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Proteolytic cleavage of LEDA-1/PIANP by furin-like proprotein convertases precedes its plasma membrane localization

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ABSTRACT

Liver endothelial differentiation-associated protein-1 (LEDA-1/PIANP) is a type-I-transmembrane protein first identified by us as a putative junctional protein in liver sinusoidal endothelial cells. Others have shown that LEDA-1/PIANP binds and activates immune inhibitory receptor PILRα in *trans*, a process that requires sialidation of LEDA-1/PIANP. Here we show that LEDA-1/PIANP is subject to O-glycosylation and sialidation as demonstrated in brain tissue as well as in LEDA-1 expressing cell lines by using anti-LEDA-1/PIANP C-terminal antibodies. In addition, analysis of LEDA-1/PIANP processing with His-tags inserted at different positions in the extracellular domain revealed that multiple steps of proteolytic cleavage occur during maturation of the protein. Proteolytic cleavage between aa59 and aa83 preceded sorting of the protein to the plasma membrane. Deletion of aa75–79 and inhibition with Furin inhibitor I confirmed that LEDA-1/PIANP is processed by a Furin-like proprotein convertase. In summary, these findings show that Furin-like proprotein convertase-dependent processing precedes plasma membrane localization of LEDA-1/PIANP that is a pre-requisite of functional receptor–ligand interactions in vitro and in vivo.

1. Introduction

Liver endothelial differentiation-associated protein-1 (LEDA-1/PIANP) was first identified by us as a putative junctional protein in liver sinusoidal endothelial cells of the rat [1]. Human and murine LEDA-1/PIANP are type-I-transmembrane proteins of 282 and 278 amino acids, respectively. LEDA-1/PIANP was shown to bind to the immune inhibitory receptor PILRα and to mediate its activation in a reporter cell system containing N-terminal PILRα fused to the intracellular domain of PILRβ [2]. Furthermore, it was shown that sialic-acid residues attached to mouse LEDA-1/PIANP at aa136 are necessary for its interaction with PILRα. Although PILRα has been shown to be involved in the inflammatory response in sepsis by modulation of neutrophil invasion [3], the functional implications of the interaction of LEDA-1/PIANP with PILRα are unknown. RT-PCR revealed the highest levels of LEDA-1/PIANP transcripts in the central nervous system (CNS) where it might play a role in the immune surveillance of the brain [2]. In addition LEDA-1/PIANP is expressed by the mouse melanoma cell line B16 implicating a potential role of LEDA-1 in tumorigenesis and tumor immunology [2].

The only known homologue of LEDA-1/PIANP is adherens-junction-associated protein-1 (AJAP-1). AJAP-1 sorts to the basolateral domain of epithelial cells and localizes to E-cadherin positive adherens junctions [4]. In addition, AJAP-1 has been shown to influence invasion and adhesion of glioblastoma cells [5] and to modulate endocytosis of E-cadherin in response to EGF in epithelial cells [6]. Basolateral sorting of AJAP-1 has been shown to rely on tyrosine-based motifs in its intracellular domain [7]. Although the C-terminal part of LEDA-1/PIANP lacks tyrosine-based motifs we were able to show that LEDA-1 also sorts to the basolateral domain in polarized epithelial cells and co-localizes with E-cadherin in adherens junctions [1]. As basolateral sorting mechanisms have been shown to be involved in a multitude of receptor–ligand interactions, basolateral sorting of LEDA-1 might also be important for its interactions with other proteins. Here, we identify and characterize post-translational modifications of LEDA-1/PIANP such as glycosylation and proteolytic processing and discuss their functional implications.

Abbreviations: LSECs, liver sinusoidal endothelial cells; LEDA-1, liver endothelial differentiation-associated protein-1; PIANP, PILR alpha associated neural protein; PILR, paired immunoglobin-like type 2 receptor; AJAP-1, adherens-junction-associated protein-1; WB, Western Blot; kDa, kilodalton.

