father and the affected grandmother. The great-grandmother also suffers from CP, but was not available for genetic testing. L14R was absent in 98 unrelated French healthy bone marrow donors, and in 340 German control subjects.

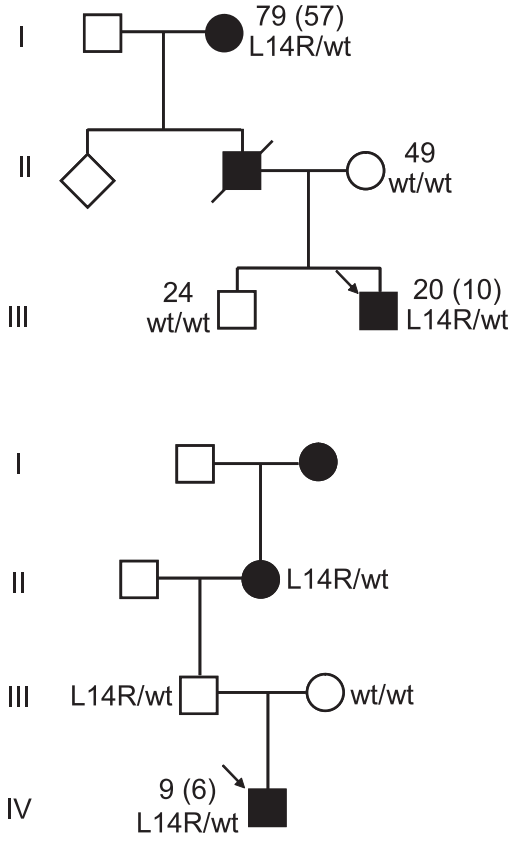

FIGURE 2. **Identification of a new *SPINK1* missense alteration p.L14R, in two families with hereditary pancreatitis. Solid symbols represent patients with pancreatitis; open symbols indicate individuals without pancreatitis; the arrow points to the index patient. The numbers next to the symbols denote the current age and the age of disease onset (in parentheses). The genotypes of family members available for genetic analysis are also indicated. Upper panel: Pedigree of the Bulgarian family living in France. Lower panel: Pedigree of the German family.**

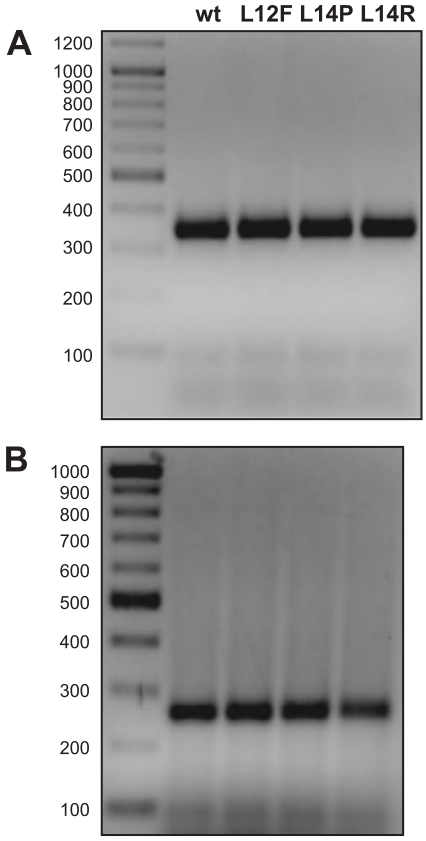

FIGURE 3. **Effect of signal peptide alterations on *SPINK1* mRNA levels. Wild-type (wt) and L12F, L14R, and L14P mutant SPINK1 were transiently expressed in HEK 293T cells and total RNA was prepared and reverse-transcribed as described in Materials and Methods. The cDNA for *SPINK1* (A) and glyceraldehyde-3-phosphate dehydrogenase (B) were PCR-amplified and the semiquantitative PCR reactions were analyzed by agarose gel electrophoresis and ethidium bromide staining.**

No other pathogenic *PRSS1*, *CFTR*, or other *SPINK1* alterations were detected in the two families with the L14R alteration.

