Impas 1 possesses endoproteolytic activity against multipass membrane protein substrate cleaving the presenilin 1 holoprotein

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Abstract Presenilins (PS1 and PS2) are supposed to be unusual aspartic proteases and components of the γ-secretase complex regulating cleavage of type I proteins. Multiple mutations in PS1 are a major cause of familial early-onset Alzheimer's disease (AD). We and others recently identified PS-related families of proteins (IMPAS/PSH/signal peptide peptidases (SPP)). The functions of these proteins are yet to be determined. We found that intramembrane protease-associated or intramembrane protease aspartic protein Impas 1 (IMP1)/SPP induces intramembranous cleavage of PS1 holoprotein in cultured cells coexpressing these proteins. Mutations in evolutionary invariant sites in hIMP1 or specific γ-secretase inhibitors abolish the hIMP1-mediated endoproteolysis of PS1. In contrast, neither AD-like mutations in hIMP1 nor in PS1 substrate abridge the PS1 cleavage. The data suggest that IMP1 is a bi-aspartic polytopic protease capable of cleaving transmembrane precursor proteins. These data, to our knowledge, are a first observation that a multipass transmembrane protein or the integral protease per se may be a primary substrate for an intramembranous proteolysis.

Key words: Presenilins; Protease; Intramembrane proteolysis; Transmembrane precursor proteins; IMPAS; Alzheimer's disease; Expression; Mammalian cells

1. Introduction

The presenilin proteins (PS1 and PS2) are implicated as critical bi-aspartic proteases for intramembranous processing of the β-amyloid precursor protein (β-APP), Notch1–4 receptors and several other type I integral proteins [1–3]. PS1 and PS2 mediate cleavage within single transmembrane domains of these proteins [1–8]. The primary extracytosolic cleavage of the substrates by other endoproteases is a prerequisite for the sequential intramembranous proteolysis regulated by PSs. Analogous two-step proteolytic mechanisms have recently been described for some other membrane-associated putative proteases and their substrates in prokaryotes and eukaryotes. This process designated as regulated intramembrane proteolysis (RIP) is an important mechanism for inter- and intracellular signaling [1,2]. Missense mutations in PS1 and PS2 and their putative β-APP substrates are a common cause of familial early-onset Alzheimer's disease (AD) [9–12]. Most if not all of these mutations in PSs increase the production of 42 amino acid Aβ-peptides via the ε-/γ-cleavage of APP, but presumably have no enhancing effect on intramembranous proteolysis of Notch1 [13,14]. The alternative non-enzymatic functions of PS proteins in trafficking of cellular proteins or as cofactors of putative intramembrane proteases have not yet been ruled out [3]. We and others found recently multiple families of proteins (termed IMPAS (family of intramembrane protease-associated or intramembrane protease aspartic proteins (IMPs)) or PSH, signal peptide peptidases (SPP)/SPPL) related to PSs by homology in several transmembrane domains [15–18]. The function of the different IMP proteins is still unknown. One of the proteins, IMP1/SPP, encoded by a gene located on human chromosome (chr) 20, may cleave short hydrophobic N-terminal signal peptide remnants, as initially demonstrated by in vitro translation experiments and by direct binding of this polytopic protein to $(Z\text{-}LL)_2$-ketone inhibitor of signal peptide proteolysis [18,19]. We found that the activity of IMP1/SPP is beyond the cleavage of the signal peptides. We have identified a previously unknown type of intramembrane proteolysis, an endoproteolytic activity of human IMP1 (hIMP1) against multipass PS1 holoprotein.

2. Materials and methods

2.1. cDNA constructs and antibodies (Abs)

The hIMP1 gene was isolated and cloned as we described (GenBank accession numbers AY169310, AY169311, AY169312) [15,16]. The PS1 full-length sequence was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) from human leukocyte and brain cDNA. The hIMP1 C-terminus was fused to V5- or c-myc targets by subcloning of full-length IMP1 into pcDNA6/V5-His A and pcDNA4/myc-His B vectors (Invitrogen). A series of missense mutations in hIMP1 gene and stop and missense mutations in PS1 gene were introduced by PCR with mismatch primer oligonucleotides directly or by using the quick-change Stratagene kit. Additional truncated PS1 432 st, PS1 441 st constructs with stop codons at the 433

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Abbreviations: Ab, antibody; AD, Alzheimer's disease; APP, amyloid precursor protein; chr, chromosome; CTF, carboxy-terminal fragment; DMSO, dimethyl sulfoxide; HD, hydrophobic domain; HEK, human embryonic kidney; IMP, intramembrane protease-associated or intramembrane protease aspartic protein; IMPAS, family of these proteins; NTF, N-terminal fragment; PS, presenilin; SPP, signal peptide peptidase; wt, wild type