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2. Materials and methods

2.1. Cloning

Mutations were introduced into human LEDA-1 cDNA in the HsLEDA-1_pEF6/V5 plasmid described previously [1] using site-directed mutagenesis technique. PCR reactions (Expand High Fidelity PCR System from Roche, Germany) were performed with the following primers: Hs_Leda1_cDNA_SpeI (forward) 5′-TGACACTAG TAA-AGGCTGAAAATCTGGG-3′, Hs_Leda1_cDNA_NotI (reverse) 5′-GTCAGCGGCCGCGGCCTCCTGCTTGGCTG-3′, del_75–79 (forward) 5′-CCTCGGGTCCCAGTCCTGCCTGGC-3′, del_75–79 (reverse) 5′-GC CAGGCAGGACTGGGACCCGAGG-3′. PCR cycling conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 60 s. PCR was finished by final elongation at 72 °C for 7 min. Final PCR products were digested with SpeI and NotI and ligated to the pEF6/V5 vector. Multiple mutations were introduced sequentially.

His-Tag was introduced into mouse LEDA-1 cDNA at different positions by PCR using the following primers: MmLeda1_NheI (forward) 5′-ACTGCTAGCAGCCGCCACCATGTGGTCT-GCTCAACTGCTG TCC-3′, MmLeda1_BstBI (reverse) 5′-GTTGTTCGAAGCATCACA GATTC-ACCAGGGGAATCCGGTTC-3′, MmLeda_37His38 (forward) 5′-GCCCATCATCATCATCAT-CATCGCCCCCCCTGCGTCCGGGGTG-3′, MmLeda_37His38 (reverse) 5′-GCGATGATGA-TGATGATGATGGG CTGGGGCTGGTGGGGATCGATGGGAG-3′, MmLeda_59His60 (forward) 5′-CCTCATCATCATCATCATCATCCGCCAAGCCGATCCCCACG GGTCCC-3′, MmLeda_59His60 (reverse) 5′-CGGATGATGATGAT-GATGATGAGGCGCCCGCTCCCAAA-CACACACATGGC-3′, MmLeda_ 83His84 (forward) 5′-CTACCCATCATCATCATCATCATCC-ATCAGGC TTTGAAGAGGGGCCTCCCTCATC-3′, MmLeda_83His84 (reverse) 5′-GGATGATGATGATGATGATGGGTAGCAGGAGGGGCAGTGCCTGG CACAAC-3′, MmLeda_149His150 (forward) 5′GACCATCATCA TCATCATCATGGGCTCATCCTTGGGGA-AACACCTGCTACCTTGAG-3′, MmLeda_149His150 (reverse) 5′-CCCATGATGATGATGAT-GATG GTCTCCGTCATCCCGCATGGAGTCTGAGTTAGG-3′. Final PCR products were digested with NheI and BstBI and ligated to the pLenti_ADR3 vector (modified pHAGE vector). All primers were designed using Vector NTI Suite 8 Software (InforMax).

2.2. Cell culture and treatments

Cell lines were obtained from ATCC (USA). MDCK cells were maintained in MEM Eagle Alpha Modification Medium (Sigma, Germany) supplemented with 10% FCS (Biochrom, Germany), 1% MEM Non-essential Amino Acids, 1% Sodium pyruvate, 1% L-Glutamine (Sigma, Germany), and 1% Penicillin/Streptomycin (Biochrom, Germany). CHO and HEK293T cells were cultivated in DMEM/F-12 or DMEM respectively (Life Technologies, Germany) with 10% FCS and 1% Penicillin/Streptomycin. Liver sinusoidal endothelial cells (LSECs) were isolated and maintained as described previously [1].

Furin inhibitor I (decanoyl-Arg-Val-Lys-Arg-chloromethylketone from Bachem, Germany) was diluted in a serum-free cell culture medium to indicated concentrations and used to treat the cells for 8 h at 37°. Collagenase P (Roche, Germany) was diluted in a serum-free cell culture medium to a concentration of 1 mg/ml and was used to treat B16F10 cells for one hour at 37°.