The c.41T>C (p.L14P) alteration was identified in 2000 in a 5-year-old German pediatric patient without a family history [Witt et al., 2000]. The mother of the index patient carried the alteration, but had no pancreatitis. In the meantime, however, the mother also developed pancreatitis at the age of 41 years. This family was also negative for *PRSS1*, *CFTR*, or other *SPINK1* alterations. L14P affects the same amino acid within the hydrophobic core of the signal peptide as the newly-found L14R alteration. The strong association with pancreatitis indicates that both the L14R and L14P alterations can be regarded as relatively severe susceptibility factors for CP.

We found another novel SPINK1 variant that altered the signal peptide, c.36G>C (p.L12F; Supplementary Fig. S1B), in 15 African subjects with idiopathic pancreatitis living in France. However, this variant turned out to be a common alteration in Africans, with an allele frequency of 11% (33/302 alleles). The L12F variation was not detected in Caucasian patients and controls from France or in 323 pancreatitis patients and 340 controls from Germany.

Signal Peptide Variants Do Not Affect mRNA Levels

Functional characterization of the effects of the signal peptide variants was performed by transiently transfecting HEK 293T cells with cDNA constructs encoding wild-type, L14P, L14R, or L12F SPINK1 variants. To measure transfection efficiency, cells were routinely cotransfected with a plasmid encoding β-galactosidase. Semiquantitative PCR analysis of cDNA preparations from transfected cells yielded products of equal intensity, indicating that mRNA levels expressed from the wild-type and the L14P, L14R, or L12F constructs are comparable (Fig. 3).

Pancreatitis-Associated Variations L14P and L14R Abolish SPINK1 Secretion

Secretion of SPINK1 protein by transfected cells was monitored by measuring the inhibitory activity of conditioned media against human cationic trypsin (Fig. 4). After transfection with wild-type or L12F *SPINK1* cDNA, trypsin inhibitory activity in the medium of HEK 293T cells steadily increased during the 2-day time-course followed, whereas media from cells transfected with the L14R and L14P mutants showed minimal or no trypsin inhibitory activity, respectively. Residual trypsin activities were converted to actual SPINK1 concentrations using a calibration curve obtained by assaying serial dilutions of purified recombinant SPINK1. The resulting concentration values were then normalized to transfection efficiency using the β-galactosidase activity of the transfected cells. Time-courses of SPINK1 secretion expressed as normalized SPINK1 concentrations are presented in Figure 5. Clearly,

