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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.

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 noncovalently 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

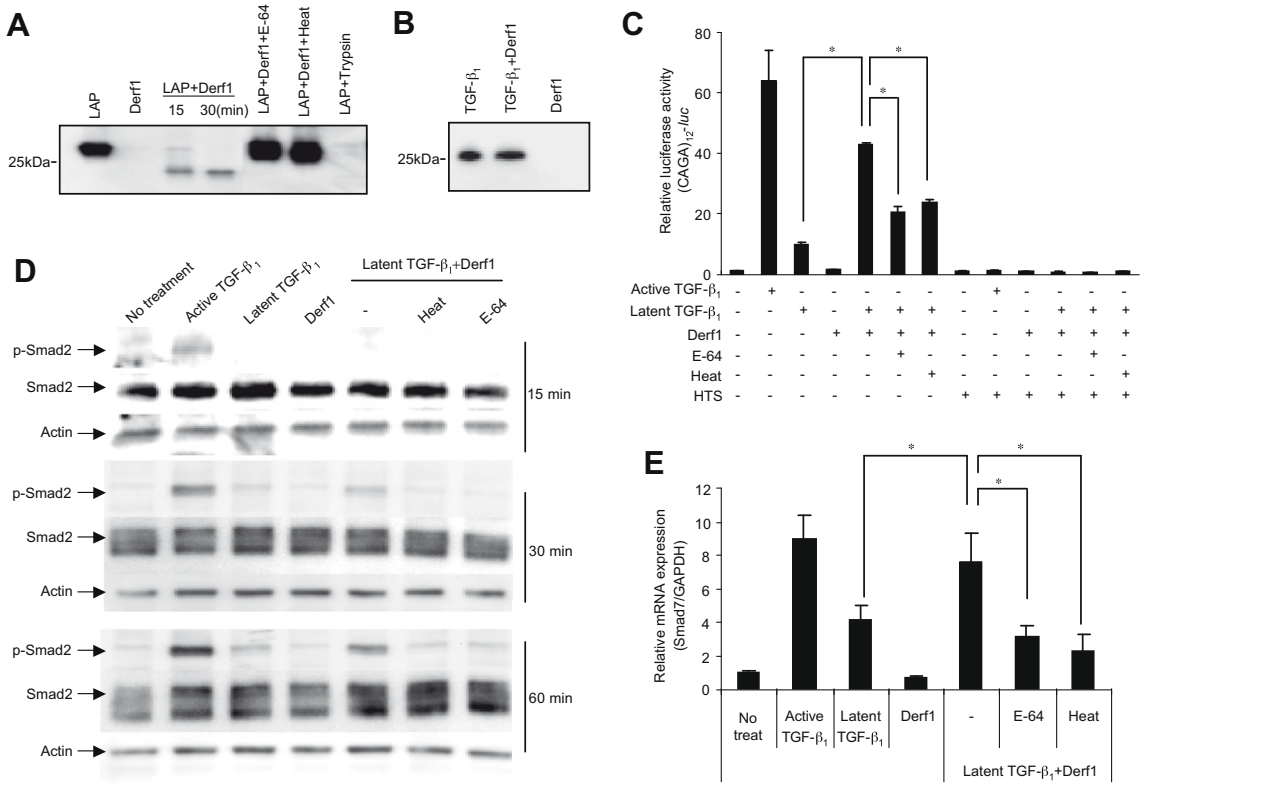

Fig. 1. Der f 1 can cleave LAP and induce activation of latent TGF-β, depending on the protease activity. (A)–(E) Recombinant LAP or TGF-β1 was incubated in the presence or absence of Der f 1 with or without E64 or was incubated with Der f 1 inactivated by heat. The reaction mixtures were then immunoblotted with anti-LAP antibody (A) or anti-TGF-β1 antibody (B) or were assayed by a reporter analysis using $CAGA_{12}$-luc plasmid in BEAS2B cells (C), or were added to BEAS2B cell culture for the indicated times and the cell lysates were subjected to a Western blot analysis with anti-phosphorylated Smad2 (pSmad2) or Smad2 antibody (D), or were added to BEAS2B cell culture for the indicated times and real-time PCR was performed for the detection of Smad7 mRNA (E). $^{*}P < 0.05$.

recombinant TGF-β1 with natural Der f 1 did not cleave the TGF-β1 protein (25 kDa) (Fig. 1B). These results indicated that Der f 1 can cleave LAP, depending on its cysteine protease activity.

