MIP-T3 associates with IL-13Rα1 and suppresses STAT6 activation in response to IL-13 stimulation

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Received 24 April 2003; revised 22 July 2003; accepted 22 July 2003

First published online 12 August 2003

Edited by Giulio Superti-Furga

Abstract To unravel the mechanism of interleukin-13 (IL-13)-specific functions, we sought to identify IL-13 receptor (IL-13R) binding molecules. A novel human IL-13Rα1 binding protein (IL13RBP1) has been identified using yeast tri-hybrid system, which was found to encode the same protein as MIP-T3 (microtubule interacting protein that associates with tumor necrosis factor (TNF) receptor associating factor-3 (TRAF3)). It constitutively associates with IL-13Rα1 and suppresses IL-4/13-induced signal transducer and activator of transcription-6 (STAT6) phosphorylation. IL-13-induced STAT6 activation was also inhibited as determined by dual luciferase assay and electrophoretic mobility shift assay (EMSA). These results suggest that MIP-T3 is a novel inhibitor of IL-13 signaling and may be a useful molecule in ameliorating various conditions in which IL-13 plays a central role.

Key words: Interleukin-13 receptor-α1 binding protein-1; Microtubule interacting protein that associates with tumor necrosis factor receptor associating factor-3; Interleukin-13; Interleukin-4; Signal transducer and activator of transcription-6

1. Introduction

Interleukin (IL)-13 is a pleiotropic immune regulatory cytokine that shares many structural and functional characteristics with IL-4 [1]. Despite their similarities, not all biological activities are mutual and overlapping due to differential expression of IL-4 and IL-13 receptor (IL-13R) complexes on various cell types. IL-13 has been shown to be necessary and sufficient for induction of asthma. In mouse models of allergic asthma, IL-13 blockade prevented allergen-induced airway inflammation while IL-13 delivery to the airway caused airway hyperresponsiveness (AHR), mucus production and eosinophilia [2–4]. IL-13 knockout mice failed to develop allergen-induced AHR; however, AHR was restored in IL-13(−/−) mice by the administration of recombinant IL-13 [5]. It has also been shown that IL-13 is more important than IL-4 as an inducer of the signal transducer and activator of transcription-6 (STAT6) in stimulating parasite expulsion [6]. In addition, IL-13 but not IL-4 is found to be an autocrine growth factor for Reed–Sternberg cells in Hodgkin lymphoma [7,8].

IL-13 signals were mediated mainly through Janus kinase (JAK)-STAT pathway and insulin receptor substrate (IRS)-1/2-phosphatidylinositol 3′-kinase (PI3k) pathway by using a complex of IL-4R and IL-13Rα1 [9–11]. But these could not offer sufficient explanation as to why IL-13 possesses distinct roles from IL-4. However, STAT6 has already been shown to be indispensable for IL-13-specific biological properties [12,13]. Therefore, identifying mechanisms of STAT6 regulation should be useful to define the distinct roles of IL-13.

In this study we have identified a novel IL-13R binding molecule by using yeast tri-hybrid system. The protein was termed as IL13RBP1 (IL-13Rα1 binding protein-1), which can constitutively associate with IL-13Rα1 chain, but not with IL-4Rα and IL-2Rγ. Interestingly, IL13RBP1 was found later to encode the same protein as MIP-T3 (microtubule interacting protein that associates with tumor necrosis factor (TNF) receptor associating factor-3 (TRAF3)), which can interact with microtubules and TRAF3 protein [14]. In our study, MIP-T3 was shown to suppress STAT6 tyrosine phosphorylation in response to IL-4/13 stimulation. However, MIP-T3 inhibits DNA binding activity and transcriptional activity in response to IL-13 but not to IL-4 stimulation. The role of MIP-T3 in IL-13 signals and its association with TRAF3 and microtubules suggest that it may work as a cross point between IL-13 and CD40 signaling pathway.