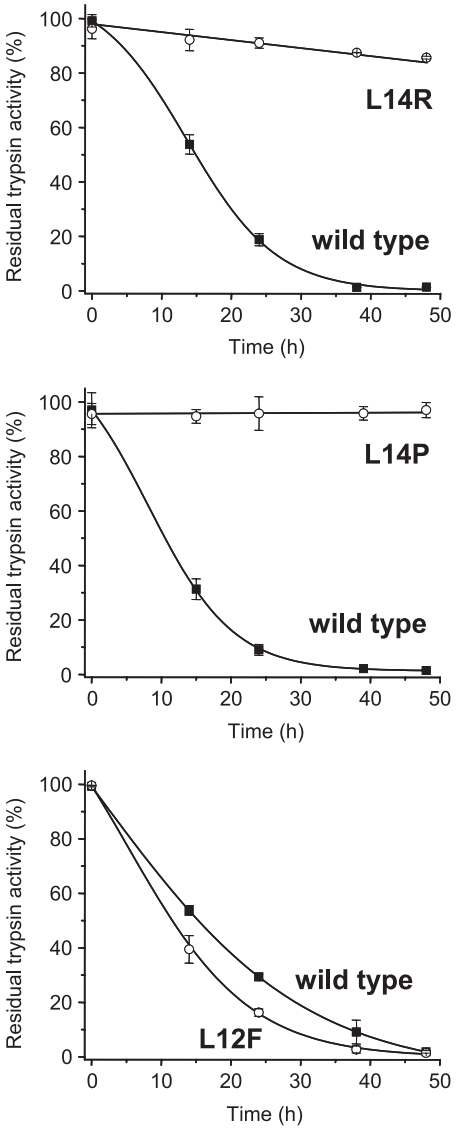

FIGURE 4. **Trypsin inhibitory activity in conditioned media of HEK 293T cells transfected with SPINK1 mutants. At the designated times, the capacity of conditioned media to inhibit human cationic trypsin was determined, as described in Materials and Methods. Residual trypsin activity is expressed as percentage of uninhibited trypsin activity. Data shown represent the mean of two parallel transfections, with the deviation from the mean indicated by the error bars. Solid squares, wild-type SPINK1; open circles, mutant SPINK1.**

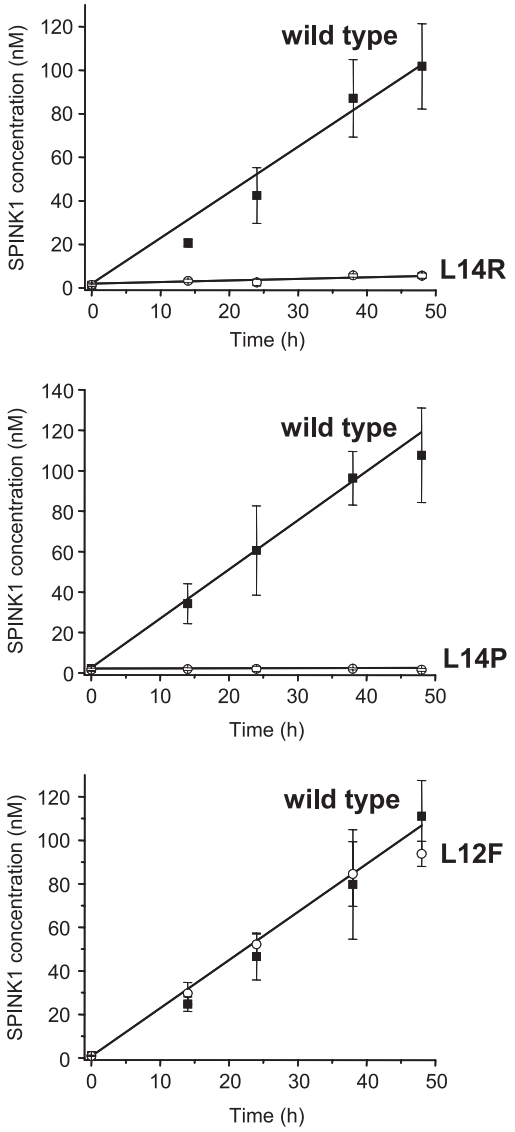

FIGURE 5. **Secretion of wild-type and mutant SPINK1 from HEK 293T cells. SPINK1 inhibitory activity was determined against human cationic trypsin as described in Materials and Methods and as shown in Fig. 4. Inhibitory activities were converted to SPINK1 concentrations using a calibration curve prepared with purified, recombinant SPINK1 and human cationic trypsin. For each transfection, a plasmid encoding β-galactosidase was cotransfected and at 48 hr β-galactosidase activity was determined from the lysed cells, as a measure of transfection efficiency. SPINK1 concentrations were then normalized to β-galactosidase activity. Data represent the mean ± SD of four experiments. Solid squares, wild-type SPINK1; open circles, mutant SPINK1.**

pancreatitis-associated alterations L14R and L14P abolish secretion, whereas the L12F variant has no effect.

To demonstrate that the lack of trypsin inhibitory activity was the result of impaired secretion, aliquots of conditioned media were analyzed by SDS-PAGE and Western blotting. First, a rabbit polyclonal antibody raised against recombinant human SPINK1 was used. As shown in Figure 6A, wild-type and L12F SPINK1 proteins were visualized at comparable levels, whereas the L14P variant was not detected, and the L14R variant exhibited markedly reduced expression. Interestingly, the faint band observed for the L14R variant migrated slower than wild-type SPINK1, indicating that signal peptide processing probably occurred at an anomalous site. Indeed, analysis of the SPINK1 signal peptide sequence with the SignalP program (Center for Biological Sequence Analysis, Technical University of Denmark; www.cbs.dtu.dk/services/SignalP) revealed that the L14R variant might be processed at the Ser18-Gly19 peptide bond resulting in a mature peptide with an additional five amino acids (GNTGA) at the N-terminus.