To investigate whether Der f 1 can activate latent TGF-β, the TGF-β-inducible $(CAGA)_{12}$-luciferase reporter construct was transfected into BEAS2B cells and then the transfected cells were stimulated with the reaction mixtures containing latent TGF-β1 (LAP + TGF-β1) and/or Der f 1 (Fig. 1C). Stimulation of the transfected cells with latent TGF-β1 increased luciferase activity to some extent possibly due to non-specific activation of latent TGF-β1 during preparation processes (e.g. by stirring). The reaction mixtures containing latent TGF-β1 and Der f 1 significantly increased the luciferase activities in comparison to the reaction mixtures containing latent TGF-β1 alone. The ability of Der f 1 to increase the luciferase activity was lost by the co-incubation of E64 with Der f 1 or by the prior inactivation of the protease activity of Der f 1 by heat. Importantly, the increase in the luciferase activity induced by the reaction mixtures containing latent TGF-β1 and Der f 1 was completely abrogated by the addition of HTS466284, a TGF-β type I receptor kinase inhibitor, thus confirming that the increase of luciferase activity totally depends on TGF-β activity.

In addition, the ability of Der f 1 to activate latent TGF-β was examined by the detection of phosphorylation of Smad2 in BEAS2B cells (Fig. 1D). The reaction mixtures containing latent TGF-β1 and Der f 1 induced phosphorylation of Smad2 in BEAS2B cells, beginning at 30 min after the stimulation. The Der f 1-induced phosphorylation of Smad2 was lost by the co-incubation of E64 with Der f 1 or by the prior inactivation of the protease activity of Der f 1 by heat.

Furthermore, the ability of Der f 1 to activate latent TGF-β was examined by the detection of endogenous Smad7, a major TGF-β target gene [12], mRNA induction in BEAS2B cells (Fig. 1E). Stimulation of the transfected cells with latent TGF-β1 (LAP + TGF-β1) increased Smad7 mRNA expression to some extent. The reaction mixtures containing latent TGF-β1 and Der f 1 significantly increased Smad7 mRNA expression in comparison to the reaction mixtures containing latent TGF-β1 alone. The ability of Der f 1 to increase Smad7 mRNA expression was lost by the co-incubation of E64 with Der f 1 or by the prior inactivation of the protease activity of Der f 1 by heat. These results indicated that Der f 1 can induce the activation of latent TGF-β via its cysteine protease activity in vitro. Given the earlier findings (Fig. 1A), it is very likely that the Der f 1-induced cleavage of LAP is responsible for the activation of latent TGF-β.

3.2. Intratracheal exposure of Der f 1 to mice induces TGF-β activity in BAL fluids and expression of TGF-β target genes in the lungs

The normal adult mouse lung expresses all isoforms of mammalian TGF-β protein (-β1, -β2, and -β3), most likely as the latent forms [13]. To determine whether Der f 1 induces the activation of latent TGF-β expressed in the mouse lung, the mice were intratracheally exposed to Der f 1 and then the BAL fluid and the lung tissue samples were immediately (6 h following the challenge) obtained in order to measure the TGF-β activity and expression levels of TGF-β target genes (TGF-β itself [14], Smad7 [12], and COL1A2 [15]), respectively.

To detect the TGF-β activity in BAL fluid samples, MFB-F11 reporter cells were used (Fig. 2A). The BAL fluid samples obtained from PBS-challenged mice did not show any increase in SEAP activity, whereas the BAL samples obtained from Der f 1-challenged mice showed a significant increase in SEAP activity. Importantly, this SEAP activity was detected without any activation of the samples, suggesting that TGF-β was already activated in the BAL fluid

samples. BAL fluid samples obtained from the mice challenged with a Der f 1 and E64 mixture showed decreased SEAP activity in comparison to those obtained from Der f 1-challenged mice. In addition, BAL fluid samples obtained from the mice challenged with Der f 1 of which the protease activity was inactivated by heat showed decreased SEAP activity in comparison to those obtained from Der f 1-challenged mice. We confirmed that these SEAP activities relied on TGF-β activity in the BAL fluid samples because the addition of HTS466284 in the MFB-F11 cell culture abolished all the SEAP activity.

