Hailey–Hailey Disease: Identification of Novel Mutations in *ATP2C1* and Effect of Missense Mutation A528P on Protein Expression Levels

Rebecca J. Fairclough,* Lorne Lonie,* Kurt Van Baelen,† Marek Haftek,‡ Colin S. Munro,§ Susan M. Burge,¶ and Alain Hovnanian*

*The Wellcome Trust Centre For Human Genetics, University of Oxford, Oxford, UK; †Laboratorium voor Fysiologie, Katholieke Universiteit Leuven, Campus Gasthuisberg O/N, Leuven, Belgium; ‡INSERM U346/CNRS, E. Herriot Hospital, Lyon, France; §Department of Dermatology, Southern General Hospital, Glasgow, UK; ¶Department of Dermatology, Churchill Hospital, Oxford, UK

***ATP2C1*, encoding the human secretory pathway Ca^{2+}-ATPase (hSPCA1), was recently identified as the defective gene in Hailey–Hailey disease (HHD), an autosomal dominant skin disorder characterized by abnormal keratinocyte adhesion in the suprabasal layers of the epidermis. In this study, we used denaturing high-performance liquid chromatography to screen all 28 exons and flanking intron boundaries of *ATP2C1* for mutations in 9 HHD patients. Nine different mutations were identified. Five of these mutations, including one nonsense, one deletion, two splice-site, and one missense mutation, have not been previously reported. Recently, functional analysis of a series of site-specific mutants, designed to mimic missense mutations found in *ATP2C1*, uncovered specific defects in Ca^{2+} and/or Mn^{2+} transport and protein expression in mutant hSPCA1 polypeptides. In order to investigate the molecular and physiological basis of HHD in the patient carrying missense mutation A528P, located in the putative nucleotide binding domain of the molecule, site-directed mutagenesis was employed to introduce this mutation into the wild-type *ATP2C1* (hSPCA1) sequence. Functional analyses of HHD-mutant A528P demonstrated a low level of protein expression, despite normal levels of mRNA and correct targeting to the Golgi, suggesting instability or abnormal folding of the mutated hSPCA1 polypeptides. Analogous to conclusions drawn from our previous studies, these results further support the theory of haploinsufficiency as a prevalent mechanism for the dominant inheritance of HHD, by suggesting that the level of hSPCA1 in epidermal cells is critical.**

Key words: denaturing high-performance liquid chromatography/familial benign chronic pemphigus/recurrent mutation/secretory pathway Ca^{2+}/Mn^{2+} ATPase
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Hailey–Hailey disease (HHD, OMIM 16960), otherwise known as familial benign chronic pemphigus, is an autosomal dominant skin disorder that typically manifests as uncomfortable erosions or lesions in flexures or at sites of trauma. The disorder, which usually presents in the third or fourth decade, is *histologically* characterized by loss of cohesion between suprabasal keratinocytes (acantholysis) and dyskeratosis of the epidermis (Lever and Schaumburg-Lever, 1983). Ultrastructural studies have revealed a breakdown of the desmosome–keratin filament complex (Wilgram *et al*, 1962; Gottlieb and Lutzner, 1970; Ishibashi *et al*, 1984; Kowalewski *et al*, 2001). HHD displays much similarity, both clinically and histologically, to Darier's disease (DD), another dominantly inherited dermatosis. Recently, the importance of Ca^{2+} in maintaining epidermal integrity was highlighted by the identification of *ATP2A2*, encoding the sarco(endo)-plasmic reticulum Ca^{2+}-transport ATPase (SERCA2), as the defective gene in DD (Sakuntabhai *et al*, 1999). This was closely followed by *ATP2C1*, encoding the related human secretory pathway Ca^{2+}/Mn^{2+} ATPase (hSPCA1), being identified as the defective gene in HHD (Hu *et al*, 2000; Sudbrak *et al*, 2000). A spectrum of missense, frameshift, splice-site, and nonsense mutations have since been reported in *ATP2C1* (Ikeda *et al*, 2001; Chao *et al*, 2002; Dobson-Stone *et al*, 2002; Yokota *et al*, 2002). Comparison between genotype and phenotype failed to yield any clear correlation between the nature of the mutation and the clinical features of HHD (Ikeda *et al*, 2001; Dobson-Stone *et al*, 2002).

