THE N-TERMINAL DOMAIN OF FILAGGRIN 2 CO-LOCALIZES, BINDS TO AND ACTIVATES THE SKIN ASPARTIC ACID PROTEASE IN THE EPIDERMIS OF HUMAN SKIN

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INTRODUCTION

Filaggrin processing is a major biological event in the production of the free amino acid component of Natural Moisturizing Factors (NMF) in skin hydration. Skin aspartic acid protease (SASPase) is one of the key enzymes involved in the early processing of filaggrin during epidermal terminal differentiation. SASPase is expressed as a 28 kDa proform and auto-activated 14 kDa form in the stratum granulosum (SG) & stratum corneum (SC) of normal human skin where it is believed to be involved in the control of epidermal differentiation and desquamation. In psoriasis, the 28 kDa form persists in the SC whereas only the 14 kDa form occurs in the upper layers of normal skin (Bernard et al 2005). SASPase deficiency in hairless mice revealed a dry skin phenotype associated with a defect in filaggrin maturation. Furthermore, SASPase cleaves the linker region between the monomer repeats in filaggrin which are a major source of NMFs in the SC (Matsui et al 2011). This suggests that SASPase is a key enzyme in the processing of filaggrin to NMFs which play an important role in maintaining hydration in the SC. In previous studies, yeast two hybrid analyses using SASPase as bait against a human reconstructed skin library identified that the N-terminal domain of filaggrin 2 (FLG2-Nter) strongly bound to the N-terminal fragment of SASPase. This study aimed at deepening our understanding of the regulation of SASPase by FLG2-Nter.

Bernard D, Mehul B, Thomas-Collignon A, Delattre C, Donovan M, et al. (2005) Identification and characterization of a novel retroviral-like aspartic protease specifically expressed in human epidermis. *J Invest Dermatol* 125: 278-287.
Matsui T, Miyamoto K, Kubo A, Kawasaki H, Ebihara T, et al. (2011) SASPase regulates stratum corneum hydration through profilaggrin-to-filaggrin processing. *EMBO Mol Med* 3:320-333

RESULTS

Figure 1: The N-terminal domain of Filaggrin 2 binds to SASPase 28.

Surface plasmon resonance (SPR) analysis using a Biacore T200 instrument shows the binding between recombinant GST-Flag-SASPase 28 and MBP-HA FLG2S100 (aa2-95). A goat polyclonal anti-GST was immobilized on a CM5 sensorchip and used to capture GST-Flag-SASPase 28 and GST, respectively. MBP-HA FLG2S100 (aa 2-95) was injected at 6 different concentrations (1 to 6 μ M). No binding was observed between GST and MBP-HA FLG2S100 (aa2-95). The kinetic association (k_a) and dissociation (k_d) rate constants were $4056 \text{ M}^{-1}\text{s}^{-1}$ and 1177 s^{-1} respectively, yielding a dissociation constant K_D of $0.29 \mu\text{M}$.

Figure 2: The binding of SASPase 28 to FLG2Nter is specific among S100 proteins.

SPR analysis of binding between recombinant GST-Flag-SASPase and several S100 proteins showed that the binding was specific between FLG2Nter and SASPase 28 with a dissociation constant K_D of $0.29 \mu\text{M}$. No binding was observed between SASPase 28 and the S100 domains (aa 2-95) of filaggrin (FLG), hornlerin or trichohyalin (TCHH).

Figure 3: SASPase28 and FLG2Nter co-localize to the stratum granulosum of human epidermis in human skin.

Confocal immunohistochemical staining of the expression of SASPase28 and the FLG2Nter on frozen sections from human skin. A) FLG2Nter detected with an Alexafluor 546 conjugated recombinant HuCAL antibody (in red). B) SASPase28 detected with monoclonal against SASPase28 and Alexafluor 488 conjugated anti mouse antibody (in green). C) Overlay showing co-localization of SASPase28/FLG2Nter in the SG of human epidermis (arrows). Nuclei were stained with Dapi (blue). Scale bar = $75 \mu\text{m}$

Figure 4: Expressed SASPase28 and FLG2Nter co-localize in cytosol of Hela cells.