2.3. Generation of transgenic cell lines

HEK293T cells were co-transfected with a modified pHAGE lentiviral vector [8] carrying murine LEDA-1 cDNA and pMD2.G L1, pMDLg/pRRE L3, pRSV rev L2, and pcDNA3.1/p35 E 71 plasmids (kindly provided by M. Leverkus, Mannheim, Germany) using X-tremeGENE 9 DNA Transfection Reagent (Roche, Germany). Conditioned supernatants containing recombinant lentiviruses were harvested 48–72 h after transfection, filtered (0.45 μm filters from Carl Roth, Germany) and concentrated using Vivaspin 20 MWCO 100,000 columns (Sartorius, Germany). The concentrated supernatants were applied to the cells. Selection was carried out with 10 μg/ml Puromycin (Invivogen).

MDCK cells were transfected with pEF6/V5 vectors carrying different modifications of human LEDA-1 cDNA using GenePORTER Gold Transfection Reagent (BioCat, Germany). Single clones were isolated and selected with 10 μg/mL Blasticidin S hydrochloride (Sigma, Germany).

2.4. Deglycosylation

Sixty micrograms of total protein were deglycosylated with Sialidase-A and O-Glycanase for 3 h at 37 °C using Enzymatic Deglycosylation Kit (Prozyme, Germany).

2.5. Antibodies

Primary antibodies: custom-made guinea pig polyclonal anti-LEDA-1 antibody [1], mouse anti-his antibody (GE Healthcare Life Science), rabbit anti-beta-actin polyclonal antibody (Sigma, Germany). Mouse monoclonal anti-LEDA-1 (C-terminus) antibody (clone 9C7) was raised against a C-terminal peptide fragment of mouse LEDA-1.

Secondary antibodies: FITC-conjugated rat anti-mouse light chain IgGκ (BD Biosciences), DyLight 488-conjugated anti-mouse IgG (Dianova, Germany), HRP-conjugated goat anti-mouse IgG (GE Healthcare, UK), HRP-conjugated donkey anti-guinea pig IgG (Dianova, Germany).

2.6. Western Blot

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as previously described [1]. Signal intensity of Luminata Forte Western HRP Substrate (Millipore, Germany) was detected by ChemoCam Imager (Intas science imaging, Germany). Anti-His Dynabead purification of His-tagged Leda-1 mutants was carried out as recommended by the manufacturer (Invitrogen, Germany).

2.7. FACS

For FACS analysis cells were washed with PBS and blocked with FACS buffer (10% FCS in 0.1% Sodium Azide). Primary antibody, i.e. mouse anti-His (GE Healthcare Life Science), was incubated on ice for 1 h. This was followed by secondary antibody (FITC Rat anti-mouse light chain, BD Biosciences) incubation on ice for 1 h. Fluorescently labeled cells were analyzed with FACS-CantoII (BD Biosciences, Heidelberg, Germany). Results were analyzed with the WinMDI Version 2.8 software (www.winmdi.software.informer.com).

2.8. Immunofluorescence

Cells were grown on 10 mm coverslips (R. Langenbrinck, Germany) and fixed for 10 min with 4%-paraformaldehyde (Fluka, Germany). The fixed cells were permeabilized with 0.1% Triton-X-100 (Sigma, Germany), blocked with 5% bovine serum albumin (Fitzgerald, USA), and incubated with primary antibodies followed by appropriate secondary antibodies.

2.9. Confocal microscopy

The preparations were analyzed by laser scanning spectral confocal microscopy (Leica, Heidelberg, Germany). The excitation wavelength was set at 488 nm. The emission maximum at 518 nm was detected to visualize DyLight 488 conjugates. Images were acquired in a sequential mode and processed with Leica confocal software.

