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House dust mite allergen Der f 1 can induce the activation of latent TGF-β via its protease activity

Yuki Nakamura [a], Masanori Miyata [a], Naomi Shimokawa [a], Yuko Ohnuma [a], Ryohei Katoh [b], Hideoki Ogawa [c], Ko Okumura [c], Atsuhito Nakao [a,c,*]

[a] Department of Immunology, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan
[b] Department of Human Pathology, Faculty of Medicine, University of Yamanashi, Yamanashi 409-3898, Japan
[c] Atopy Research Center, Juntendo University School of Medicine, Tokyo 113-8421, Japan

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ABSTRACT

A major house dust mite allergen Der f 1 belongs to the papain-like cysteine protease family. This study investigated whether Der f 1 can cleave the latency-associated peptide (LAP) of transforming growth factor (TGF)-β via its proteolytic activity and activate latent TGF-β. We found that Der f 1 can cleave LAP and induce the activation of latent TGF-β, leading to functional Smad signaling. Importantly, these actions of Der f 1 were inhibited by cysteine protease inhibitor E64 or inactivation of the protease activity by heat. Thus, latent TGF-β may be a direct target of Der f 1 protease activity.
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1. Introduction

Der f 1 and Der p 1, derived from the house dust mites *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*, are major allergens associated with asthma, atomic dermatitis, and allergic rhinitis [1]. Both Der f 1 and Der p 1 belong to the papain-like cysteine protease family and their proteolytic activity has been suggested to be linked to the potent allergenicity of house dust mites [2] For instances, Der p 1 can cleave the tight-junction adhesion protein occludin, thereby disrupting epithelial barrier function [3].

Transforming growth factor-β (TGF-β) is a fibrogenic cytokine that is involved in the pathophysiology of asthma [4]. TGF-β is secreted in a latent complex in which TGF-β homodimers are non-covalently associated with homodimers of the pro-peptide called the latency-associated peptide (LAP) [5,6]. The release of TGF-β from its LAP is required for binding of TGF-β to the cellular receptors and subsequent activation (phosphorylation) of intracellular signaling mediators of Smad2 and Smad3 [7]. Extensive work on the activation of latent TGF-β led to two classes of putative TGF-β activators which liberate TGF-β from the constraints of LAP through a conformational change or LAP proteolysis [5,6]. For instances, extremely low or high pH activates latent TGF-β by altering the structure of LAP, whereas certain proteases activate latent TGF-β through proteolytic digestion of LAP.

Given that both Der f 1 and TGF-β are involved in the pathophysiology of asthma, we hypothesized that there might be a functional link between Der f 1 and TGF-β. This study thus investigated whether Der f 1 can cleave LAP via the protease activity and activate latent TGF-β in vitro. We then determined whether the intratracheal challenge of Der f 1 induces the activation of latent TGF-β in the mouse lung, resulting in increases in Smad promoter activity and the expression of TGF-β target genes.

2. Materials and methods

2.1. Reagents

Natural Der f 1 (Asahi Breweries, Tokyo, Japan), recombinant human TGF-β1 LAP, TGF-β1, and latent TGF-β1 (TGF-β1 associated with its LAP) (R&D Inc., Minneapolis, MN), HTS466284, a selective small molecule inhibitor of TGF-β type I receptor kinase [8] (Calbiochem, San Diego, CA), and E64 (Peptide Institute, Osaka, Japan) were purchased.

Abbreviations: LAP, latency-associated peptide; SBE, Smad binding element; SEAP, secreted alkaline phosphatase; COL1A2, collagen type I α2 chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

* Corresponding author. Address: Department of Immunology, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. Fax: +81 55 273 9542.
E-mail address: anakao@yamanashi.ac.jp (A. Nakao).

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2.2. Der f 1/LAP/latent TGF-β1 incubations

Reaction mixtures containing recombinant LAP, TGF-β1, latent TGF-β1, and/or Der f 1 (1 μg for each) with or without 1000-fold molar excess of E64 were incubated for the indicated times at 37 °C in 100 μl PBS. For some experiments, Der f 1 (1 μg) was incubated at 98 °C for 30 min to inactivate the enzymatic activity and then was added to the reaction mixtures.

2.3. Western blotting

For the detection of LAP protein, recombinant LAP, Der f 1, or reaction mixture containing LAP and Der f 1 with or without E64 were boiled for 3 min in SDS sample buffer containing 5% 2-mercaptoethanol (2-ME) and subjected to SDS–PAGE. Proteins (50 ng/well) were then electrotransferred to nitrocellulose membrane and subjected to immunoblotting with anti-human TGF-β1 LAP antibody (R&D Inc.) which can detect both homodimeric (75 kDa) and monomeric forms (20–29 kDa) of LAP protein. For the detection of TGF-β protein, the samples were dissolved in 2-ME-free SDS sample buffer without boiling and subjected to immunoblotting with anti-human TGF-β1/2/3 antibody (1D11) (R&D Inc.) because the antibody can detect only TGF-β homodimers (25 kDa). For the detection of phosphorylated Smad2 in BEAS2B cells, the whole cell extracts (10 μg) were subjected to immunoblotting with anti-phosphorylated Smad2 antibody, anti-Smad2/3 antibody, or β-actin antibody (all from Cell Signaling Technology Inc., Danvers, MA).