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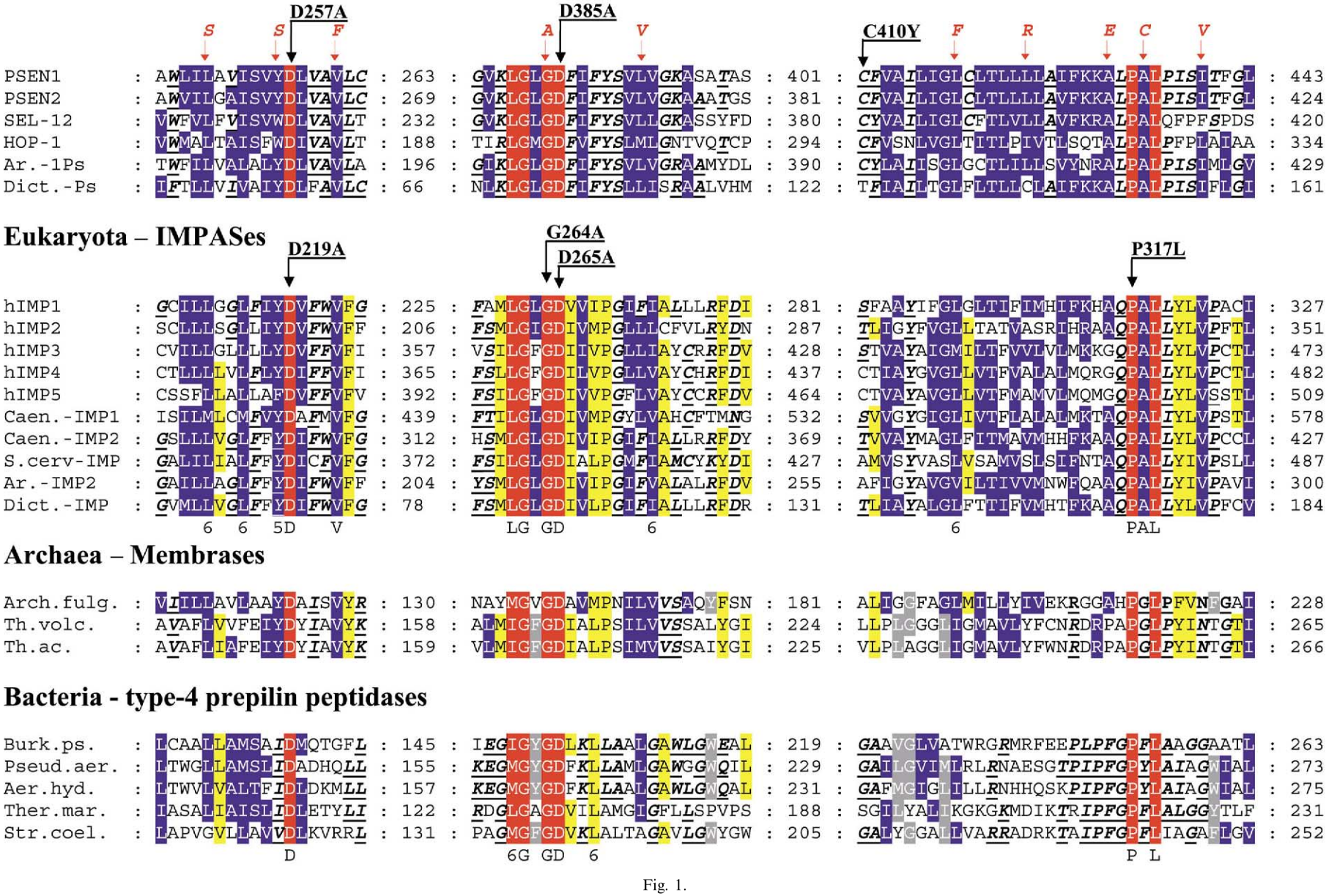
Eukaryota – presenilins
D257A
D385A
C410Y
S
S
F
A
V
F
R
E
C
V
PSEN1 : AWLILAVISVYDLVAVLC : 263 : GVKLGLGDFIFYSVLVGKASATAS : 401 : CFVAILIGLCLTLLLLAIFKKALPALPISITFGL : 443
PSEN2 : AWVILGAISVYDLVAVLC : 269 : GVKLGLGDFIFYSVLVGKAAATGS : 381 : CFVAILIGLCLTLLLLAVFKKALPALPISITFGL : 424
SEL-12 : VWFVLFVISVWDLVAVLT : 232 : GVKLGLGDFIFYSVLIGKASSYFD : 380 : CYVAILIGLCFTLVLLAVFKRALPALQFPFSPDS : 420
HOP-1 : VWMALTAISFWDIVAVLT : 188 : TIRLGMGDFVFYSLMLGNTVQTCP : 294 : CFVSNLVGLTITLPIVTLSQTALPALPFPLAIAA : 334
Ar.-1Ps : TWFILVALALYDLVAVLA : 196 : GIKLGLGDFIFYSVLVGRAAMYDL : 390 : CYLAIISGLGCTLILLSVYNRALPALPISIMLGV : 429
Dict.-Ps : IFTLLVIVAIYDLFAVLC : 66 : NLKLGLGDFIFYSLLISRAALVHM : 122 : TFIAILTGLFLTLLCLAIFKKALPALPISIFLGI : 161
Eukaryota – IMPASes
D219A
G264A
D265A
P317L
hIMP1 : GCILLGGLFIYDVFWVFG : 225 : FAMLGLGDVVIPGIFIALLLRFDI : 281 : SFAAYIFGLGLTIFIMHIFKHAQPALLYLVPACI : 327
hIMP2 : SCLLLSGLLIYDVFWVFF : 206 : FSMLGIGDIVMPGLLLCFVLRYDN : 287 : TLIGYFVGLLTATVASRIHRAAQPALLYLVPFTL : 351
hIMP3 : CVILLGLLLLYDVFFVFI : 357 : VSILGFGDIIVPGLLIAYCRRFDV : 428 : STVAYAIGMILTFVVLVLMKKGQPALLYLVPCTL : 473
hIMP4 : CTLLLLVLFLYDIFFVFI : 365 : FSLLGFGDILVPGLLVAYCHRFDI : 437 : CTIAYGVGLLVTFVALALMQRGQPALLYLVPCTL : 482
hIMP5 : CSSFLLALLAFDVFFVFV : 392 : FSILGFGDIVVPGFLVAYCCRFDV : 464 : CTVAYAVGLLVTFMAMVLMQMGQPALLYLVSSTL : 509
Caen.-IMP1 : ISILMLCMFVYDAFMVFG : 439 : FTILGLGDIVMPGYLVAHCFTMNG : 532 : SVVGYGIGLIVTFLALALMKTAQPALIYLVPSTL : 578