3. Results and discussion

The LEDA-1/PIANP protein was previously identified by us by Western Blot (WB) with a custom-made polyclonal antibody in rat liver sinusoidal endothelial cells (LSECs) as a protein of a molecular weight of approximately 24 kDa [1]. Here, we generated a monoclonal mouse antibody (9C7) against a C-terminal peptide of LEDA-1/PIANP which proved to be highly specific and sensitive on WB detecting similar protein bands as the previously described polyclonal antibody. With the anti-LEDA-1 antibodies, a major band of approximately 38 kDa was detected by WB of rat and mouse brain lysates, rat liver lysates, and B16F10 mouse melanoma cells (Fig. 1A and B). In contrast, WB of primary LSECs again showed a major band of approximately 24 kDa (Fig. 1A). The apparent difference in the molecular weight of LEDA-1/PIANP in isolated primary LSECs as opposed to whole liver lysates could be explained by the action of collagenase P which is used as part of the isolation protocol of primary rat LSECs. Indeed, collagenase P

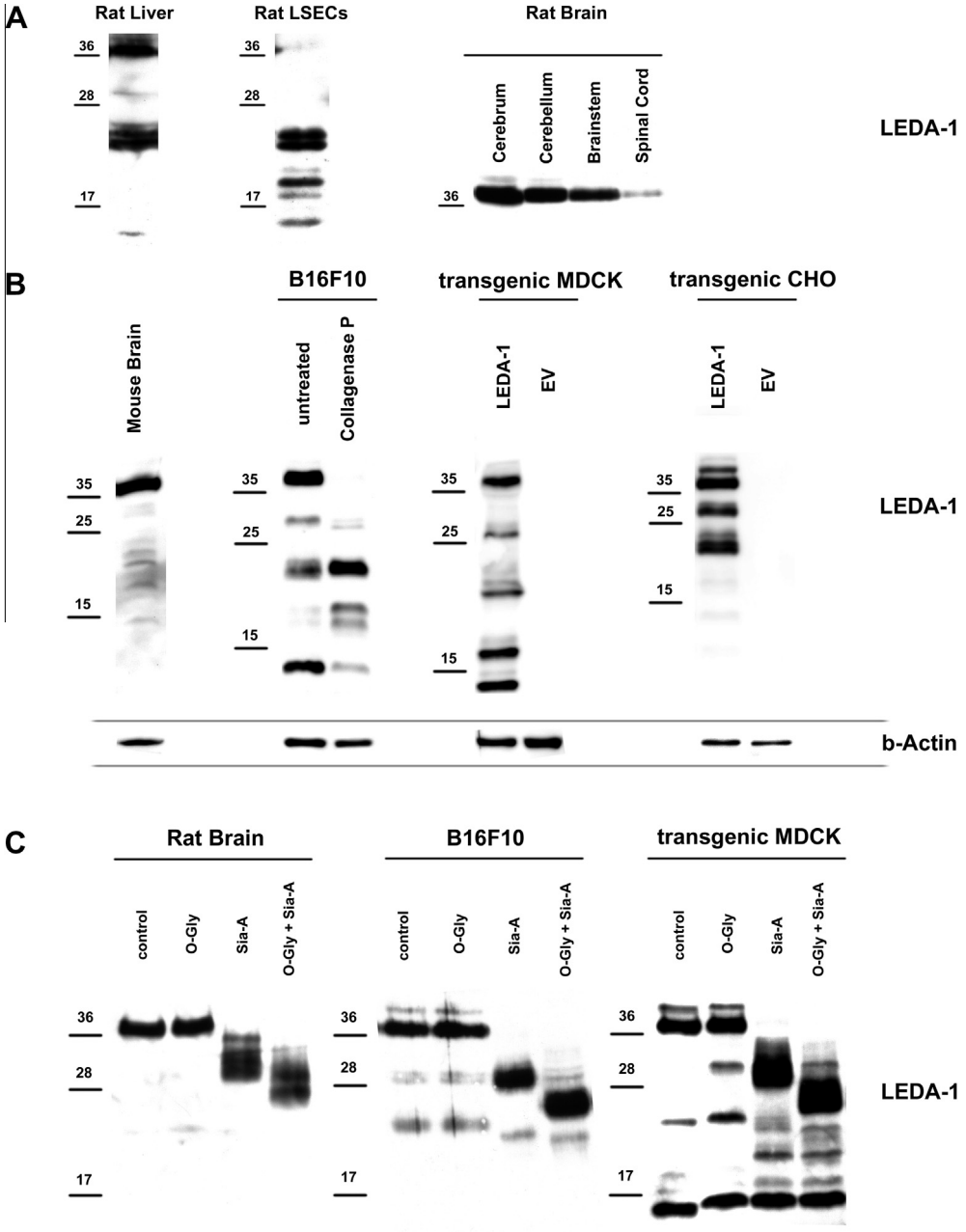

