Sos recruitment system for the analysis of the interaction between sulfatase-modifying factor 2 subtypes and interleukin-13

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ABSTRACT. Interleukin (IL)-13 is a central mediator in allergic asthma. Our previous results have indicated that sulfatase-modifying factor 2 (SUMF2) interacts with IL-13 and inhibits its secretion. In this study, we investigated the interactions between SUMF2 subtypes and 2 types of IL-13. Wild type IL-13 (wh-IL-13) and its mutated counterpart (mh-IL-13) were analyzed and cloned into pSos yeast expression vectors. Protein was expressed in host cdc25H yeast strains. A quartet of agar growth plates was prepared for the yeast two-hybrid system, which was used to detect IL-13 and SUMF2 subtype interactions. Both yeast expression vectors, pSos/whIL-13 and pSos/whIL-13, and recombinant expression vectors for the 5 subtypes of SUMF2 (pMyr/SUMF2-Vx) were constructed. Our data showed that all of the SUMF2 subtypes bound to whIL-13 and mhIL-13 in the CytoTrap system. Five SUMF2 subtypes - SUMF2-V2, SUMF2-V3,

SUMF2-V4, SUMF2-V5, and SUMF2-V7 - interacted with whIL-13 and mhIL-13. These subtypes may contribute to allergic asthma by mediating IL-13 release.

Key words: Sulfatase modifying factor 2; Interleukin 13; Subtypes; Allergic asthma; Pathogenesis

INTRODUCTION

Allergic asthma is a complex disorder characterized by local and systemic allergic inflammation and reversible airway obstruction (Grünig et al., 1998). The importance of interleukin-13 (IL-13) in allergic disorders in humans has been evidenced in numerous reports of excessive IL-13 production in asthma, atopic rhinitis, allergic dermatitis, and chronic sinusitis (Madala et al., 2011; Walsh et al., 2011). Through its combined actions on epithelial and smooth muscle cells, IL-13 can induce all of the pathological features of asthma independently of traditional effector cells such as mast cells and eosinophils.

Evidence suggests that IL-13 is the initial inducer of the pathological features of asthma, which adds to the theory that mast cells and eosinophils are the main players in asthma pathogenesis (Wills-Karp and Chiaramonte, 2003). Numerous studies have focused on the molecular mechanisms that regulate IL-13 production and its downstream effector pathways. However, these mechanisms remain unknown.

The sulfatases are a large family of prokaryotic and eukaryotic enzymes that catalyze the hydrolysis of ester sulfates (Hopwood and Ballabio, 2003). Sulfatases undergo a unique post-translational modification in which a cysteine located within their active sites is converted into C-alpha-formylglycine (FGly), which is essential for the catalytic activities of the sulfatases. FGly is generated by the protein product of sulfatase-modifying factor 1 (SUMF1) (Cosma et al., 2003). The primary protein sequences of SUMF1 and SUMF2 are nearly identical except for a region between amino acids 303 and 351 in SUMF1 (Dierks et al., 2003). This region is likely the catalytic domain of SUMF1. Thus, SUMF2 binds to SUMF1 and sulfatases and controls the generation of FGly-activated sulfatases (Zito et al., 2005).

Our previous studies have shown that SUMF2 binds to IL-13 and inhibits the secretion of IL-13 independently of IL-13 glycosylation. When we induced transient SUMF2 expression in lymphocytes, we found that amounts of the 12-kDa form of intracellular IL-13 were significantly increased, whereas levels of IL-13 in lymphocyte culture supernatants were significantly reduced. In addition, blocking N-glycosylation via treatment with tunicamycin eliminated the 17-kDa form of intracellular IL-13 but failed to promote the secretion of IL-13 by bronchial smooth muscle cells (Liang et al., 2009).