Because the L14R and L14P alterations occur in a heterozygous form in patients, we have also performed cotransfection experiments with wild-type and variant *SPINK1* cDNAs. Consistently, we found a slight to moderate increase in the secreted SPINK1 activity, which appeared to be due to increased secretion of the

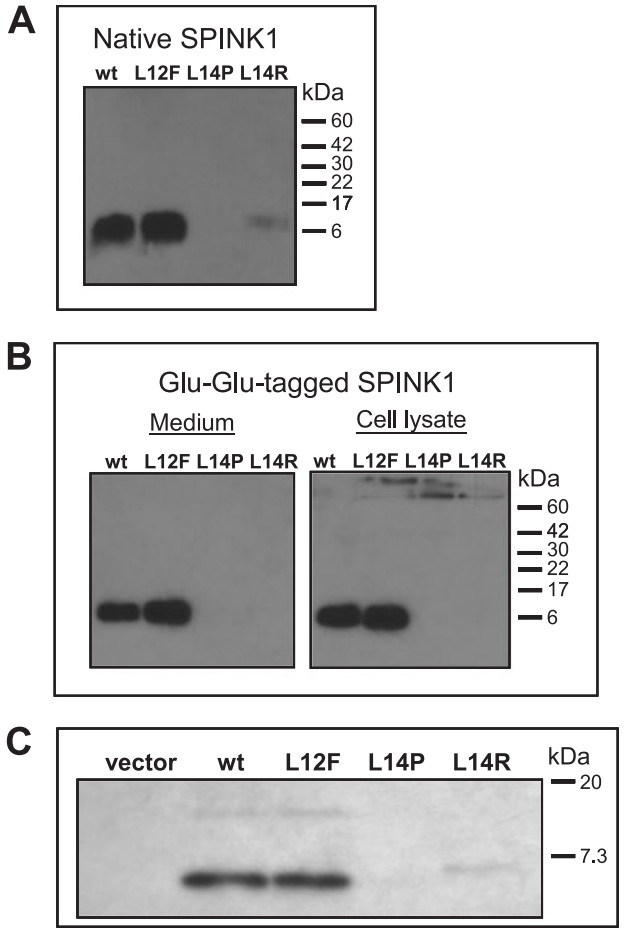

FIGURE 6. **Western blot analysis of SPINK1 expression in conditioned media and cell lysates of transfected HEK 293T cells. Aliquots of conditioned media (5 μL) and cell lysates (10 μL; ~3.5 mg/mL protein concentration) harvested 48 hr posttransfection were subjected to polyacrylamide gel electrophoresis, transferred to Immobilon P membranes, and immunodetection was performed as described in Materials and Methods. A: SPINK1 in conditioned media was detected with a polyclonal rabbit antibody raised against recombinant human SPINK1. B: Expression of epitope-tagged SPINK1 proteins in conditioned media and cell lysates was visualized with a commercial anti-Glu-Glu tag antibody (Abcam). C: SPINK1 in the conditioned media of CHO cells was detected with the PSKAN2 monoclonal mouse IgG antibody against human SPINK1 (Mobitec; Göttingen, Germany).**

wild-type form, as judged by immunoblot analysis of differentially tagged SPINK1 proteins (not shown). These observations may indicate that in the presence of an inactive allele, compensatory mechanisms increase expression of the wild-type allele. On the other hand, cotransfection experiments do not appropriately model chromosomal biallelic expression and other interpretations of the results (e.g., homologous recombination between transfected plasmids) are also possible.