2. Materials and methods

2.1. Cell lines, cytokines and antibodies

THP-1 cells were cultured in RPMI-1640, 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and COS-7 cells were cultured in minimal essential medium (MEM) containing 10% fetal bovine serum. Recombinant human IL-4 (hIL-4) and human IL-

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Abbreviations: AHR, airway hyperresponsiveness; EMSA, electrophoretic mobility shift assay; IL-13R, interleukin-13 receptor; JAK, Janus kinase; MIP-T3, microtubule interacting protein that associates with tumor necrosis factor receptor associating factor-3; STAT, signal transducer and activator of transcription; TRAF, tumor necrosis factor receptor associating factor

0014-5793/03/$22.00 © 2003 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00860-3

13 (hIL-13) were purchased from PEPRO TECH EC Ltd (UK). Anti-c-myc (9E10), Omni-probe (M-21) (anti-His), anti-STAT6 (S-20), anti-IL-4Rα (C-20) and anti-IL-2Rγ (N-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotin-conjugated anti-phosphotyrosine (4G10) and streptavidin-horseradish peroxidase (HRP) were from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Yeast tri-hybrid cloning

pBTM-JH-IL-13Rα1 (cytoplasmic domain) was constructed and used as a bait to screen human fetal liver cDNA library (Clontech, USA) [15]. Positive yeast clones were selected by prototrophy for histidine or expression of β-galactosidase, and then subjected to sequence analysis to search for novel IL-13Rα1 binding proteins. Association between IL-13Rα1 and new proteins was confirmed in yeast two-hybrid system: Plasmid containing target gene and pAS21-IL-13Rα1 (cytoplasmic domain) were transformed into CG1945 strain. Histidine and β-galactosidase expression was determined to detect the association. The whole length of cDNA was cloned from human testis cDNA library (Clontech) by using 5′-rapid amplification of complementary deoxyribonucleic acid ends-polymerase chain reaction (RACE-PCR).

2.3. Immunoprecipitation and Western blot

Cells were transfected by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction. After transfection, cells were harvested and stimulated with hIL-4 or hIL-13 at 37°C for 15 min. Cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 7.4), 300 mM NaCl, 0.1% NP-40, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were incubated with antibody-conjugated protein G/A-Sepharose 4B (Sigma Aldrich, USA) overnight at 4°C. Immunoprecipitated proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Japan). Immunoblots were incubated with antibodies as required. Signal was detected by using enhanced chemiluminescence (ECL) Western detection reagent and exposed to Hyperfilm™ ECL (Amersham Pharmacia Biotech, UK).

To detect STAT6 tyrosine phosphorylation, cells were harvested in lysis buffer containing phosphatase inhibitor (1 mM Na_3VO_4, 25 mM NaF, 10 mM Na-pyrophosphate and 1 mM ethylenediamine tetraacetic acid (EDTA)). Immunoblot was reacted with diluted biotin-4G10 for 4 h and HRP-conjugated streptavidin for 30 min at room temperature. Finally ECL detection was performed and images were recorded by using Luminescent Image Analyzer LAS1000 plus (FUJIFILM, Japan).

2.4. Dual luciferase assay

TPU474 reporter gene was used for dual luciferase assay to determine STAT6 transcriptional activity. It contains four copies of individual oligonucleotide (C/EBP-N4) and a truncated thymidine kinase promoter (TK) [16]. N4 is a STAT6-specific binding site and TK drives expression of luciferase reporter gene (LUC). TPU474 and pSRHisA-MIP-T3 were transfected to COS-7 cells by using Lipofectamine 2000. pRL-TK vector (Promega, USA) expressing Sea pansy luciferase was co-transfected with every sample as internal control. After 24 h, cells were stimulated with recombinant hIL-4 (50 ng/ml) or hIL-13 (100 ng/ml) at 37°C for 6 h. Luciferase activity was measured by using Pica Gene Dual luciferase kit (Toyo Ink, Tokyo, Japan). Every sample was done in duplicate and each experiment was repeated twice. Expression of MIP-T3 in every sample was determined by immunoblotting analysis using Omni-probe.