Fig. 1. LEDA-1/PIANP protein expression and glycosylation in organs and cell lines. (A) Western Blot of rat liver, LSECs, and brain protein lysates with the guinea pig anti-LEDA-1 polyclonal antibody. (B) Western Blot of protein lysates of mouse brain, B16F10, and transgenic cell lines with the mouse anti-LEDA-1 (C-terminus) monoclonal antibody. (C) Western Blot of protein lysates of rat brain, B16F10 and transgenic MDCK before and after enzymatic deglycosylation. O-Gly–O-Glycanase, Sia-A–Sialidase-A. Primary antibody-guinea pig anti-LEDA-1 polyclonal IgG.

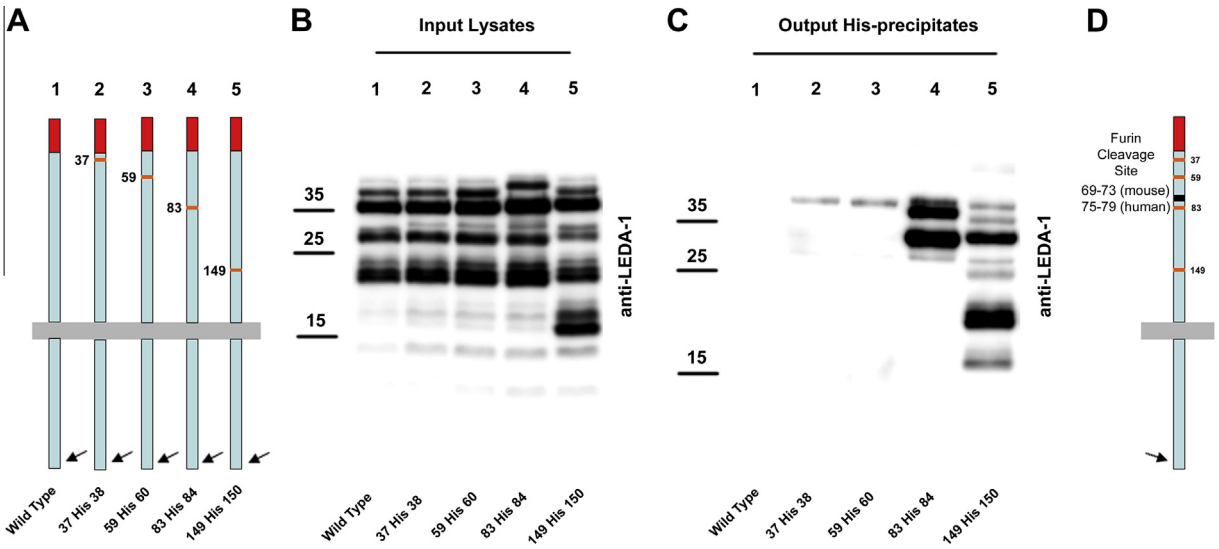

Fig. 2. LEDA-1/PIANP is cleaved between aa59 and aa83. (A) Schematic representation of the used mouse LEDA-1 constructs. Positions of His-tags are indicated. The binding site of anti-LEDA-1-antibodies is indicated by an arrow. (B) Western Blot of protein lysates of transgenic CHO cells expressing His-tagged LEDA-1 as indicated in A. Primary antibody-mouse anti-LEDA-1 (C-terminus) monoclonal IgG. (C) Western Blot of LEDA-1 immunoprecipitated with anti-His dynabeads from protein lysates of transgenic CHO cells expressing His-tagged LEDA-1 as indicated in A. Primary antibody applied in Western Blot – mouse anti-LEDA-1 (C-terminus) monoclonal IgG. (D) Schematic representation of the putative furine cleavage site in the mouse LEDA-1. The binding site of anti-LEDA-1-antibodies is indicated by an arrow.