To date, the best-characterized member of the secretory pathway Ca^{2+}/Mn^{2+} ATPase (SPCA) class is the *Saccharomyces cerevisiae* homologue, named somewhat confusingly PMR1 for plasma membrane ATPase-related. Following its identification as a probable Ca^{2+}/Mn^{2+} ATPase (Rudolf *et al*, 1989; Lapinskas *et al*, 1995), the protein was localized to the Golgi compartment (Antebi and Fink, 1992). Null yeast strains defective in *PMR1* illustrated a pleiotropic

Abbreviations: DD, Darier's disease; DHPLC, denaturing high performance liquid chromatography; HHD, Hailey–Hailey disease; (h)SPCA1, (human) secretory pathway Ca^{2+}/Mn^{2+} ATPase; nt, nucleotide; PMR1, plasma membrane ATPase-related; PTC, premature termination codon; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase

effect on Golgi function including impaired proteolytic processing, incomplete glycosylation, and abnormal pre-, post- and intra-Golgi translocation of secreted proteins. These defects resulted in defective cell wall morphogenesis (Antebi and Fink, 1992).

More recently, human SPCA1 was localized to the Golgi in transfected yeast and Chinese Hamster Ovary cells. Its capacity to complement the *PMR1* null mutation and restore Ca^{2+} and Mn^{2+} transport in yeast was also demonstrated (Ton *et al*, 2002). In a recent study, we have reported functional defects in Ca^{2+} and/or Mn^{2+} binding, phosphorylation, dephosphorylation, or protein expression in HHD mutants generated by site-directed mutagenesis (Fairclough *et al*, 2003). In this study, we provide a wider understanding of the molecular basis of the disease, by identifying *ATP2C1* mutations in nine unrelated families and analyzing the effect of a disease-causing point mutation on hSPCA1 protein function.

Results

Spectrum of mutations The entire coding and flanking intronic sequence of *ATP2C1* was amplified from genomic DNA by PCR, and screened for mutations by DHPLC, in six families and three sporadic cases with HHD. Sequencing of PCR products showing aberrant DHPLC running profiles led to identification of nine different mutations, five of which were novel (Table 1).

Nonsense mutations Two different nonsense mutations resulting in premature termination codons (PTCs) were identified in this study (Table 1). Family OX25 has a C → T transition at nt 457, which results in arginine codon 153 (CGA) being substituted for a stop codon (TGA). A T → A transversion at nt 2141 in sporadic case OX29 results in TTA leucine codon 714 being substituted for a stop codon (TAA).

Insertion/deletion mutations Three deletions were identified in the coding sequence of *ATP2C1*, all of which created a shift in the reading frame and introduced a PTC (Table 1). Family OX27 has a 1 bp deletion (335delT), resulting in the introduction of a PTC 19 codons downstream of the deletion site. Families OX26 and PL1 both have 4 bp deletions (1089delTCAC and 2374delTTTG), resulting in introduction of PTCs 21 and 9 codons, respectively, downstream of the deletion site. A guanine deletion, together with a large insertion of approximately 24 adenines followed by a thymine (76delGins ~ 24), was identified in family NC13. Sequence analysis of eight subclones of this region from all available affected family members revealed the number of inserted adenines to vary between 20 and 25, making the precise consequence of this mutation difficult to predict.