2.5. Electrophoretic mobility shift assay (EMSA)

Plasmid DNA was transfected into semiconfluent cells using GenePORTER transfection reagent (Gene Therapy Systems, USA) according to the manufacturer's instructions. 2 days later, cells were

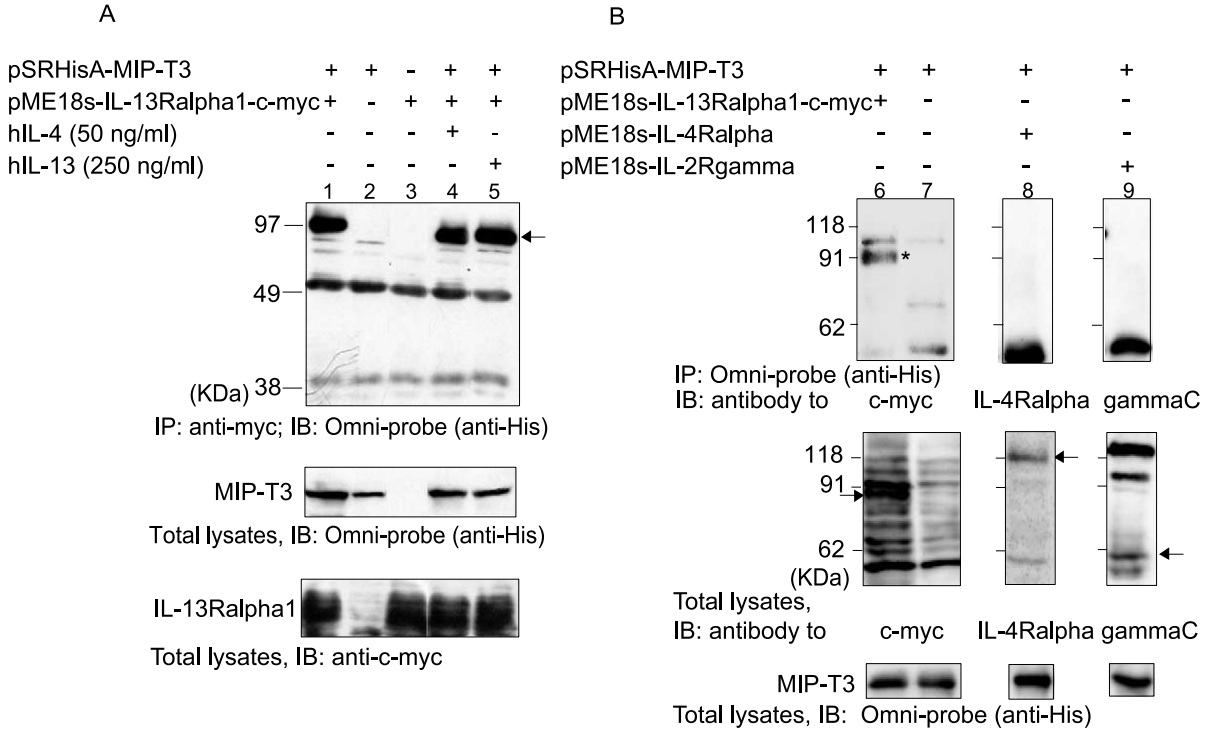

Fig. 1. Specific association between MIP-T3 and IL-13Rα1. A: Plasmids encoding IL-13Rα1 (10 μg) and MIP-T3 (15 μg) were transfected to 293T cells in a 90 mm dish by using Lipofectamine 2000. 24 h later, cells were stimulated with or without hIL-4 or hIL-13 at 37°C for 15 min. Cell lysates were immunoprecipitated (IP) with anti-c-myc antibody and immunoblotted (IB) with Omni-probe (top panel). Expression levels of MIP-T3 (middle panel) and IL-13Rα1 (lower panel) in total cell lysates were determined by immunoblotting with Omni-probe or anti-c-myc. Arrow indicates position of co-precipitated MIP-T3 protein. B: 293T cells were transfected with plasmids encoding various receptors (10 μg) and MIP-T3 (15 μg). Cell lysates were immunoprecipitated with Omni-probe and immunoblotted with anti-c-myc, anti-IL-4Rα (C-20), or anti-IL-2Rγ (N-20) antibody to detect co-precipitated receptors (top panel). Expression levels of receptors (middle panel) and MIP-T3 (lower panel) in total cell lysates were determined by immunoblotting with corresponding antibodies. * indicates co-precipitated IL-13Rα1 protein. Arrows indicate positions of receptors.