Caen.-IMP2 : GSLLLVGLFFYDIFWVFG : 312 : HSMLGLGDIVIPGIFIALLRRFDY : 369 : TVVAYMAGLFITMAVMHHFKAAQPALLYLVPCCL : 427
S.cerv-IMP : GALILIALFFYDICFVFG : 372 : FSILGLGDIALPGMFIAMCYKYDI : 427 : AMVSYVASLVSAMVSLSIFNTAQPALLYIVPSLL : 487
Ar.-IMP2 : GAILLAGLFFYDIFWVFF : 204 : YSMLGLGDIVIPGIFVALALRFDV : 255 : AFIGYAVGVILTIVVMNWFQAAQPALLYIVPAVI : 300
Dict.-IMP : GVMLLVGLFFYDIFWVFG : 78 : FSMLGLGDIVLPGIFIALLLRFDR : 131 : TLIAYALGLFTTIFVMHTFKAAQPALLYLVPFCV : 184
6 6 5D V
LG GD 6
6
PAL
Archaea – Membrases
Arch.fulg. : VIILLAVLAAYDAISVYR : 130 : NAYMGVGDAVMPNILVVSAQYFSN : 181 : ALIGGFAGLMILLYIVEKRGGAHPGLPFVNFGAI : 228
Th.volc. : AVAFLVVFEIYDYIAVYK : 158 : ALMIGFGDIALPSILVVSSALYGI : 224 : LLPLGGGLIGMAVLYFCNRDRPAPGLPYINTGTI : 265
Th.ac. : AVAFLIAFEIYDYIAVYK : 159 : VLMIGFGDIALPSIMVVSSAIYGI : 225 : VLPLAGGLIGMAVLYFWNRDRPAPGLPYINTGTI : 266
Bacteria - type-4 prepilin peptidases
Burk.ps. : LCAALLAMSAIDMQTGFL : 145 : IEGIGYGDLKLLAALGAWLGWEAL : 219 : GAAVGLVATWRGRMRFEEPLPFGPFLAAGGAATL : 263
Pseud.aer. : LTWGLLAMSLIDADHQLL : 155 : KEGMGYGDFKLLAMLGAWGGWQIL : 229 : GAILGVIMLRLRNAESGTPIPFGPYLAIAGWIAL : 273
Aer.hyd. : LTWVLVALTFIDLDKMLL : 157 : KEGMGYGDFKLLAALGAWLGWQAL : 231 : GAFMGIGLILLLRNHHQSKPIPFGPYLAIAGWIAL : 275
Ther.mar. : IASALIAISLIDLETYLI : 122 : RDGLGAGDVILAMGIGFLLSPVPS : 188 : SGILYALIKGKGKMDIKTRIPFGPFLALGGYTLF : 231
Str.coel. : LAPVGVLLAVVDLKVRRL : 131 : PAGMGFGDVKLALTAGAVLGWYGW : 205 : GALYGGALLVARRADRKTAIPFGPFLIAGAFLGV : 252
D
6G GD 6
P L