However, whether SUMF2 subtypes bind to wild type IL-13 (whIL-13) or the mutated form of IL-13 (mhIL-13) remained unclear. Thus, in this study, we analyzed IL-13 and its mutant by cloning them into pSos yeast expression vectors to express the protein in host cdc25H yeast strains. A quartet of agar growth plates was prepared for the CytoTrap yeast 2-hybrid system to detect IL-13 and SUMF2 subtype interactions. The Sos recruitment system is shown in Figure 1.
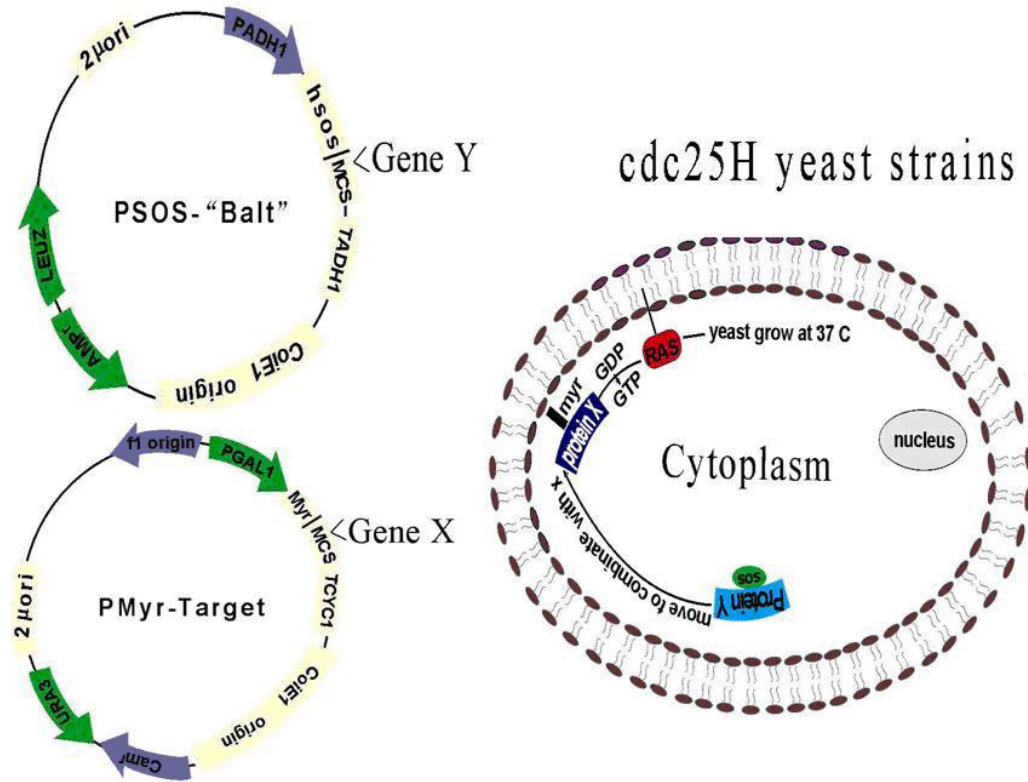
Figure 1. Summary of the Cytotrap yeast two-hybrid system based on the Sos recruitment system. Protein baits were cloned into the pSos plasmids and target part cloned into the pMyr plasmid. Protein–protein interactions have a temperature-sensitive mutation, allowing yeast to grow at the non-permissive temperature.

MATERIAL AND METHODS

Reagents and plasmids

Restriction enzymes and reverse transcriptase Moloney murine leukemia virus (RNase H-) were from Takara Biotechnology Co., Ltd. (Japan). The yeast 2-hybrid screening used a CytoTrap Vector Kit (Stratagene). Vectors pSos, pMyr, and control plasmid pMyr MAFB (positive control for CytoTrap Vector Kit), pSos MAFB (positive positive control for CytoTrap Vector Kit), pMyr Lamin C (negative control for CytoTrap Vector Kit), and pSos Col l (negative control for CytoTrap Vector Kit) were provided with the CytoTrap Vector Kit. We used yeast *Saccharomyces cerevisiae* temperature-sensitive mutant strain cdc25H(α) containing a point mutation at amino acid residue 1328 of the cell division cycle 25 homolog A gene. This yeast homologue of the Sos gene encodes for a guanyl nucleotide exchange factor that binds to and activates Ras, which initiates the Ras signal transduction pathway.

Plasmid construction

Total RNA was isolated from human bronchial epithelial cells using Trizol® reagent (Invitrogen) according to manufacturer instructions. Primers (Table 1) were designed to be complementary to a target sequence and introduce desired restriction sites. Polymerase chain reaction was performed using Phusion high-fidelity DNA polymerase (Finnzymes) according to manufacturer instructions. The target sequences of SUMF2 subtypes and restriction sites were all included in the pUC plasmids, which were designed by Shanghai SANGON Biological Engineering Technology Services Limited. The sequences of the pUC plasmids are shown in Figure 2.