To investigate the intracellular fate of SPINK1 variants, epitope-tagged versions of SPINK1 constructs were expressed and analyzed. Epitope tagging became necessary because the rabbit polyclonal antibody gave strong nonspecific and/or cross-reaction signals when tested on cell lysates. We placed the so-called Glu-Glu tag (EYMPME) at the C-terminus of SPINK1. The epitope-tagged SPINK1 construct was as efficiently secreted as untagged wild-type SPINK1 and the trypsin inhibitory activity was also uncompromised. In activity assays, conditioned medium from cells expressing epitope-tagged versions of L14P and L14R SPINK1 exhibited no trypsin inhibition, whereas medium with epitope-tagged L12F and wild-type SPINK1 inhibited trypsin comparably (not shown). On Western blots, the wild type and L12F constructs gave a comparably strong signal, while equal amounts of L14P and L14R media yielded no detectable SPINK1 (Fig. 6B). Similarly, analysis of L14P and L14R cell lysates failed to reveal immunoreactive protein, indicating rapid intracellular degradation of these SPINK1 variants. As expected, wild-type and L12F SPINK1 proteins were clearly detected from cell lysates. Finally, to investigate whether the observed effects are dependent on the cell line used, CHO cells were also transfected with SPINK1 cDNAs. Western blot analysis of conditioned media revealed the same secretion defect for the L14P and L14R variants, whereas the L12F variant was secreted as well as wild-type SPINK1 (Fig. 6C).

DISCUSSION

Here we report that SPINK1 alterations that cause a complete block in secretion are associated with hereditary pancreatitis in heterozygotes. This is the first clear genetic-biochemical demonstration that severe loss-of-function *SPINK1* alterations are associated with a high risk of CP; therefore, these alterations should be regarded as disease-causing rather than disease-modifiers. Potential examples of other severe *SPINK1* alterations in association with hereditary pancreatitis have been described previously [Witt et al., 2000; Le Maréchal et al., 2004; Masson et al., 2006]. The results presented here support the notion that this group of SPINK1 alterations should be classified as "severe" and should be differentiated from "mild" alterations, particularly from the relatively frequent, archetypal N34S variant [Witt et al., 2001a; Le Maréchal et al., 2004; Masson et al., 2006]. Mild alterations confer a smaller risk of pancreatitis, are typically found in association with idiopathic pancreatitis, and additional genetic and environmental risk factors are believed to influence their expression to a significant degree.

The L14R alteration is a novel variant, which we identified in two unrelated families, a Bulgarian family living in France, and a German family originating from the northern part of Germany. In both families, the alteration is inherited through several generations, thus establishing the diagnosis of autosomal dominant hereditary pancreatitis. The penetrance of the L14R alteration is high but incomplete, which is in agreement with the typical penetrance of dominant *PRSS1* (cationic trypsinogen) alterations associated with classic hereditary pancreatitis [Whitcomb et al., 1996; Le Maréchal et al., 2006]. The newly described L14R alteration affects the same amino acid as the previously reported L14P alteration [Witt et al., 2000]. The L14P alteration was originally identified in a 5-year-old boy without a family history of pancreatitis. Interestingly, since that time, the mother, who also carried the alteration, developed pancreatitis. In addition to the two disease-causing alterations within the SPINK1 signal peptide, we also found a frequent polymorphic variant, L12F, in pancreatitis patients of African origin as well as in African controls, but not in Caucasian subjects. This variant served as an important control, demonstrating that not all signal peptide alterations are associated with pancreatitis and a direct relationship exists between the functional effects of the alterations and disease-association.

Functional analysis using transient expression in HEK 293T and CHO cells demonstrated that although mRNA expression was unaffected, both L14P and L14R abolished SPINK1 secretion, whereas L12F was secreted normally. The SPINK1 signal peptide

TABLE 1. Mutations That Affect the Hydrophobic Core of the Signal Peptide in Human Diseases[†]