Fig. 1.

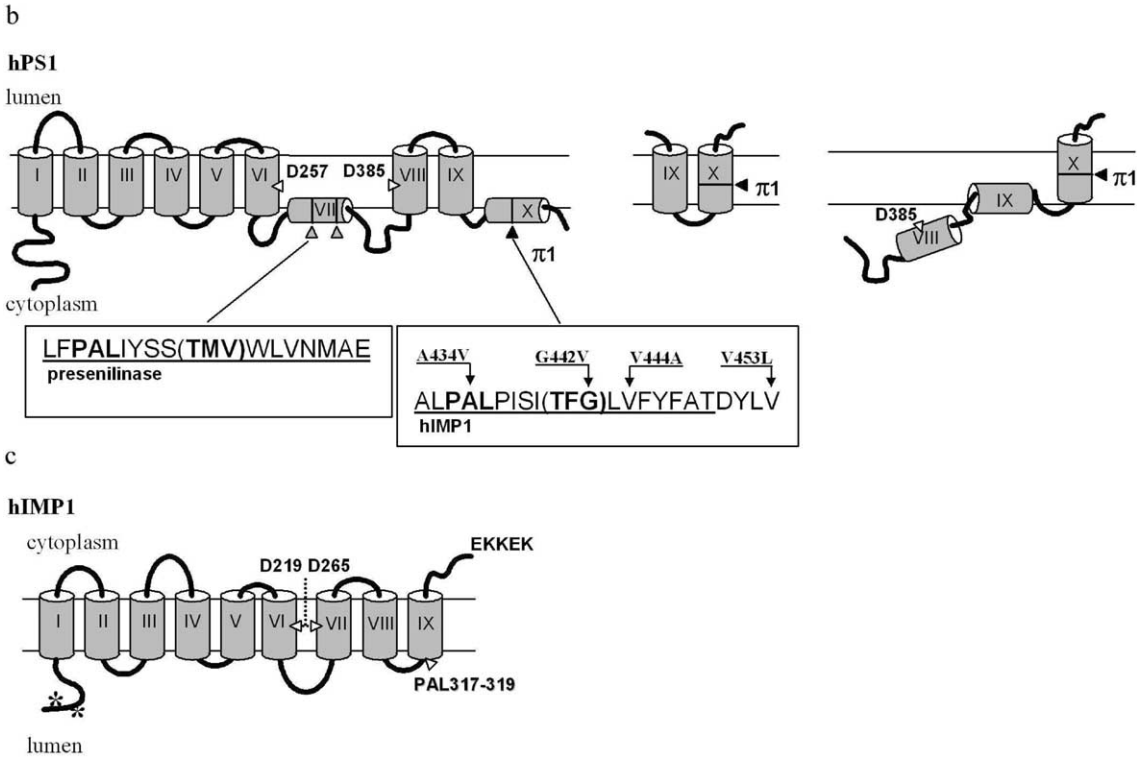

Fig. 1. Structure of PS and PS-related IMP proteins. a: Multiple alignments of most conservative domains of protein families: eukaryotic PSs (PSEN1,2, *Homo sapiens*; SEL-12, HOP-1, *Caenorhabditis elegans*; Ar.-1PS, *Arabidopsis thaliana*; Dict.-PS, *Dictyostelium discoideum*); IMPASes (hIMP1–5, *H. sapiens*; Caen.-IMP-1, Caen.-IMP-2, *C. elegans* C36B1.12, T05E11.5; S. cerv., *Saccharomyces cerevisiae*; Ar.-IMP, *A. thaliana* AAC34490; Dict. IMP, *D. discoideum*); related proteins from Archaea (Arch.fulg., *Archaeoglobus fulgidus* AAB89302; Th.volc., *Thermoplasma volcanium* NP_110537; Th.ac., *Thermoplasma acidophilum* CAC11210); and bacterial type 4 prepilin peptidases (Burk.ps., *Burkholderia pseudomallei*; Pseud.aer., *Pseudomonas aeruginosa*; Aer.hyd., *Aeromonas hydrophila*; Ther.mar., *Thermotoga maritime*; Str.coel., *Streptomyces coelicolor*). Shading, amino acid with similarity inside protein families $\geq 50\%$ (for Archaea $\geq 30\%$) common for: (1) all protein families are shown in red; (2) PSs and any other protein family are shown in blue; (3) IMPs and Archaea or bacteria proteins are shown in yellow; (4) Archaea and bacteria proteins are shown in gray. Amino acid with similarity $\geq 50\%$ only inside any of the protein families are underlined. Beneath amino acids identical in all members of the IMPAS/PS suprafamily or bacterial peptidases are depicted. Groups of similar amino acids: 1-D,N; 2-E,Q; 3-S,T; 4-K,R; 5-F,Y,W; 6-L,I,V,M (Blosum 62 scoring matrix). Red arrows denote AD-associated amino acid substitutions in conservative sites of PS and IMP proteins. Black arrows indicate mutations in IMP1 and PS1 used in this work or catalytically critical aspartic residues in PS1. b,c: Predicted membrane topology of HD of human PS1 (hPS1) and hIMP1 proteins. b: PS1 topology. HD X is shown in possible structure conformations as membrane-associated or membrane-spanning domain. White arrowheads denote conservative aspartate (D) residues and PAL motif. The PAL motives in HD VII and X are shown in bold. Gray arrowheads indicate the sites for 'PSase'-dependent cleavages in HD VII [21]. The predicted cleavage (291–293) and similar motif in HD X are in parentheses. Black arrowheads indicates hIMP1-dependent π1-cleavage of PS1wt. Sequences of HD are underlined and mutations in HD X used in this work are denoted. c: hIMP1 topology. White arrowheads denote conservative aspartates (D) and PAL motif. The predicted glycosylation (asterisks) and endoplasmic reticulum (ER) membrane retention signals are shown.

and 442 residue positions, omitting also spliced VRSQ residues, were initially obtained from Dr. A. Goate's laboratory. For the experiments described here, these PS1 isoforms were subcloned into the wild type of PS1 (PS1wt) harboring VRSQ. The deletion in the 5′-encoding region of the PS1 gene was introduced using PCR with a 5′-oligonucleotide primer missing the internal sequence. The resulting gene construct (PS1 5′-del) encoded a protein lacking a segment of 23 amino acids (Δ5–27 amino acids) following the first MTEL residues in PS1. Polyclonal IMP1-N Abs were raised against the hIMP1 N-terminus as we described [16]. Abs against V5 and c-myc epitopes were obtained from Invitrogen and Sigma. Sensitive polyclonal rabbit Abs (PS1 N-M) were raised against hPS1 N-terminal polypeptide (1–93 amino acids). The 9N14 Abs against the first 1–14 amino acids in N-terminal PS1 were kindly provided by Dr. A. Goate and PS1 G1L3 Abs against PS1 carboxy-terminal fragment (CTF) (epitope between hydrophobic domains (HDs) VII and VIII) were previously described [20].

2.2. Transfection and Western/immunoblot detection

Human embryonic kidney (HEK)293 or Chinese hamster ovary (CHO) cells were transiently cotransfected with hIMP1 and PS1 constructs or mock plasmids using LipofectAMINE PLUS reagent (Invitrogen). Cells lysates or membrane fractions were prepared in 24 h after transfection. Proteins were electrophoresed in polyacrylamide gels. To resolve small molecular mass differences between C-terminally truncated 40–45 kDa PS1 isoforms the protein extracts were loaded onto sodium dodecyl sulfate (SDS)–polyacrylamide gels, and electrophoresis was run until the 36 kDa marker fragment almost reached the bottom of the gel. In all figures presented, Tris–glycine SDS–polyacrylamide gels were used, except in Fig. 2e where tricine–polyacrylamide gel was used to detect CTF. The PS1 and hIMP1 proteins were detected by immunoblots with the Abs described above using standard procedures [16]. Whenever indicated, autographs were scanned and images were analyzed using Quantity One Version 4.3.0 quantification software (Bio-Rad).

2.3. Intramembrane protease inhibitors

The $(Z\text{-LL})_2$-ketone inhibitor, L-685,458, and *N*-*S*-phenyl-glycine-*t*-butyl ester (DAPT) compounds were purchased from Calbiochem. Cell lines transfected with PS1 and hIMP1 isoforms or mock plasmids were incubated in culture cell media containing different concentrations of the inhibitors for 24 h before lysis procedures. The cell lysates were analyzed by Western blots as described above.