A proximity ligation assay was performed in Hela cells in which recombinant proteins for SASPase28 and FLG2Nter were overexpressed. Fixed cells were incubated with a specific monoclonal antibody to SASPase28 and a recombinant antibody to FLG2Nter conjugated with Duolink probe marker oligonucleotide PLUS. Visualization of interacting proteins was performed using a Duolink kit and nuclei were stained with Dapi. In the presence of both antibodies both proteins are co-localized (red staining & white arrows) in the cytosol of the cells.

Figure 5: SASPase interactome in normal human keratinocytes.

A proximity mediated BioID2 assay was performed in NHK cells to identify interacting proteins with SASPase. NHKs were transfected with a Tet-on 3G BioID2/SASPase28 vector, selected with puromycin and induced with 10 ng/ml doxycycline and grown to confluence in the presence of $50 \mu\text{m}$ biotin. A) Western blot analysis for SASPase in protein cell lysates shows the induced expression of the chimeric BioID2-SASPase 28 protein (65 kDa). B) Streptavidin-HRP blot showing biotinylated bands in induced cells at 76, 38 and 30 kDa.

Figure 6: FLG2Nter & SASPase14 are present in the Stratum Corneum of human skin.

Western blot analyses of SASPase and FLG2Nter in soluble protein extracts from the SC, human epidermis and reconstructed epidermis (RHE) shows: A) 15 kDa band (blue arrow) representing FLG2Nter in SC, human epidermis and RHE. B) SASPase28 (red arrow) and its 14 kDa catalytic form (green arrow) in the same samples. (RHE = reconstructed epidermal skin; NE = normal skin epidermis; PSC = planar stratum corneum; SC = stratum corneum from varnish stripping (VS) samples; rec = recombinant FLG2Nter. C) SASPase14 (green arrow) in repetitive VS samples of SC from the leg: outer most layer (O) to inner most layer (I). D) FLG2Nter (15 kDa band - blue arrow) in the same samples.

Figure 7: FLG2Nter activates the auto-activation and proteolytic activity of SASPase28 in vitro

A) In vitro microplate assay using recombinant SASPase28 and FLG2Nter (aa 2-213) and FLG2Nter (aa 81-213) at different mass ratios with a peptide Dabcyt-QIDRIMEK-Glu(Edans)-NH2 at pH5 & 37°C showed that FLG2Nter (aa 2-213) enhanced SASPase enzymatic activity. The truncated FLG2Nter (aa 81-213) did not stimulate SASPase. B) Overlapping peptides (13 aa) representing the FLG2Nter (aa 1-212) were evaluated in the assay. Peptide (# 25) LMIFKLTMACNKV in the EF hand domain of FLG2Nter significantly stimulated SASPase activity. C) Kinetic analysis of SASPase28 auto-activation at pH 5.2 & 37°C with peptide LMIFKLTMACNKV at different mass ratios. SASPase Western blot analysis at time points 0 to 60 mins showed that the peptide enhanced SASPase28 auto-activation

Figure 8: Model for the role of FLG2Nter in the auto activation of SASPase and the maturation of FLG and FLG2.

CONCLUSION

- The binding of FLG2Nter to SASPase28 was confirmed by SPR and appears to be specific among related S100 proteins.
- Confocal immunohistochemical studies showed that SASPase and FLG2Nter co-localized within the stratum granulosum. Their interaction was confirmed by PLA in cultured cells.
- Western blots confirmed the expression of FLG2Nter in the epidermis and in SC. Both forms of SASPase are expressed in the epidermis while SASPase 14 appears to be major form of SASPase present in SC - at least in samples taken from the legs. However, further experiments are required to determine if this is true in SC from other body sites.
- In vitro enzymatic assays and western blots showed that the FLG2Nter and a peptide from the EF-Hand of this region enhanced SASPase activity and its auto-activation to its 14 kDa active form.
- The data suggest that FLG2Nter can regulate SASPase auto-activation, which may be a key event in filaggrin processing to NMF in human skin.