Consistent with these findings, the increases in the transcription of major TGF-β target genes TGF-β2, Smad7, and COL1A2 were observed in the lungs obtained from mice challenged with Der f 1, but not with PBS (Fig. 2B). The Der f 1-induced increases in TGF-β2, Smad7, and COL1A2 mRNAs were abrogated by the treatment of Der f 1 with E64.

We found that the lung tissue sections obtained from Der f 1-challenged mice at this point (6 h after the challenge) did not show any overt pathological changes (e.g. inflammation) (Fig. 2C). Thus, the increases in TGF-β activity and target gene expressions in the mouse lung following Der f 1 challenge are likely to reflect the activation of latent TGF-β, which is not associated with inflammation.

To further confirm that Der f 1 leads to the activation of TGF-β signaling in vivo, SBE-luc mice were used. These mice harbor a

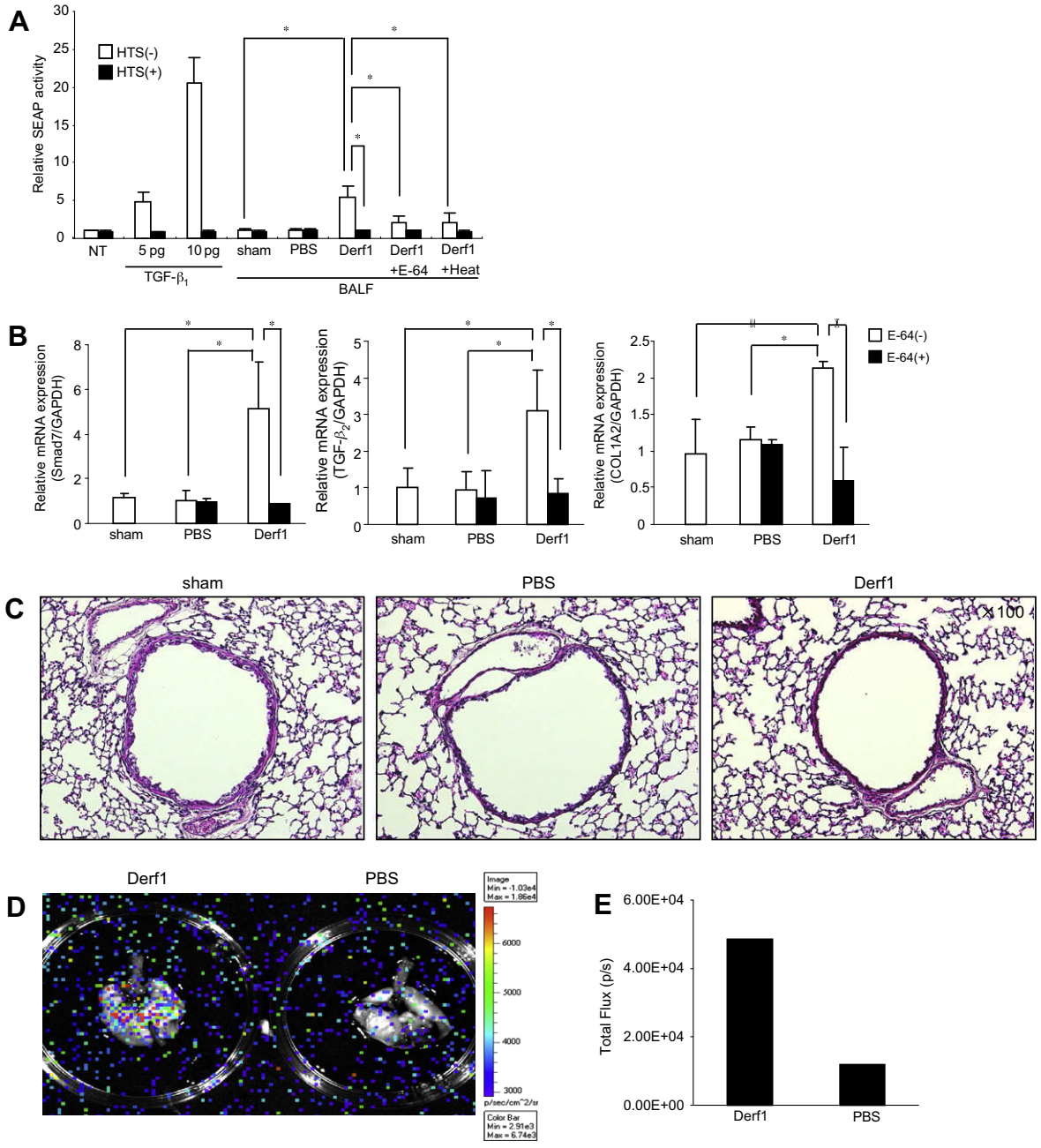

Fig. 2. Intratracheal exposure of Der f 1 induces the activation of latent TGF-β in the mouse lung, depending on the protease activity. (A)–(D) BALB/c mice were intratracheally challenged with Der f 1 (Der f 1) or PBS (PBS) with or without E64 or challenged with heat-inactivated Der f 1 (Der f 1 + Heat). Six hours after the challenge, BAL fluid and lung tissue samples were collected. (A) SEAP activity of the BAL fluid samples measured using MFB-F11 cells with or without HTS466284 (HTS) in the culture system. (B) Real-time RT-PCR for the detection of Smad7, TGF-β2, and collagen type I α2 chain (COL1A2) and GAPDH mRNAs. (C) HE staining of the lung tissue sections. Sham: sham-treated mice; PBS: PBS-treated mice; Der f 1: Der f 1-treated mice. (D) and (E) SBE-luc mice were intratracheally challenged with Der f 1 (Der f 1) or PBS (PBS). Six hours after the challenge, rapidly dissected mouse lungs were imaged with a bioluminescence imaging system for 1 min (D) and the quantification of bioluminescence in the mouse lungs was performed (E). $^{*}P < 0.05$.