3. Results

First, we determined the common sites conserved throughout evolution of the suprafamily of PSs and PS-related proteins (IMPAS). Comprehensive alignment was developed (Fig. 1a) demonstrating that IMP and PS proteins share the most identities in three conserved HDs corresponding to PS1 HD VI, HD VIII and HD IX/HD X. A significant homology between IMPAS/PS families and the bacterial type 4 prepilin peptidases was found in these domains. The data suggested that PSs (found in a primary form in Protista [16]) and IMP (found in yeast) might originate from an ancient prototype eventually evolved into diverged subfamilies of related proteins. The D-6G*x*GD-P*x*L (*x*, any amino acid; 6, aliphatic non-polar hydrophobic, e.g. L, I, V or M amino acid) is an invariant signature of the IMPAS/PS suprafamily and bacterial peptidases (Fig. 1a), which may be critically required for their proteolytic properties.

The predicted topology and structure of α-helical transmembrane domains in hIMP1 and PS1 are similar. In addition, PSs have a HD (HD VII) cleaved by 'presenilinase' (PSase) activity. hIMP1 forms complexes and is glycosylated

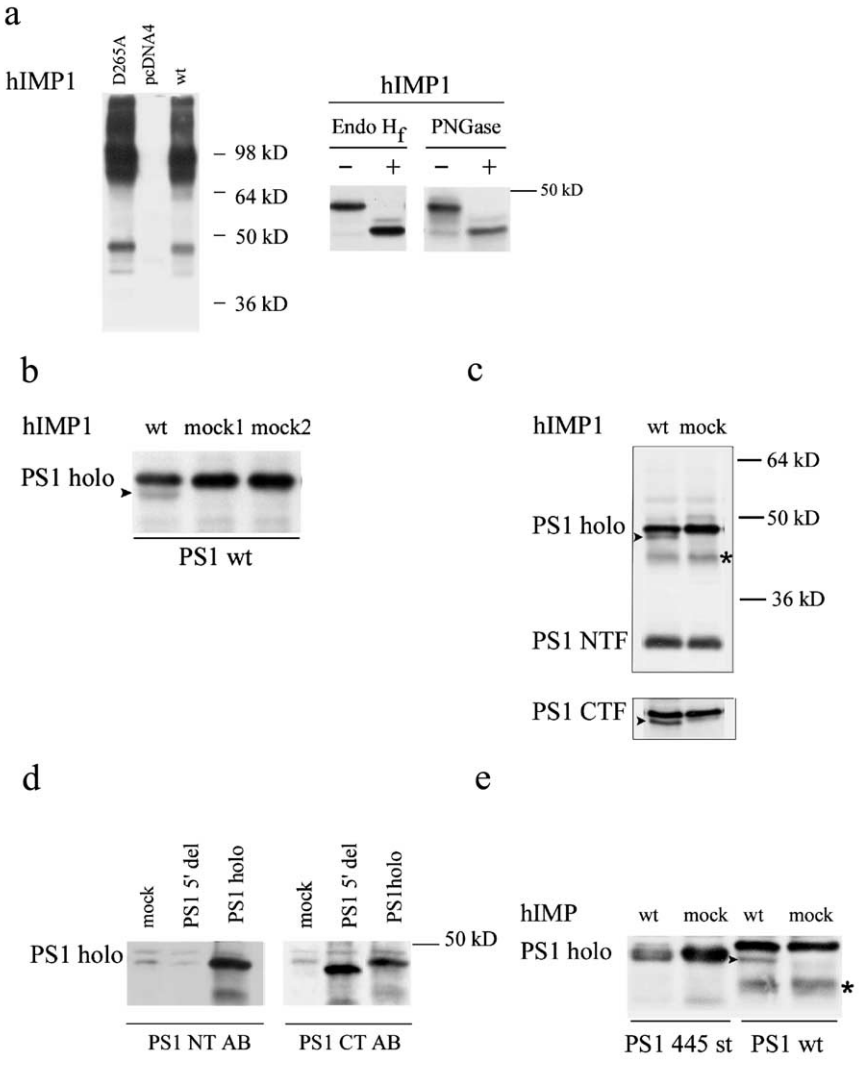

Fig. 2. Expression of hIMP1 induces cleavage of PS1 protein. a: hIMP1 is glycosylated in mammalian cells and is detected by immunoblot as two major modified protein fractions (45–50 and 70–100 kDa). b: Coexpression of PS1wt holoprotein with hIMP1wt (but not with mock 1 and 2 (pcDNA4 or pcDNA6 plasmids)), in HEK293 cells induces cleavage of the PS1 holoprotein. c: Appearance of additional derivative for CTF, but not for NTF, suggests the cleavage occurred in the carboxy-terminal part of PS1. d,e: Analysis of PS1 isoforms with 23 amino acids deletion in N-terminus (PS1Δ5–27) and 23 amino acids deletion in C-terminus (PS1Δ445–467). d: The N-terminal PS1 NT (PS1 N-M) Abs were unable to detect the PS1 omitting the N-terminal amino acid, whereas C-terminal PS1 CT (G1L3) Ab detected this isoform. e: In cells co-expressing PS1 and hIMP1 the π1-cleaved PS1Δ-C fragment was detected for PS1wt holoprotein, but not for PS1 lacking the C-terminal tail. f: AD mutations in PS1 substrate did not abridge the IMP1-induced proteolysis. The comparison of PS1wt and PS1 C410Y (AD mutant) is shown. g: Mutation analysis of the last HD of PS1 (HD X) predicted as a target for hIMP1 cleavage. Mutation G442V in a motif predicted for cleavage abolished the endoproteolysis, whereas the more distantly located mutations did not affect the cleavage. h: Mapping of π1-cleavage site by comparison of π1-cleaved PS1Δ-C fragment with PS1 carrying systematic deletions in C-terminus. Protein lysates from cells coexpressing (1) PS1wt and hIMP1wt and (2) series of PS1 truncated isoforms were run in 10% Tris–glycine SDS–polyacrylamide gel. The comparison of the sizes of the proteolytic fragment and the truncated isoforms was performed in replications. The cleavage site was predicted in HD X between 432 and 445 nearby 441 residue (comparison with series of other C-terminal deletions is not shown). Asterisks indicate non-specific band.