Table 1. List of primers used to perform PCR-based cloning and plasmid sequencing.

Primer	Restriction enzyme	Use
5'-CA**GGATCC**ATGGCCCTGTGCCTCCCTC-3'	*Bam*HI	pSos-whIL-13(-)
5'-CACC**CCATGG**TCAGTTGAACCGTCCCTCG-3'	*Nco*I	pSos-whIL-13(+)
5'-CA**GGATCC**ATGGCCCTGTGCCTCCCTC-3'	*Bam*HI	pSos-mhIL-13(-)
5'-CACC**CCATGG**TCAGTTGAACTGTCCCTCG-3'	*Nco*I	pSos-mhIL-13(+)

(+) = forward primer; (-) = reverse primer. The bold part is the restriction site for cloning.

Figure 2. Target sequence of SUMF2 and the restriction sites *Eco*RI and *Xho* were all designed in the puc plasmids, which were completed by Shanghai SANGON Biological Engineering Technology Services Limited.

Polymerase chain reaction products, pUC plasmids, and vectors were then digested with the indicated restriction enzymes, gel purified, ligated using T4 DNA ligase, and transformed in chemically competent JM109 *Escherichia coli* cells. Selection for successful transformants used media containing 100 μg/mL ampicillin or 30 μg/mL chloramphenicol for pSos and pMyr constructs, respectively. Transformants were amplified, and plasmids were extracted using commercial kits according to manufacturer instructions.

Verification of yeast host

The cdc25H host yeast strain, which has a temperature-sensitive phenotype, was obtained from a glycerol stock after scraping off solid ice with a sterile inoculating stick or sterile wire loop and streaking the splinters onto a YPAD (yeastextract,peptone,adenine,and dextrose) agar plate. At the same time, each of 4 "dropout" plates was stocked with agar yeast streaked from -80°C glycerol. The dropout plates were made using appropriate 10X dropout solutions and agar plates with the following nutritional requirements: histidine, tryptophan, uracil, and leucine. These plates were prepared to test the cdc25H yeast strain. They were incubated at room temperature (22°-25°C) for 3-4 days. After the phenotype was verified, colonies from the YPAD plate were picked to inoculate medium for yeast competent cells.

Competent cdc25H yeast cells and bait plasmid transformation

Yeast competent cells were generated as described previously using the CytoTrap yeast 2-hybrid system. Twenty microliters of salmon sperm DNA was pre-boiled and added to each transformation tube, and the SUMF2 subtypes constructed in pMyr vectors were co-transformed with the bait plasmid in yeast cells with a transformation efficiency of 1.75×10^3 cfu/mg DNA (30 μL each). The DNA mixture and 1 mL polyethylene glycol/LiAc solution (50 μL total) were added to the competent cells. The DNA-cell mixture was incubated on a rotator at 25°C for 30 min, and then 100 μL dimethyl sulfoxide was added to the transformation mix and heated at 42°C for 10 min. The cell mixture was centrifuged for 1 min. The supernatant was discarded and the cell pellet was resuspended with 1 M sorbitol and plated on SD/glucose (-L) plates and incubated for 4-6 days at 24-25°C. Three colonies were picked and resuspended in 24 μL double-distilled water, after which 2.5 μL each suspension was spotted onto an SD/glucose (-UL) agar plate and an SD/galactose (-UL) agar plate incubated at 37°C for 7-10 days to observe whether yeast colonies appeared.

RESULTS

Construction of pSos-IL-13 yeast expression vectors

The whIL-13 and mhIL-13 genes were cloned in pSos (pSos-IL-13) yeast expression vectors. Restriction endonuclease *Bam*HI/*Nco*I digestion patterns showed that a 205-bp fragment had been correctly inserted into these vectors. The whIL-13 and mhIL-13 gene sequences were analyzed by Shanghai Biotechnology Co., Ltd., and conformed to the IL-13 gene sequence in a gene bank (Mammalian Gene Collection; 116787). The 5 SUMF2 subtype

genes were abstracted from pUC plasmids and cloned in pMyr vectors. Their sequences were analyzed by Shanghai Biotechnology Co., Ltd.