Protein	Mutation	Signal peptide[a]	Disease association
Prepro-parathyroid hormone (preproPTH); MIM *168450	C18R	MIPAKDMAKVMIVMLAI**C**FLTKSDG	Familial isolated hypoparathyroidism; MIM #146200; Arnold et al. [1990]
Coagulation factor IX (Christmas factor)	I17N	MQRVNMIMAESPSLIT**I**CLLGYLLSA	Hemophilia B; MIM #306900; Green et al. [1993]
Bilirubin UDP-glucuronosyltransferase; MIM *191740	L15R	MAVESQGGRPLVLG**L**LLCVLGPVVSHA	Crigler-Najjar disease type II; MIM #606785; Seppen et al. [1996]
Coagulation factor VII (Morioka)	L13P	MVSQALRLLCLL**L**GLQGCLA	Hereditary factor VII deficiency; MIM #227500; Ozawa et al. [1998]
Human sonic hedgehog (SHH); MIM *600725	L17P	MLLLARCLLLVLVSSL**L**VCSGLA	Holoprosencephaly 3; MIM #142945; Kato et al. [2000]
Platelet glycoprotein IX (GPIX); MIM *173515	L7P	MPAWGA**L**FLLWATAEA	Bernard-Soulier syndrome; MIM #231200; Lanza et al. [2002]
Dentine sialophosphoprotein (DSPP); MIM *125485	Y6D	MKIIT**Y**FCIWAVAWA	Dentine dysplasia type II; MIM #125420; Rajpar et al. [2002]
Calcium-sensing receptor (CaSR); MIM +601199	L11S; L13P; T14A	MAFYSCCWVL**L**A**LT**WHTSA	Hypocalciuric hypercalcemia; MIM #145980; Pidasheva et al. [2005]

[†]The OMIM database numbers and literature references are also indicated.
[a]The bold and underlined letters indicate the positions of the mutations.

conforms to the general paradigm of secretory signal peptides and comprises three functionally important regions; a positively charged N-terminal domain (five amino acids), a hydrophobic central core (12 amino acids), and a polar C-terminal region (six amino acids), including the signal-peptidase cleavage site [von Heijne, 1990]. The alterations studied here all disrupt the hydrophobic core region and thus are expected to block translocation of preSPINK1 into the lumen of the endoplasmic reticulum. Alterations that affect the hydrophobic core of the signal peptide have been described and characterized in association with various human diseases. As shown in Table 1, the Leu → Pro change is a relatively frequent occurrence and a Leu → Arg alteration has also been detected. Functional analyses invariably demonstrated that these variations block secretion by inhibiting translocation of the preprotein into the endoplasmic reticulum. Therefore, signal peptide alterations that disrupt the hydrophobic core result in a loss of protein expression and consequently a loss of function.

Two potential caveats need to be discussed here. First, it is formally possible that the L14P and L14R mutations exert their effect at the level of translation. Although not tested directly, this scenario seems very unlikely, given the fact that the mutations are more than 40 nucleotides downstream of the translation initiation site. Second, it is arguable as to whether the constitutive secretory pathway of HEK or CHO cells used in our experiments adequately model the regulated secretory pathway of the pancreatic acinar cells. Unfortunately, there are no human acinar cell lines available that we could have used to characterize regulated secretion. The widely used rat acinar cell line AR42J [Jessop and Hay, 1980] exhibits diminished secretory function and its utility as a model to study acinus-related secretory phenomena is questionable. Given the difficulty in testing the SPINK1 variants in authentic acinar cells, we utilized epithelial cell lines that carry out constitutive secretion. In these cell lines, the alterations clearly impair secretion of SPINK1, which we interpret as a defect in targeting to the endoplasmic reticulum. Because targeting and translocation of secretory proteins to the endoplasmic reticulum is a universally conserved mechanism, we posit that the same defect is expected to occur in pancreatic acinar cells, eventually leading to decreased SPINK1 secretion through the regulated pathway as well. Direct measurements of SPINK1 levels from pancreatic juice samples of L14R or L14P carriers would provide strong corroborating evidence for our findings. However, juice samples from the patients described in this study were not available for analysis.

In summary, the present study demonstrates that the p.L14R and p.L14P signal peptide alterations result in essentially complete loss of SPINK1 secretion. Clinically, these loss-of-function alterations are associated with autosomal dominant hereditary pancreatitis, confirming the notion that severe *SPINK1* alterations represent strong risk factors for CP.

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