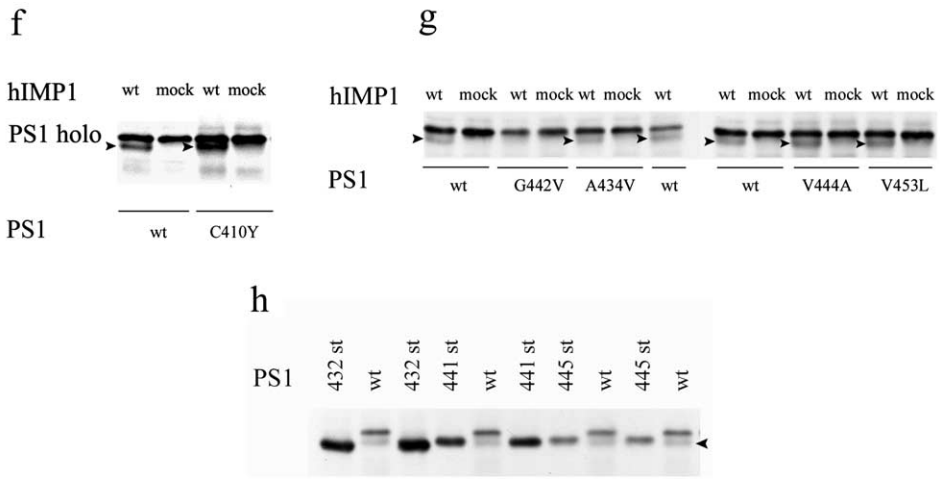

Fig. 2 (*Continued*).

in mammalian cells and does not undergo the intense cleavage (Fig. 2a) described for 'PSase'-regulated processing of PSs [16,21]. However, the less efficient proteolytic processing of hIMP1 in some cells may not be excluded. We made the mutations in the evolutionary invariant residues in each of the most conserved domains of hIMP1 (Fig. 1) and searched for potential proteolytic substrates for the hIMP1.

We unexpectedly found, in coexpression experiments using live mammalian cells, that a PS1wt may be processed via the activity of hIMP1wt (Fig. 2). The Western immunoblot with Ab against N-terminus of PS1 demonstrated production of an additional PS1 fragment (PS1wt-ΔC) in HEK293 cells coexpressing hIMP1wt and PS1wt. The molecular mass of this product is slightly lower than of PS1 holoprotein, suggesting that the cleavage (designated as π1) may occur near the end of the PS1 protein. We found that neither the PS1 holoprotein nor PS1wt-ΔC was phosphorylated or glycosylated (not shown) and, apparently, the PS1wt-ΔC fragment was a proteolytic product of the PS1 holoprotein (Fig. 2b,c,e).

We next addressed the question whether naturally occurring AD mutations in PS1 may affect the cleavage of PS1 regulated by hIMP1wt. We found that hIMP1 induces cleavage of the PS1 AD mutant PS1 holoprotein (C410Y) and produces a proteolytic derivative similar to that found for PS1wt (Fig. 2f). These data demonstrate that the AD mutations do not abridge PS1 holoprotein as a substrate for the hIMP1-mediated cleavage. The PS1 cleavage was consistently detected by direct immunoblot analysis of PS1 in cell lysates or membrane fractions in multiple replicate experiments using cells transiently coexpressing PS1 and hIMP1.

To assess further what part (C- or N-terminal) of PS1 is a substrate for this cleavage we employed a variety of Abs to C-terminal and N-terminal epitopes of PS1. Both the N-terminal PS1 N-M and the 9N14 Abs used in this study are unable to detect PS1 with small deletions in N-terminus (Fig. 2d). Consequently, these Abs would not detect the proteolytic derivative if cleavage occurred in the N-terminus. We reasoned, therefore, that the detectable PS1-ΔC proteolytic fragment was likely generated by cleavage at the C-terminal part of PS1. This prediction was confirmed further by the observation that the PS1-ΔC derivative was not found for the PS1 isoform (PS1 445 tr) omitting small portion of C-terminus (Fig. 2e). Furthermore, no additional bands for processed 28–30 kDa PS1 N-terminal fragment (NTF) were found, but an extra fragment was observed for 18–20 kDa PS1 CTF (Fig. 2b). These data provided evidence that π1-cleavage occurs in the C-terminal part of the PS1 holoprotein. It remains to be determined whether hIMP1 may promote π1-cleavage of the processed PS1 CTF or if this π1-cleavage always preludes 'PSase' processing of the PS1 holoprotein between domains VI and VIII (Fig. 1b).

To determine more precisely the location of the cleavage site, we employed a series of PS1 cDNA constructs encoding PS1 holoproteins with systematic stop codons or deletions in the C-terminus with average size differences of 4–9 amino acid residues. The analysis with PS1 isoforms harboring stop codons, identified to be closest to the predicted π1-cleavage site, is shown in Fig. 2h. The putative cleavage site for PS1wt was mapped between 432 and 445 amino acids in the middle of the last HD X of PS1 (Figs. 1b and 2h). In addition, we designed series of constructs with mutations in or nearby the PS1 HD X domain (Fig. 2g). We found that only one mutation, G442V located in a small predicted SITFG region of π1-cleavage, completely suppressed the endoproteolysis. The data demonstrate that helix-breaking residues at the center of the membrane-embedded or membrane-spanning domain X are critical for hIMP1-mediated endoproteolysis of PS1.