Yeast host strain marker phenotype

After spotting on 4 SD agar plates [SD/glucose (-His), SD/glucose (-Trp), SD/ glucose (-Ura), and SD/glucose (-Leu)], yeast colonies did not appear until 7 days at 25°C, although numerous colonies appeared on the YPAD plate (see Table 1). The genotype of the cdc25H(α) strain was MATαhis3-200 ura3-52 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal+. Thus, the phenotype of this yeast strain was verified (growth on a YPAD plate but no growth on any of the 4 SD agar dropout plates). The colonies from the YPAD plate could be used as competent yeast cells. These results are summarized in Table 2.

Table 2. Test on the phenotype ability of cdc25H(α).

	SD/glucose(-His)	SD/glucose(-Trp)	SD/glucose(-Ura)	SD/glucose(-Leu)	YPAD
cdc25H(α)	-	-	-	-	+

(+) = cells grow well; (-) = cells fail to grow.

Screening for interactions between IL-13 and SUMF2 subtypes by CytoTrap

White madid patch colonies grew at room temperature on fresh SD/glucose (-UL) plates containing 12 groups until day 5. Six days later, the candidates that produced patches did not grow on SD/glucose (-UL) plates at 37°C. The negative control group co-transforming pSosMAFB and pMyr LaminC did not grow on SD/galactose (-UL) plates, but patches could be detected for other groups (Table 3).

Table 3. Test on the selfactivation and fixing ability of the bait vector.

Group	Plasmids transformed	SD/(-UL) 25°C	SD/(-UL) 37°C	
		Glucose	Glucose	Galactose
1	pSosMAFB + pMyrMAFB	+	-	+
2	pSosMAFB + pMyr laminC	+	-	-
3	pSosIL-wh13 + pMyr SUMF2V2	+	-	+
4	pSosIL- wh 13 + pMyr SUMF2V3	+	-	+
5	pSosIL- wh 13 + pMyr SUMF2V4	+	-	+
6	pSosIL- wh 13 + pMyr SUMF2V5	+	-	+
7	pSosIL- wh 13 + pMyr SUMF2V7	+	-	+
8	pSosIL- mh13 + pMyr SUMF2V2	+	-	+
9	pSosIL- mh 13 + pMyr SUMF2V3	+	-	+
10	pSosIL- mh 13 + pMyr SUMF2V4	+	-	+
11	pSosIL- mh 13 + pMyr SUMF2V5	+	-	+
12	pSosIL- mh 13 + pMyr SUMF2V7	+	-	+

(+) = cells grow well; (-) = cells fail to grow.

These results indicated that every SUMF2 subtype interacted with whIL-13 and that mhIL-13 combined with the 5 SUMF2 subtypes (Figure 3).
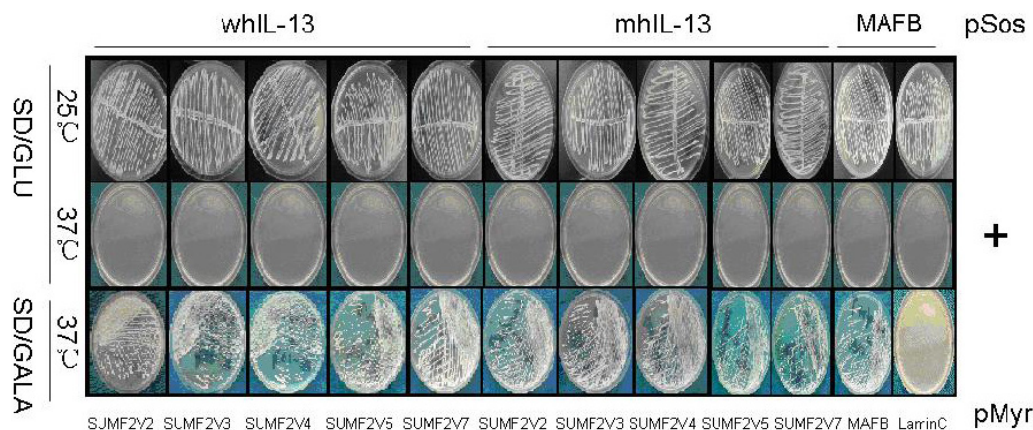
Figure 3. Detection of the interaction between whIL-13 (mhIL-13) and the subtypes of SUMF2 by the Sos recruitment assay. Saccharomyces cerevisiae strain cdc25H was transformed with the plasmid combinations indicated. pMyr-MAFB and pSos-MAFB were used as positive controls, and pMyr-Lamin C and pSos-MAFB were used as negative controls.