TGF-β/Smad-responsive luciferase reporter gene and have been shown to reliably indicate activation of TGF-β/Smad signaling [11]. Consistent with the earlier results, the intratracheal challenge of Der f 1 to SBE-luc mice resulted in an increase in the emission of bioluminescence from the lungs (Fig. 2D and E).

Natural Der f 1 had the ability to activate latent TGF-β in vitro without any manipulations known to reduce oxidized catalytic cysteine residue of cysteine proteases such as adding cysteine or DTT (Fig. 1). This is consistent with the recent report by Ogawa et al. who showed that natural non-activated Der f 1 can induce GM-CSF and IL-8 production in human keratinocytes [16]. They speculated that the oxidized catalytic cysteine residue of Der f 1 could be reduced by interaction with the cells or components in the culture supernatant including cell-derived factors. Similar speculation could be applied to the current experimental system.

Where and how the cysteine protease activity of Der f 1 (or Der p 1) can be activated in vivo also remain critical issues in this field. Herbert et al. speculated that Der p 1 could be activated by reduction with glutathione present in airway tissue on the basis of their in vitro results [17]. These issues remain to be clarified in future studies.

TGF-β plays an important role for the pathophysiology of asthma, particularly in fibrotic changes of asthmatic airways termed "airway remodeling" [4]. Recent evidence suggests that airway remodeling may occur very early in asthma pathogenesis, independent of inflammation [18]. For instances, there were increases in the number of phosphorylated Smad2-positive epithelial cells in mucosal biopsy specimens obtained at 24 h after allergen challenge in patients with asthma along with fibrotic changes of the airways [19]. The identification of latent TGF-β as a possible direct target of Der f 1 protease activity might provide an experimental support for the notion that airway remodeling may occur early in asthma pathogenesis and shed new light on the pathogenesis of airway remodeling in asthma or remodeling observed in other house dust mite-related allergic diseases.

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