The IMP proteins share multiple sites identical to PSs, including amino acid residues mutated in AD, as well as evolutionary invariant sites occurring in bacterial peptidases, which are supposed to be essential for their catalytic activity (Fig. 1a). Therefore, we tested next whether analogous mutations affect proteolytic properties of hIMP1. We constructed series of mutant hIMP1 isoforms and found that D219A, D265A and P317L mutations in hIMP1 completely abolished the hIMP1-promoted proteolysis of PS1wt. Conversely, hIMP1 with the AD mimic mutation (G264A) retains proteolytic activity (Fig. 3). Lastly, it was of essential interest to determine whether γ-secretase inhibitors, thought to specifically target proteolytic properties of PS1, affect also hIMP1 activity. Indeed, we found that the L-685,458 γ-secretase inhibitor of PS efficiently inhibited hIMP1-mediated π1-cleavage. We found, however, that another γ-secretase inhibitor, DAPT, was ineffective in the suppression of IMP1 proteolytic activity on PS1wt. The $(Z\text{-}LL)_2$-ketone, inhibitor of SPP activity [18], showed also a high inhibitory potency for the π1-cleavage of

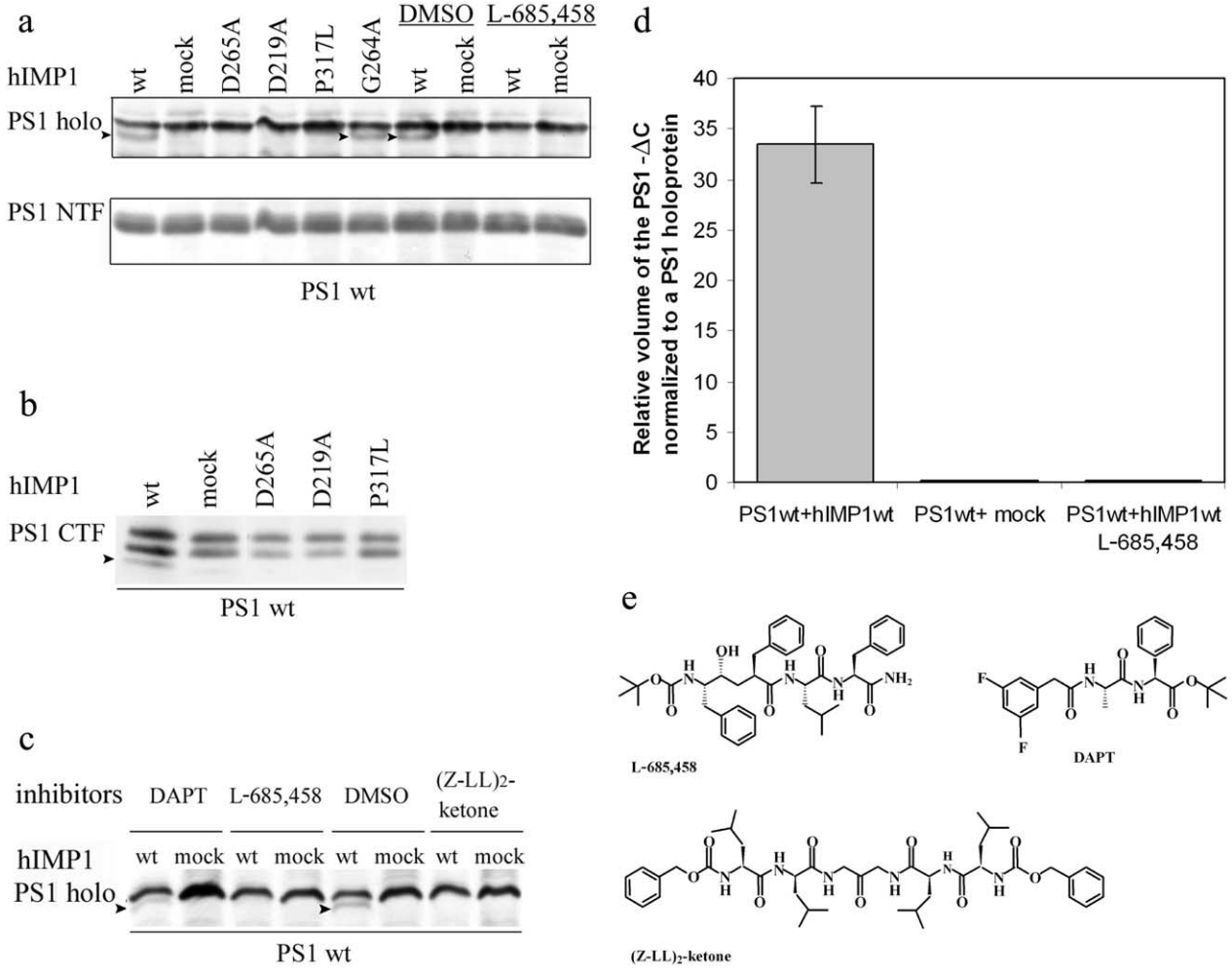

Fig. 3. Inhibition of the PS1 endoproteolysis by mutations in hIMP1 and by chemical compounds. a: Mutations in evolutionary invariant aspartates (putative catalytic sites) and proline residue (PAL motif) in hIMP1 and γ-secretase inhibitor abolished the cleavage of PS1wt holoprotein. The AD mimic G264A mutation in hIMP1 did not suppress the PS1 endoproteolysis. b: Analysis of CTF also revealed the proteolytic fragment for PS1wt, but not for the aspartate or proline mutants. Two common CTF bands are detected in high resolution SDS–polyacrylamide gel electrophoresis (PAGE). c: Determination of compounds inhibiting the hIMP1-mediated endoproteolysis of PS1 protein. HEK293 cells coexpressing PS1 isoforms with hIMP1wt or mock were incubated with dimethyl sulfoxide (DMSO) or 75 μM DAPT, 7.5 μM L-685,458 and 38 μM $(Z\text{-}LL)_2$-ketone, in concentrations described to be efficient to inhibit intramembrane proteolysis [18,22,23]. The protein lysates were analyzed by immunoblot. DAPT or control DMSO did not abolish the cleavage of PS1wt, but the L-685,458 and $(Z\text{-}LL)_2$-ketone completely inhibited the cleavage. d: Histogram showing evaluation of the π1 proteolytic cleavage of PS1wt. The PS1 protein fragments were directly detected in protein lysates from HEK293 cells by immunoblots. The proteolytic PS1Δ-C fragments were quantities as percent ratio to uncleaved PS1 holoprotein. In the absence of transfected hIMP1 the PS1 cleavage is undetectable in HEK293 cells by this assay. The γ-secretase inhibitor L-685,458 strongly inhibited the cleavage. Mean values ± S.E.M. in three independent experiments are shown. e: Structure of chemical compounds used in the experiments.