DISCUSSION

We sought information on SUMF2 localization and whether its subtypes could interact with whIL-13 and mhIL-13. The Sos recruitment system takes advantage of Ras-activation through membrane-localized hSos in yeast (Kruse et al., 2006; Aronheim et al., 2006). The SUMF2 subtypes are particularly well suited for this test, as they can rescue mutations in the yeast homolog *YJL097w/PHS1* (Bellec et al., 2002).

IL-13 is produced by activated T helper 2 cells, mast cells, and other cell types. IL-13 induces B cells to release immunoglobulin E, increases vascular cell adhesion molecule 1 expression (Wills-Karp et al., 1998), and is important for the recruitment of eosinophils to airway tissue. Furthermore, IL-13 can stimulate fibroblast proliferation, mast cell hyperplasia, mucus production, airway remodeling, and airway hyperresponsiveness (Wills-Karp et al., 1998; Kumar et al., 2004; Horvat et al., 2010). Although IL-13 is significant in the pathology of allergic asthma, little is known about its capability to modulate IL-13 secretion.

The catalytic sites of prokaryotic and eukaryotic sulfatases contain a unique amino acid, FGly. FGly is an aldehyde hydrate in the catalytic cleavage of sulfate esters and is therefore essential for the activities of sulfatases. In humans, the FGly-generating enzyme is encoded by the SUMF1 gene. SUMF2 is found only in vertebrates and shares a 48% amino acid identity and a 62% similarity with SUMF1 (Cosma et al., 2003). Although SUMF2 has no FGly-generating enzyme activity, it does co-localize and interact with SUMF1 in the endoplasmic reticulum, and it controls the generation of FGly-activated sulfatases (Zito et al., 2005). Our previous studies have shown that in addition to interacting with IL-13 receptors, SUMF2 interacts with IL-13. Furthermore, SUMF2 inhibits the secretion of IL-13 independently of IL-13 glycosylation (Liang et al., 2009).

Previous studies have identified many genetic variations in the IL-13 gene in both coding and non-coding regions. Among these various single-nucleotide polymorphisms, Ar-

g130Gln is the best known. Numerous studies have shown that it is closely associated with increased serum immunoglobulin E and asthma. In this study, we designed 2 plasmids, pSos-whIL-13 and pSos-mhIL-13 (Arg130Gln), and examined whether they interacted with SUMF2.

The National Center for Biotechnology Information identifies 5 SUMF2 subtypes: SUMF2 variant 2, SUMF2 variant 3, SUMF2 variant 4, SUMF2 variant 5, and SUMF2 variant 7. SUMF2 is a protein discovered during a genome-wide scan performed in 2003. Its function remains uncertain, as does that of the SUMF2 subtypes. pMyr-SUMF2-V2, pMyr-SUMF2-V3, pMyr-SUMF2-V4, pMyr-SUMF2-V5, and pMyr-SUMF2-V7 plasmids were designed to investigate the interaction of these subtypes with IL-13.

We found that all of the yeast strains except the negative control grew on SD/gal plates at 37°C but did not grow on glucose plates at 37°C (see Figure 3). These results indicated that all SUMF2 subtypes interacted with both whIL-13 and mhIL-13. However, whether all the SUMF2 subtypes interacted equally with IL-13 was unknown. Thus, additional research is required to determine whether all of these subtypes inhibit IL-13 secretion.

In summary, we confirmed that 5 SUMF2 subtypes, SUMF2-V2, SUMF2-V3, SUMF2-V4, SUMF2-V5, and SUMF2-V7, interacted with whIL-13 using a CytoTrap yeast system. Similarly, mhIL-13 may combine with these SUMF2 subtypes. We believe that the binding region is within a common domain of these subtypes. Thus, understanding the interaction at this binding site in the common domain would provide a foundation for the study of the role of SUMF2 in asthma.

ACKNOWLEDGMENTS

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