2.4. Transcriptional reporter assay

A transcriptional reporter assay using $(CAGA)_{12}$-luciferase reporter plasmid ($CAGA_{12}$-luc), which is exclusively activated by Smad3 and Smad4 [9], in human bronchial epithelial cell line BEAS2B cells was performed as previously described [9]. Briefly, 24 h after transfection of the reporter plasmid, the cells were stimulated with the reaction mixtures containing latent TGF-β1 and/or Der f 1 (1 ng/ml for each) with or without E64, or 1 ng/ml TGF-β1 in the presence or absence of 10 μM HTS466284. Ninety-six hours after the stimulation, the luciferase activities were measured.

2.5. Detection of endogenous Smad7 mRNA in BEAS2B cells

BEAS2B cells (1×10^6 $well^{-1}$) were stimulated with the reaction mixtures containing latent TGF-β1 and/or Der f 1 (1 ng/ml for each) with or without E64, or 1 ng/ml TGF-β1. Three hours after the stimulation, total RNA was extracted and a real-time RT-PCR for human Smad7 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed.

2.6. In vivo Der f 1 exposure

BALB/c mice (Japan SLC, Tokyo, Japan) were challenged intranasal with 20 μl of Der f 1 (100 μg/ml) incubated with 50 μM E64 or PBS for 30 min at 37 °C or incubated at 98 °C for 30 min to inactivate the enzymatic activity prior to the challenge. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Yamanashi.

2.7. Histology

Six hours after the Der f 1 challenge with or without E64, mouse lungs were removed, fixed, and the tissue sections were stained with hematoxylin and eosin (H&E).

2.8. Measurement of TGF-β activity in bronchoalveolar lavage (BAL) fluid

BAL fluid samples were collected 6 h after Der f 1 challenge. TGF-β activity in the BAL fluid samples was then determined using MFB-F11 cells stably transfected with the reporter plasmid containing 12 CAGA boxes (Smad binding element, SBE), fused to a secreted alkaline phosphatase (SEAP) reporter gene [10]. Briefly, MFB-F11 cells (4×10^4 cells/well) in 96-well flat-bottom tissue culture plates (BD Falcon, San Jose, CA) were incubated in 50 μl serum-free DMEM for 2 h and then BAL fluid samples or TGF-β (5 and 10 pg/ml) were added in 50 μl volume in the presence or absence of 10 μM HTS466284. Following 24 h incubation, SEAP activity in the culture supernatants was measured using Gene Light 55 (Microtec nition, Chiba, Japan).

2.9. Quantitative real-time RT-PCR using mouse lung samples

Six hours after Der f 1 challenge with or without E64, the right lung was homogenized and total RNA was extracted. A real-time RT-PCR analysis for mouse TGF-β2, Smad7, collagen type I α2 chain (COL1A2), and GAPDH was then performed.

2.10. Bioluminescence imaging

Bioluminescence was detected with the In Vivo Imaging System (IVIS; Xenogen, Alameda, CA) using transgenic mouse lines T9-7F or T9-55F harboring a SBE-luc transgene [11]. This transgene consists of 12 SBE repeats fused to a herpes simplex virus/thymidine kinase minimal promoter upstream of firefly luciferase followed by a simian virus 40 late polyadenylation signal. Six hours after an intratracheal challenge with Der f 1, the mice were anesthetized and injected i.p. with 150 mg/kg D-luciferin (Sigma–Aldrich). Five minutes later, the mice were anesthetized with an overdose of Halothane and killed by cervical dislocation. The lungs were then rapidly dissected, placed in 24-well culture discs and imaged exactly 10 min after the initial luciferin administration. Photons emitted from the lungs were acquired as photons per s/cm^2 per steridian (sr) by using LIVINGIMAGE software (Xenogen) and integrated over 5 min. For photon quantification, a region of interest was manually selected; the signal intensity was converted into photons per s/cm^2 per sr.

2.11. Data analysis

The data are summarized as the mean ± S.D. Statistical analysis was performed using a non-parametric Mann–Whitney *U*-test to compare data in different groups. A value of $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Der f 1 can cleave LAP and induce activation of latent TGF-β in vitro

To investigate whether Der f 1 can cleave LAP, recombinant TGF-β1 LAP (LAP) was incubated with natural Der f 1 and then analyzed by Western blotting with anti-LAP antibody (Fig. 1A). Following incubation, LAP (29 kDa) was cleaved at 15 min after incubation and the cleaved LAP protein fragment (approximately 20 kDa) appeared. As a control, complete cleavage of LAP by incubation with trypsin was observed. Importantly, the ability of Der f 1 to cleave LAP was lost by the co-incubation of E64, a cysteine protease inhibitor, with Der f 1 or by the prior inactivation of the protease activity of Der f 1 by heat. In contrast, the incubation of

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