harvested and stimulated with hIL-4 (50 ng/ml) or hIL-13 (250 ng/ml) for 10 min. Cells were then washed with cold phosphate-buffered saline (PBS) and solubilized with cold whole cell extraction buffer (10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 20 mM HEPES (pH 7.0), 10 mM KCl, 300 mM NaCl, 0.5 mM dithiothreitol (DTT), 0.1% NP-40, 1 mM PMSF, 1 mM Na_3VO_4, and 20% glycerol). 50 μg of sample proteins were incubated in 20 μl of binding buffer (10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 10% glycerol, 0.05% NP-40, and 0.05 mg/ml poly(dI-dC)^2) for 20 min at room temperature with 1 ng of ^{32}P-labeled double stranded oligonucleotide probe SBE-1 [10]. Samples were applied to 4% non-reducing polyacrylamide gel and run at 150 V for 2.5 h. Gels were dried for 2 h and autoradiographed.

3. Results

3.1. Cloning of IL13RBP1 using yeast tri-hybrid system

To clarify the molecular and cellular events involved in IL-13-specific signaling pathways, we searched for novel IL-13Rα1 binding proteins by using yeast tri-hybrid system. Among the positive clones obtained, one fragment encoding 125 amino acids was found to associate with the cytoplasmic domain of IL-13Rα1 independent of tyrosine phosphorylation in yeast two- and tri-hybrid system.

By using this fragment as probe, the entire gene was cloned from human testis cDNA library, which has a whole length of 2568 bp and encodes a protein of 691 amino acids. It was named IL13RBP1 and submitted to GenBank (GenBank Accession Number AF242456). Coincidentally, another protein named MIP-T3 (GenBank Accession Number NM_015650) was found to be the same one as IL13RBP1. MIP-T3 was reported to constitutively associate with TRAF3 and to interact with microtubule, while in our studies it was cloned as an IL-13Rα1 binding protein.

3.2. Interaction of MIP-T3 with IL-13Rα1 in mammalian cells

To confirm the association between MIP-T3 and IL-13Rα1 in mammalian cells, pSRHisA-MIP-T3 was constructed and transfected to 293T cells with pME-IL-13Rα1-c-myc [10]. Association was detected by immunoprecipitation. MIP-T3 can be co-precipitated with IL-13Rα1 only when both proteins were expressed indicating their specific association (Fig. 1A, top panel, lane 1). In addition, the effects of hIL-4/13 on the association between MIP-T3 and IL-13Rα1 were investigated. In Fig. 1A (lanes 4, 5), band intensities of co-precipitated MIP-T3 did not change before and after hIL-4/13 stimulation. Therefore, the association between MIP-T3 and IL-13Rα1 was not affected by IL-4/13.

Besides IL-13Rα1, we also checked whether MIP-T3 associates with IL-4Rα or IL-2Rγ. Plasmids encoding different receptors were transfected into 293T cells with pSRHisA-MIP-T3. MIP-T3 protein was immunoprecipitated with Omni-probe and co-precipitated receptors were detected with related antibodies. Results in Fig. 1B showed that only IL-13Rα1 (lane 6) could be pulled down with MIP-T3, while IL-4Rα (lane 8) and IL-2Rγ (lane 9) could not.

The above results suggest that MIP-T3 may be a specific IL-13Rα1 binding protein and its association is not affected by hIL-4 or hIL-13.

3.3. Suppression of hIL-4/13-induced STAT6 tyrosine phosphorylation by MIP-T3

To gain an insight into functional importance of MIP-T3 association with IL-13Rα1, we studied whether STAT6 phosphorylation was affected by MIP-T3. COS-7 cell line was used as it expresses all necessary IL-4/13 signal molecules [17]. As shown in Fig. 2A, after introduction of MIP-T3, STAT6 phosphorylation was partially inhibited in response to hIL-4 and hIL-13 stimulation, but MIP-T3 suppression of IL-4-induced STAT6 phosphorylation (lanes 3, 4) was weaker than that of hIL-13 (lanes 5, 6).