Splice-site mutations Two mutations were predicted to affect mRNA splicing, through alteration of the invariant splice acceptor site consensus sequence GT (Table 1). Sporadic case NC12 has a G → A transition in intron 2 (118(−1G-A)), whereas a 1 bp thymine insertion (GTT) was

Table 1. *ATP2C1* mutations identified in HHD patients

Patients[a]	DNA change[b]	Protein change[b]	Location	Putative protein domain[c]	Verification method
Nonsense					
OX25 (f)	457C → T	R153X	Exon 7	Actuator	*Ava*I site
OX29 (s)	2141T → A	L714X	Exon 23	M5	Sequencing
Insertions/deletions					
NC13 (f)	76delGins ~ 24	Unknown[d]	Exon 2	N terminal	3% agarose gel
OX27 (f)	335delT	111LfsX19[e]	Exon 5	M2	*Hpy* CH4 IV
OX26 (f)	1089delTCAC	363TfsX21	Exon 13	Phosphorylation	Sequencing
PL1 (f)	2374delTTTG	791LfsX9	Exon 24	M7	*Tth*III, sequencing
Splice-site					
NC12 (f)	118(−1G → A)	(aberrant splicing)	Intron 2	N terminal	*Alu*I site
NC11 (f)	1840(−2insT)	(aberrant splicing)	Intron 19	ATP-binding—hinge	*Nla*III site
Missense					
OX28 (s)	1582G → C	A528P	Exon 18	Phosphorylation	Sequencing

[a]The bracketed letter after the patient name refers to the sporadic (s) or familial (f) nature of HHD.
[b]Nucleotide and amino acid numbering is based on the cDNA sequence reported by Hu *et al* (2000), with the adenosine of the initiation codon assigned position 1.
[c]Putative protein domain prediction is based on the position of the equivalent residue within the structure of SERCA1a.
[d]As the precise number of bases inserted could not be determined by analysis of genomic DNA alone, it is not known whether this mutation creates a shift in the reading frame or not.
[e]Mutation nomenclature is such that 111LfsX19 indicates a frameshift where Leu^{111} is the first affected amino acid, followed by a PTC introduced 19 codons downstream.
HHD, Hailey–Hailey disease; PTC, premature termination codon.

found in intron 19 in family NC11 (1840(−2insT)). Unfortunately, in the absence of RNA from the affected patients, the exact consequence of these mutations could not be predicted.

Missense mutations A G → C transversion was identified at nt 1582 in sporadic case OX28, which resulted in the amino acid substitution A528P. This mutation was not found in 50 controls using the verification methods outlined in Table 1. It occurs at an amino acid residue located in the putative nucleotide-binding domain of hSPCA1, which is invariant across a range of SPCA1 orthologues (Fig 1), suggesting that it may play an important structural/functional role in the protein.

Consequence of mutation G2582C (A528P) on protein and mRNA levels To investigate the precise effect on protein function, site-directed mutagenesis was employed to introduce mutation G2582C into the previously described wild-type *ATP2C1* mammalian expression clone (Fairclough *et al*, 2003). Following over-expression in COS-1 cells, a rabbit polyclonal antibody, raised against the large cytoplasmic domain of hSPCA1 (Fairclough *et al*, 2003), was used to compare A528P mutant protein expression and cellular localization with that of the wild-type, as the introduction of minor structural abnormalities by the mutation could lead to mistargeting or protein degradation (Fig 2). Immunocytochemical staining of wild-type and A528P-transfected COS-1 cells clearly demonstrated that the mutant protein is correctly targeted to the Golgi, by co-localization with the key Golgi marker TGN46 (Fig 2*A*). But a comparison of wild type and A528P protein levels, by western blot analysis of total protein extracted from transfected COS-1 cells, demonstrated the low expression of A528P, which was calculated at approximately 30% of the wild-type protein expression level (Fig 2*B*, *top row*). As previously noted, the immunoreactive band migrated slightly lower than the predicted M_r of 104 kDa (Fairclough *et al*, 2003). The low level of hSPCA1 reactivity in non-transfected COS-1 cells indicates that the hSPCA1 antiserum cross-reacts with the low level of endogenous COS-1 SPCA1 homologue. Equal protein loading was confirmed by re-staining the membrane for α-tubulin (Fig 2*B*; middle row). Lightcycler-based real-time PCR, performed in the presence of SYBR Green 1, confirmed that the low level of protein expression observed for A528P was not a result of a reduced mRNA expression level. *ATP2C1* mRNA copy numbers were calculated to be over 200-fold higher in COS-1 cells transfected with the wild-type cDNA, compared with endogenous levels. Mutant *ATP2C1* mRNA copy number did not differ significantly from the wild type (*data not shown*). PCR products isolated from the LightCycler capillaries after cycling are shown in Fig 2*C*.