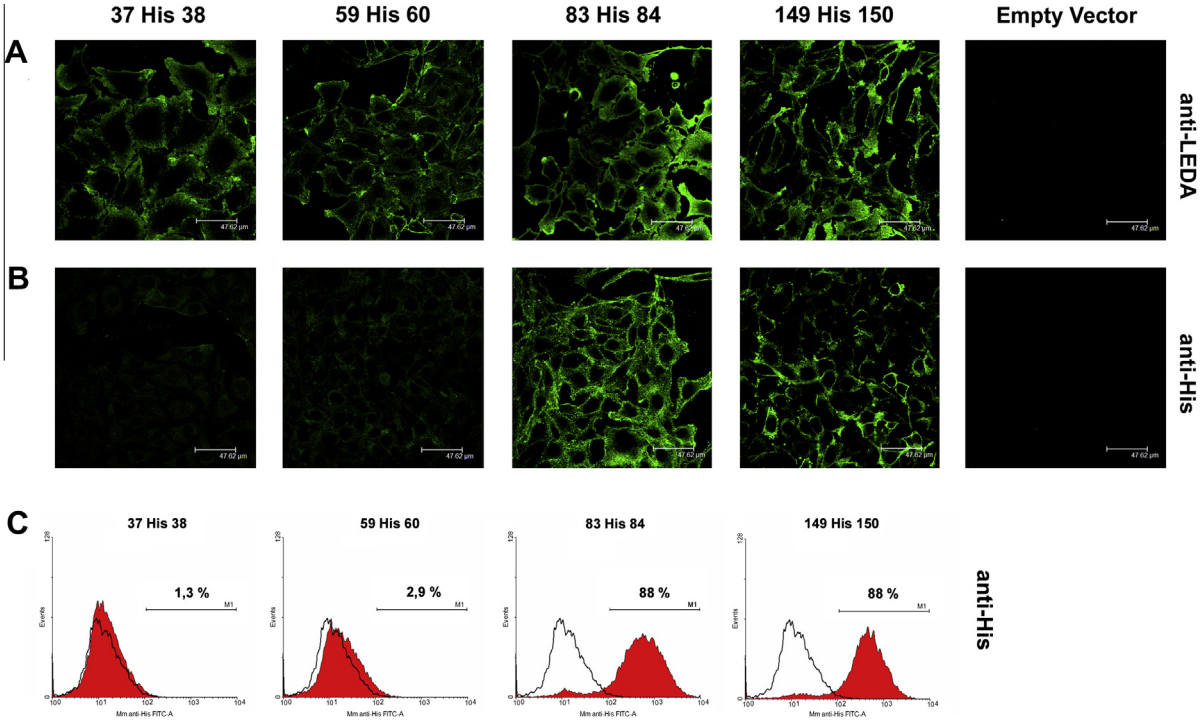

Fig. 3. LEDA-1/PIANP cleavage between aa59 and aa83 occurs before localizing to the plasma membrane. (A, B) Immunohistochemistry of transgenic CHO cells expressing LEDA-1 with his-tags at indicated positions. Primary antibodies-mouse anti-LEDA-1 (C-terminus) monoclonal IgG (A) mouse anti-His IgG (B) Images were acquired using laser scanning confocal microscopy. Bars 47, 62 μm. (C) FACS of transgenic CHO cells expressing LEDA-1 with his-tags at indicated positions. Primary antibody-mouse anti-His IgG.

treatment of B16F10 cells reduced the size of the major LEDA-1/PIANP band to approximately 24 kDa (Fig. 1B). In complement to these findings, we were able to detect recombinant mouse and human LEDA-1/PIANP in transgenic cell lines (CHO, MDCK) that do not naturally express LEDA-1/PIANP. A band pattern similar to rat brain and liver lysates suggests that the majority of LEDA-1/PIANP is similarly processed in vivo and in the analyzed cell lines in vitro.