To confirm the above results, we introduced various amounts of pSRHisA-MIP-T3 to COS-7 cells and detected STAT6 phosphorylation. As shown in Fig. 2B, with increasing amounts of transfected plasmid, STAT6 phosphorylation in response to hIL-13 was suppressed gradually. IL-4-induced STAT6 phosphorylation was determined in the same experiment. STAT6 phosphorylation was suppressed by MIP-T3, but the effect was modest compared to that of IL-13 (Fig.

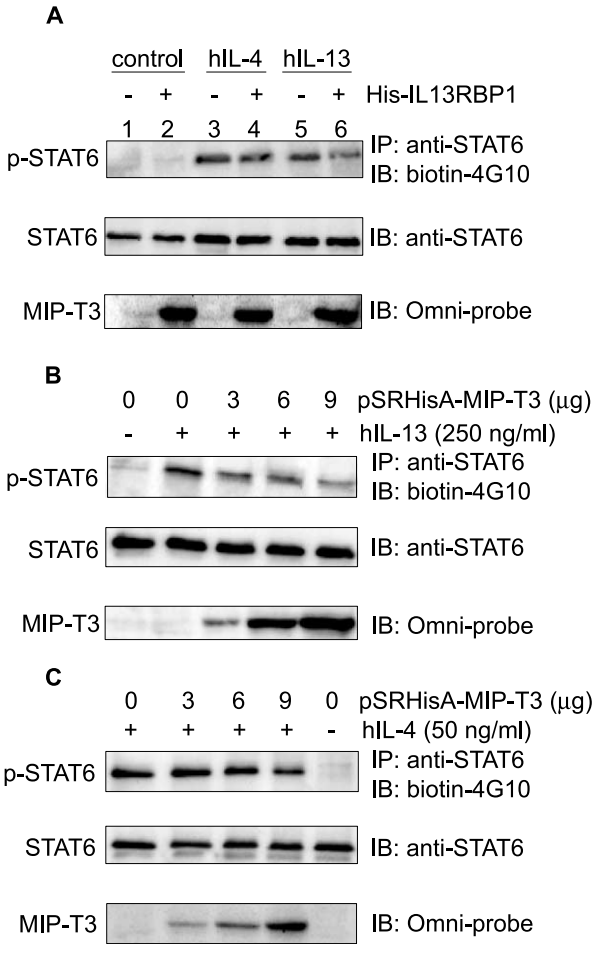

Fig. 2. Suppression of STAT6 phosphorylation by MIP-T3 in response to hIL-4/13. A: COS-7 cells in a 90 mm dish were transfected with 6 μg of pSRHisA-MIP-T3 or pSRHisA by using Lipofectamine 2000. After 24 h, cells were stimulated with or without hIL-4 (50 ng/ml) and hIL-13 (250 ng/ml) at 37°C for 15 min. Cell lysates were immunoprecipitated (IP) with anti-STAT6 antibody and immunoblotted (IB) with biotin-4G10 (top panel). Immunoblot was stripped and reprobed with anti-STAT6 antibody to check the amount of immunoprecipitated STAT6 protein (middle panel). Expression level of MIP-T3 in whole cell lysates was determined by immunoblotting (IB) with Omni-probe (lower panel). B, C: COS-7 cells were transfected with various amounts of pSRHisA-MIP-T3 or pSRHisA. After 24 h, cells were harvested and stimulated with hIL-13 (B) or hIL-4 (C) at 37°C for 15 min. STAT6 phosphorylation and MIP-T3 expression level were determined as described in A.

2C). Therefore, MIP-T3 can suppress STAT6 tyrosine phosphorylation especially in response to hIL-13.