Discussion

In this study, we screened the *ATP2C1* gene in nine HHD patients and identified nine different mutations likely to cause disease. Five of these mutations have not been previously reported, demonstrating the substantial allelic heterogeneity of the disease, consistent with findings reported in other mutation studies. Mutations did not cluster but were scattered across the length of the *ATP2C1* gene. Four of the mutations identified here were previously described in other studies. The OX25 mutation, 76delGins ∼ 24, was previously reported in our earlier study (NC2, (Sudbrak *et al*, 2000)). Consistent with their common geographical origin in northern England, haplotype analysis, using microsatellite markers flanking *ATP2C1*, suggested mutation that 76delGins ∼ 24 was inherited from a single ancestral gene, by revealing a shared disease haplotype in families OX25 and NC2, (*data not shown*). Other mutations identified in this study that have been previously reported include C457T (JHHD-Su, (Hu *et al*, 2000); JHHD-1, (Ikeda *et al*, 2001); patient 1(Yokota *et al*, 2002), 1089delTCAC (OX22, (Dobson-Stone *et al*, 2002)) and 2374delTTTG (OX7, (Dobson-Stone *et al*, 2002); FR5 (Sudbrak *et al*, 2000); and HHD-Du and HHD-Ho, (Hu *et al*, 2000). Unfortunately, inaccessible DNA made investigations into the recurrent or sporadic nature of these mutations impossible.

Predicted effects of *ATP2C1* mutations on function Six out of the nine mutations (66%) identified in this study are

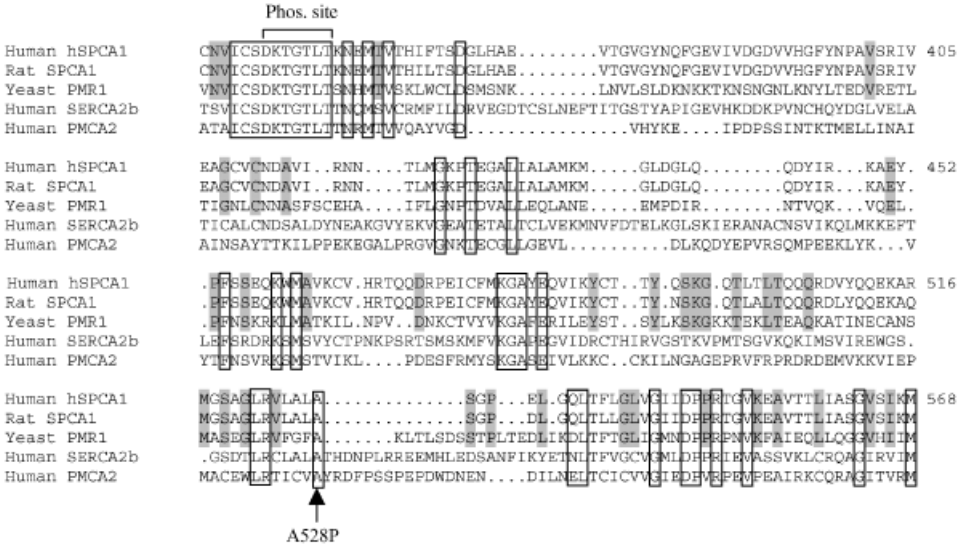

Figure 1
Partial amino acid sequence alignment of hSPCA1 with the Golgi SPCA pumps from yeast (Pmr1) and rat (SPCA1), human SERCA2b, and human PMCA2 showing the location and conservative nature of A528. Identical residues in all family members are boxed. Those conserved only in Golgi pumps are shown in gray. The sequence of part of the large cytoplasmic domain, encompassing the phosphorylation and nucleotide-binding domains, between transmembrane segments M4 and M5 is shown to highlight the location of mutation A528P. The position of the highly conserved phosphorylation site is also indicated.