PS1 holoprotein (Fig. 3a–d). L-685,458 is a compound containing hydroxyethylene dipeptide isostere which functions as a transition state analog inhibitor of aspartate protease. The L-685,458 and $(Z\text{-}LL)_2$-ketone directly bind to protease active sites, whereas the mechanisms of interaction of the non-transition state analog DAPT inhibitor are not yet well determined (Fig. 3e) [18,22,23].

4. Discussion

Our data demonstrate that the recently identified IMP1/SPP is a protease capable of cleaving large transmembrane precursor proteins. In cultured mammalian cells, IMP1 induced the intramembrane cleavage of structurally related PS1 holoprotein. To substantiate proteolytic activity of hIMP1 against PS1 substrates, we employed here analysis of protein extracts from live cells. Detection assay of the proteolytic fragments in cells transiently coexpressing these proteins was more efficient than analysis in stably transfected cells, and demonstrated completely reproducible results in multiple replicate experiments. The detection of cleavage directly by immunoblot in this assay (Section 2) required cells overexpressing both hIMP1 and PS1 holoproteins. The endogenous PS1 holoprotein is rapidly processed and generally undetectable in mammalian cells on a constitutive level by Western blot procedures [21,24,25]. Therefore, the role of hIMP1 in the cleavage of endogenous PS1 remains yet to be determined. Nevertheless, the data suggest that IMP1 possesses or induces endoprotease activity and proposes a simple assay to monitor proteolytic properties of IMP1 isoforms. To our knowledge, this finding

is the first evidence that a multipass protein may be a direct substrate for intramembrane proteolysis. Beyond the biological significance of this novel intramembrane-type proteolysis, these data imply that polytopic proteases may not only have enzymatic activity, but be themselves substrates for other intramembrane proteases.

We previously identified and named these PS-related proteins as IMPAS (IMP) and classified them by hierarchical homology to the most conserved member IMP1: IMP1 (chr 20), IMP2 (chr 12), IMP3 (chr 15), IMP4 (chr 19), IMP5 (chr 17) [15,16]. Other terms have also been proposed for the same proteins (PSH/SPPL) and, especially, SPP (identical to IMP1) to designate its specific SPP activity [17,18]. It may be pointed out that the signal peptide protease and SPP terms have also been in use for other types of unrelated proteases, cleaving bacterial or chloroplast proteins [26]. The signal peptides are likely also not exclusive substrates for SPP/IMP1 or other bi-aspartic intramembrane proteases. For example, the bacterial Eep, resembling human intramembrane metalloprotease S2P, regulates cleavage of signal peptides in bacteria [1]. The SPP-type cleavage requires prior endoproteolysis by signal peptidase (SP) as described for RIP two-step proteolysis [1,18]. The data reported here demonstrated putative proteolytic activity of the IMP1/SPP beyond the cleavage of signal peptides. The primary extracytosolic cleavage is not expected to be a prerequisite for this type of intramembrane proteolysis. It was assumed that the initial cleavage of the extracytosolic fragment of both type I and type II proteins to short segment (<30 amino acids) is a common prerequisite for the secondary cleavage by intramembrane proteolysis [1]. Based on the data reported here, we postulate that the holoprotein harboring HD, adjusted to short C-terminal tail, may undergo intramembrane cleavage without extracytosolic proteolysis (the process which may be termed as direct intramembrane proteolysis (DIP)). This proteolytic cleavage activity may result in production of C-terminal truncated PS1 isoform lacking >25 C-terminal residues. Other putative substrates for IMP1/SPP with similar protein structure may be anticipated.

Interestingly, the last hydrophobic C-terminal PS1 domain HD X cleaved by hIMP1 strictly resembles domain HD VII of PS1 holoprotein, which is constitutively cleaved by a not yet precisely elucidated 'PSase'. The similarities between these domains include hydrophobicity and location predictions at the end of mature protein derivates (NTF or CTF), common conserved motif (PAL) and the location of helix-breaking residues in the middle of the domains (Fig. 1). It might be of interest to investigate further whether members of IMPAS proteins contribute also to 'PSase' cleavage. The HD X may be either a membrane-inserted, but not -spanning, or lipid bilayer-spanning domain, according to different models (Fig. 1) [27,28]. The topology, and thereby orientation, of the PS1 C-terminus to lumen or cytosolic space may possibly be dynamically regulated [27] and affect the efficiency of cleavage by hIMP1. Mutation analysis and testing with chemical compounds demonstrated here and elsewhere [8,18] imply that IMP/SPP, as its distant PS homologs, are likely bi-aspartic proteases with similar catalytic but differential pharmacological potency.

Finally, it was shown by systematic deletion analysis that truncation of only a few last C-terminal residues in PS1 AD mutant isoforms abolishes the overproduction of Aβ42 [20]. Therefore, the role of the intramembrane proteolysis in regulation of PSs and AD modulation have to be investigated. It would be important to determine further whether IMP/PSH/SPP proteins, similarly to PS1, regulate neurodegeneration processes or normal development in vivo. Because of the abundant presentation of the IMP-like homologs in all major taxons, including single cellular organisms, the identification of the evolutionary conserved functional pathway of these new putative intramembrane proteases is of great interest.

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