Beside the major band at approximately 38 kDa, several smaller bands were detectable in all cell lines analyzed. As LEDA-1/PIANP is known to be O-glycosylated and sialidated, tissue and cell lysates were subjected to deglycosylation with O-Glycanase and Sialidase. A shift of the major band to approximately 26 kDa was detected after deglycosylation of recombinant mouse and human Leda-1 (Fig. 1C). O-Glycanase was active, only in the presence of Sialidase-A. Based on the modes of action and specifity of these

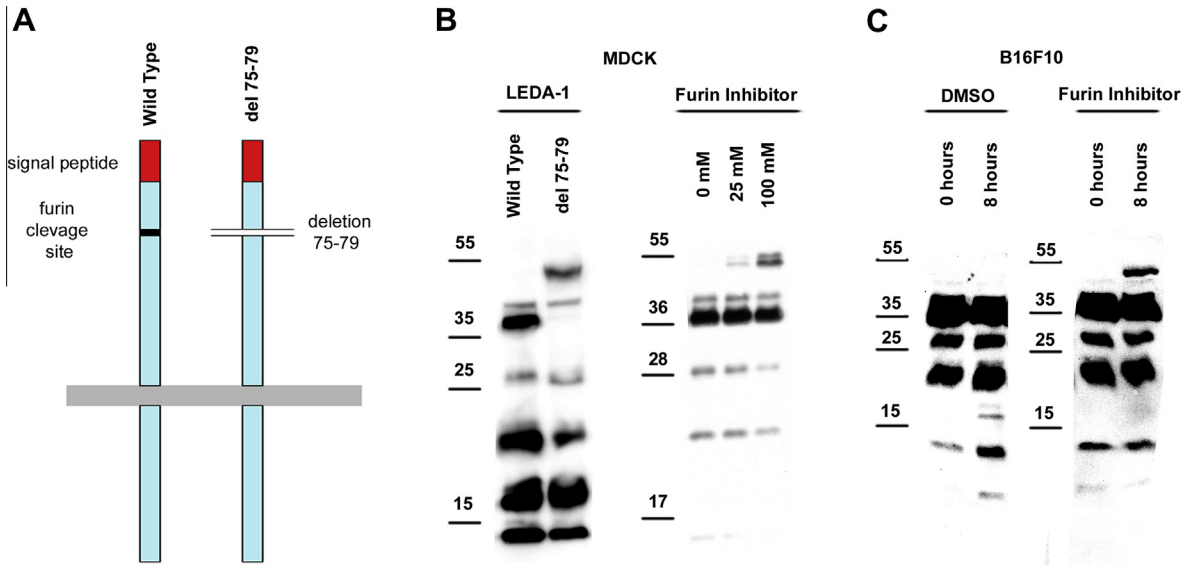

Fig. 4. LEDA-1/PIANP processing takes place at a Furin cleavage site at aa75–79 (human) or aa69–73 (mouse). (A) Schematic representation of the putative furine cleavage site and the introduced deletion in the human LEDA-1. (B) Western Blot of protein lysates of transgenic MDCK cells expressing wild type and mutated LEDA-1 (left) and transgenic MDCK cells treated with the Furin inhibitor I at indicated concentrations (right). (C) Western Blot of protein lysates of murine B16F10 cells naturally expressing LEDA-1. The cells were treated with the Furin inhibitor I (100 mM) or a vehicle for indicated time.

enzymes [9] our data indicate that poly-sialylated galactose-β(1-3)-N-acetylgalactosamine-α1-linked core 1 oligosaccharides are attached to human, mouse, and rat LEDA-1. However, glycosylation alone was not able to explain the multiple bands seen on Western Blot.