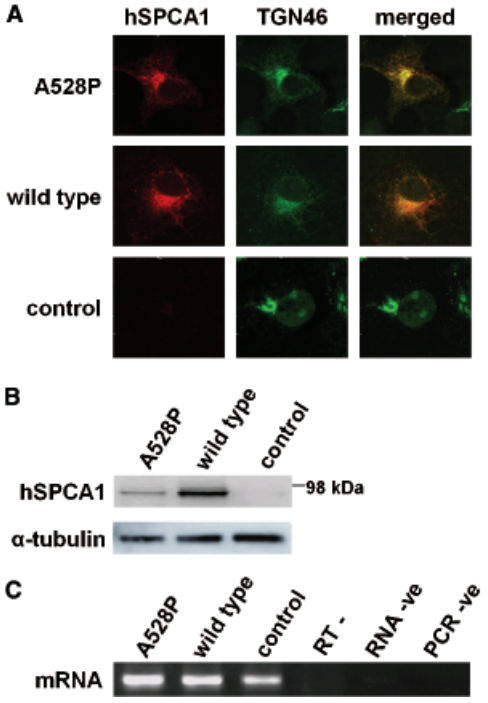

Figure 2
A528P protein and mRNA expression in COS-1 cells. (*A*) Immunocytological staining of COS-1 cells (× 100 magnification) transiently transfected with A528P, wild-type hSPCA1, and empty vector (control). Cells were incubated with polyclonal antibodies against hSPCA1 (1:1500) and the resident Golgi marker TGN46 (1:300). Secondary incubation was with FITC-conjugated donkey anti-sheep and goat anti-rabbit antibodies (1:1000). The superimposable localization of hSPCA1 and TGN46, in a juxtanuclear concentration characteristic of Golgi, is illustrated by the merged image. (*B*) Western blot analysis of protein (25 μg) isolated from COS-1 cells transiently transfected with wild-type or HHD-mutant A528P and empty vector (control). Antibodies used for incubations included: primary, anti-hSPCA1 (1:500); and secondary, alkaline phosphatase-conjugated goat anti-rabbit (1:8000). The size of the protein bands is indicated to the right of the image. The blot was then stripped and immunostained with mouse anti-α-tubulin (1:2000) followed by anti-mouse secondary antibody as above. A fluoroimager model STORM 840 (Molecular Dynamics) was used for blot analysis. (*C*) Products generated by Lightcycler-based real-time PCR with *ATP2C1* primers C7F and C7R were isolated and visualized after electrophoresis on 1.5% agarose gels. Control samples, where no reverse transcriptase was added (−RT enz), were included in all experiments to control for DNA contamination. Negative controls were also included during reverse transcription (RT −ve) and PCR (PCR −ve). Results represent those obtained from three closely similar independent experiments.

nonsense or frameshift insertion/deletions that lead to PTCs. The predicted effect of such mutations is an absence or significant reduction in the level of mutant hSPCA1 protein expression as a consequence of nonsense-mediated mRNA decay. Alternatively, truncated hSPCA1 proteins could be recognized and targeted for endoplasmic reticulum-associated degradation by the cell. Nevertheless, the observation that a high proportion of the *ATP2C1* mutations (53%) reported to date lead to PTCs suggests that haploinsufficiency is a prevalent mechanism for the dominant inheritance of HHD. The possibility, however, that some PTCs may lead to truncated proteins that cause disease through a dominant-negative mechanism cannot be discounted. In the absence of RNA from affected family members, it is difficult to predict the effect of the two splice-site mutations on hSPCA1 protein expression levels. But it seems highly likely that such mutations will not result in proteins that function correctly.