3.4. Suppression of STAT6 transcriptional activity by MIP-T3

After demonstrating the inhibitory effect of MIP-T3 on STAT6 phosphorylation, STAT6 transcriptional activity was determined by dual luciferase assay using COS-7 cells. Upon hIL-4 or hIL-13 stimulation, 4–5-folds induction of luciferase activity was observed in control cells. However, after transfection of pSRHisA-MIP-T3, folds induction in response to hIL-13 decreased while there was no obvious change in hIL-4-stimulated cells (Fig. 3A), although expression levels of MIP-T3 were proved to be equal among all samples (Fig. 3B). The decreased folds induction demonstrated that STAT6 transcriptional activity in response to hIL-13 stimulation can be suppressed by MIP-T3.

3.5. Inhibition of activated STAT6 DNA binding activity by MIP-T3

Besides dual luciferase assay, EMSA was performed to determine the effect of MIP-T3 on STAT6 DNA binding activity by using THP-1 cells. SBE-1 probe was used for binding assay, which specifically bound to STAT6 protein as shown in the right panel of Fig. 4A. When cells were introduced with MIP-T3 gene and stimulated with hIL-13, intensity of STAT6 band was lowered to nearly 30% of that in control samples (Fig. 4A, lanes 2, 4). The effect of MIP-T3 on STAT6 in response to hIL-4 was also examined; however, there was no change before and after introduction of MIP-T3 (Fig. 4B, lanes 6, 8). In addition, cells were transfected with different amounts of pSRHisA-MIP-T3 plasmid and stimulated with hIL-13. We found that 6 μg of MIP-T3 plasmid was already enough to suppress STAT6 DNA binding activity in THP-1 cells (Fig. 4C). These results confirmed the inhibitory effect of MIP-T3 on IL-13-induced STAT6 DNA binding activity.

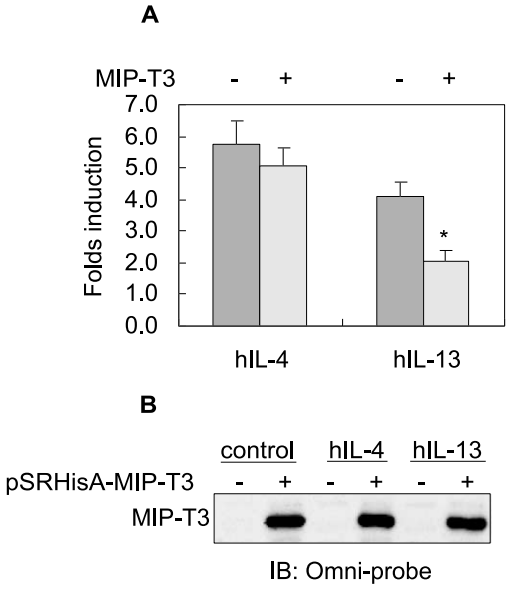

Fig. 3. Effects of MIP-T3 on STAT6 transcriptional activity in response to hIL-4/13 stimulation. A: COS-7 cells in a 12-well plate were transfected with 0.4 μg of TPU474, 1.6 μg of pSRHisA-MIP-T3 (or pSRHisA) and 0.02 μg of pRL-TK per well by using Lipofectamine 2000. After 24 h, cells were stimulated with hIL-4 (50 ng/ml) or hIL-13 (100 ng/ml) at 37°C for 6 h. Cells were harvested and measured for luciferase activity. Luciferase activity was normalized by Sea pansy activity to eliminate the influence of transfection efficiency and cell viability. Fold induction was calculated as the ratio of relative luminescence units (RLU) of stimulated cells to that of non-stimulated cells. Values are expressed as means of four independent experiments. $*P < 0.05$, versus control. B: Expression level of MIP-T3 was determined by loading an equal amount of cell lysates to SDS–PAGE and immunoblotting with Omni-probe.