To further scrutinize processing of LEDA-1/PIANP, His-tags were inserted at several positions (aa37, aa59, aa83, aa149) in the extracellular domain of mouse LEDA-1/PIANP. CHO cells, which do not express LEDA-1/PIANP naturally, were transfected with His-tagged LEDA-1 using a lentiviral vector. Pull-down of the tagged constructs from CHO cells with anti-His dynabeads revealed that the majority of LEDA-1/PIANP protein has to be cleaved between aa59 and aa83 and that several steps of subsequent processing occur between aa83 and aa149 (Fig. 2). This may be explained by the presence of a putative furine cleavage site identified by an in silico prediction algorithm [10] at the amino acid position 69–73 in mouse and 75–79 in human LEDA-1.

To analyze the subcellular distribution of the differently processed variants of LEDA-1/PIANP, immunocytochemistry (ICC) and FACS analyses were performed with an anti-His and an anti-LEDA-1/PIANP antibody using transgenic CHO cells expressing LEDA-1/PIANP with His-tags at different positions (aa37, aa59, aa83, aa149). On ICC the antibody binding the C-terminus of LEDA-1/PIANP facing the cytoplasm did not show any differences in intracellular localization of these His-tagged constructs (Fig. 3A). In contrast, anti-His antibody binding to His-tags located in the extracellular N-terminal part of LEDA-1 revealed membranous staining only in constructs tagged at aa83 and aa149. His-Tags at aa59 and aa83 which are located further away from the plasma membrane could not be recognized at the cell surface (Fig. 3B). These findings suggest that cleavage between aa59 and aa83 takes place early during LEDA-1 processing. Similarly, FACS analysis demonstrated cell surface expression of His-tagged LEDA-1/PIANP proteins only in cells expressing LEDA-1 with His-tag at aa83 or aa149 and not for constructs tagged at aa37 or aa59 (Fig. 3C).

These data suggest that LEDA-1/PIANP protein is proteolytically processed between aa59 and aa83 before it is sorted to the plasma membrane. Early proteolytic processing of type-I-transmembrane proteins often occurs by proprotein convertases such as Furin, PC1, PC4 or PC7 [11]. Indeed, we identified a putative Furin cleavage site at aa69–73 and aa75–79 in the sequence of murine and human LEDA-1/PIANP, respectively (Figs. 2D and 4A). To analyze whether proprotein convertase processing takes place at this particular cleavage site, human LEDA-1/PIANP was mutated by deletion of aa75–79 and transfected into MDCK cells (Fig. 4A). Comparison of wildtype (WT) LEDA-1 and LEDA-1-del75–79 revealed a major band of approximately 52 kDa for the deletion mutant as compared to 38 kDa for WT (Fig. 4B). To further test our hypothesis, B16F10 and LEDA-1/PIANP-transfected MDCK cells were treated with Furin Inhibitor I (Dec-RVKR-CMK) which also led to the accumulation of a protein of a molecular weight around52 kDa (Fig. 4B and C). Although Furin-mediated proteolytic cleavage may also occur later during protein processing such as after endocytosis, mutational analysis, processing dynamics and inhibition experiments suggest that LEDA-1/PIANP is proteolytically cleaved by Furin-like proprotein convertases at aa75–79 in human and aa69–73 in mouse cells before it gets to the plasma membrane. In addition, multiple steps of proteolytic cleavage of LEDA-1 were observed after its localization to the plasma membrane as evidenced by multiple bands on Western Blot. In general, these findings may suggest that soluble fragments of LEDA-1/PIANP could be generated by proteolytic cleavage and shed into the extracellular space thus activating receptors such as PILRα not only by direct cell–cell contact, but also at distant sites. In summary, these findings improve our knowledge of the complex processing of LEDA-1/PIANP and promote future investigation of LEDA-1/PIANP processing, interactions and functions in vitro and in vivo.

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