Missense mutation A528P was not found in 50 individuals (100 control chromosomes), suggesting that it does not represent a benign polymorphism. The functional effect of mutation A528P, located in the nucleotide-binding pocket of the large cytosolic domain between transmembrane segments 4 and 5 of hSPCA1, however, cannot be predicted *a priori*. As with the majority of other missense mutations identified in *ATP2C1*, A528P occurs at a residue that is invariant across a range of hSPCA1 orthologues and paralogues (Fig 1). This high degree of conservation suggests that the residues involved are essential for correct protein function. We have recently provided functional evidence that missense mutations in *ATP2C1* affect levels of hSPCA1 protein expression, or cause lack of ion transport through specific alterations to the partial reactions of the catalytic cycle (Fairclough *et al*, 2003). Analogous to the conclusions drawn from that study for HHD mutants L341P, C344Y, C411R, T570I, and G789R, we have now shown that the low expression level of mutant protein A528P is not the result of impaired mRNA levels. Instead, we suggest that the non-conservative change A528P introduces structural perturbations into hSPCA1. This could result in either abnormal protein folding or destabilization of the correctly folded protein, thus making it sensitive to endoplasmic reticulum-mediated quality control. Such an effect was previously observed when deletions or specific substitutions of select residues were made in the N-terminal region of SERCA1a (Daiho *et al*, 1999). Although these mutations had no effect on transcription, translation, or integration of the protein into the membrane, protein degradation was shown to be induced at a rate substantially faster than the wild type. Moreover, detection of some A528P that had correctly localized to the Golgi in COS-1 cells supports the hypothesis that this amino acid substitution may induce a more rapid rate of mutant protein degradation, as it indicates that at least some of the mutant protein does escape degradation following production in the endoplasmic reticulum. Nevertheless, by suggesting that epidermal cells are sensitive to levels of hSPCA1, these findings further support the theory of haploinsufficiency, discussed above, as a mechanism for the dominant inheritance of HHD.

Materials and Methods

Patients Six families and three sporadic cases were included in this study. All patients were Caucasian and of British origin, with the exception of one familial case of Polish (PL1) origin. In each case, HHD was diagnosed by a dermatologist based on family history, clinical, and histopathological findings. Peripheral blood samples were taken from patients for DNA isolation in adherence to the Declaration of Helsinki Guidelines, following their informed consent and the relevant ethics committee approval for this study.

Mutation and haplotype analysis Genomic DNA was extracted from peripheral blood leukocytes using standard methods (Sambrook *et al*, 1989). *ATP2C1* was amplified from proband and control DNA by PCR using 28 pairs of oligonucleotide primers spanning all 28 translated exons together with flanking splice sites (primer sequences available upon request). Standard reaction mixes containing 2.5 mM $MgCl_2$ and a 9:1 combination of

AmpliTaq Gold (Perkin-Elmer Life Sciences, Boston, Massachusetts) and Pfu Turbo (Stratagene, Amsterdam, the Netherlands) polymerases were used for PCR. Cycling conditions consisted of 14 touch-down cycles followed by 25 amplification cycles, using annealing temperatures of 54–58°C, as described previously (Bitoun *et al*, 2002). PCR amplification was checked by 2.5% agarose gel electrophoresis. Denaturing high-performance liquid chromatography (DHPLC) mutation analysis was essentially performed as described before (Dobson-Stone *et al*, 2000). Proband samples showing abnormal DHPLC running profiles, in comparison with controls, were sequenced as previously described (Bitoun *et al*, 2002). The presence of each mutation was later confirmed in all available family members by sequencing or restriction digestion.

Site-specific mutagenesis and analysis of protein and mRNA expression Mutation G1582C was introduced into the sequence of the previously described full-length *ATP2C1d* cDNA cloned into mammalian expression vector pMT2, using the Quikchange XL Site-Directed Mutagenesis Kit (Stratagene) as described in detail in an earlier study (Fairclough *et al*, 2003). Transient expression of the subsequent mutant protein A528P in COS-1 cells, immunostaining, RNA preparation, and Lightcycler-based real-time PCR analyses (Roche Applied Science, E. Sussex, UK) were also performed using previously described methods (Fairclough *et al*, 2003).

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Address correspondence to: Alain Hovnanian, INSERM U563, Purpan Hospital, Pavillon Lefebure, place du Dr Baylac, 31059 Toullouse, France. Email: alain.hovnanian@toullouse.inserm.fr

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Appendix: Electronic Database Information Accession numbers and URLs for data in this article are as follows:

GenBank at The National Centre for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/ (GenBank accession no. AY268375 for *ATP2C1d*/hSPCA1d and CA942187 for GAPDH).

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Hailey–Hailey Disease 16960).