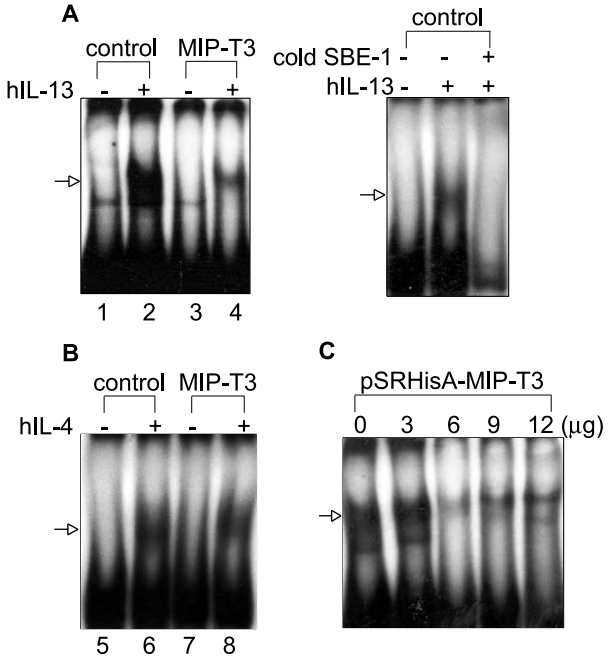

Fig. 4. Effects of MIP-T3 on activated STAT6 DNA binding activity in response to hIL-4/13 stimulation. A, B: THP-1 cells in a 90 mm dish were transfected with 12 μg of pSRHisA-MIP-T3 or pSRHisA. C: THP-1 cells were transfected with various amounts of pSRHisA-MIP-T3 (0–12 μg). 2 days later, cells were incubated with hIL-13 (A, C) (250 ng/ml) or hIL-4 (B) (50 ng/ml) for 10 min, solubilized with cold whole cell extraction buffer, and 50 μg of sample protein was incubated with 1 ng of ^{32}P-labeled SBE-1 probe (or with non-radiolabeled SBE-1 probe, A, right panel) in binding buffer for 20 min. DNA–protein interaction was analyzed by PAGE analysis. Arrows indicate STAT6 positions.

4. Discussion

We have identified a new IL-13Rα1 binding protein (IL13RBP1) by using yeast tri-hybrid system and found that IL13RBP1 is the same protein as MIP-T3, which also interacts with TRAF3 and microtubules. Besides the association, we found that transient expression of MIP-T3 partially inhibited IL-13-induced STAT6 phosphorylation, DNA binding activity and transcriptional activity.

To clone IL-13Rα1 binding proteins, yeast tri-hybrid system was used because tyrosine phosphorylation was widely utilized for cytokine signaling. However, MIP-T3 was found to constitutively associate with IL-13Rα1 independent of tyrosine phosphorylation, which is proven in both yeast system (data not shown) and mammalian cells (Fig. 1A). Besides IL-13Rα1, IL-4Rα is also utilized for IL-4/13 signaling and IL-2Rγ is utilized for IL-4 signaling, but they do not associate with MIP-T3 (Fig. 1B). Thus MIP-T3 may be a specific IL-13Rα1 binding protein.

We have investigated the role of MIP-T3 in IL-13 signals. STAT6 has been shown to be indispensable for IL-13-specific

biological functions [18,19] but the detailed mechanism is still unknown. We studied the influence of MIP-T3 on STAT6 using transient expression system and found that STAT6 phosphorylation was suppressed by MIP-T3 (Fig. 2). Interestingly, the effect of MIP-T3 on hIL-13-induced STAT6 phosphorylation was stronger than that of hIL-4.

The effect of MIP-T3 on hIL-4/13-induced STAT6 transcriptional activity was also examined (Fig. 3). Corresponding to inhibited STAT6 phosphorylation in response to hIL-13 stimulation, STAT6 transcriptional activity was suppressed by MIP-T3. But MIP-T3 did not inhibit STAT6 activation in response to hIL-4. This result was further confirmed in EMSA (Fig. 4), in which MIP-T3 only suppressed STAT6 DNA binding activity in response to hIL-13, but not hIL-4. These results suggest that MIP-T3 may selectively inhibit IL-13 signaling.

Although we have cleared that MIP-T3 inhibits IL-13-induced STAT6 phosphorylation and activation, its mechanism remains unclear. No direct association was detected between MIP-T3 and STAT6 protein (data not shown). Suppressor of cytokine signaling (SOCS) proteins have been shown to be important inhibitors of JAK-STAT pathway [20]. SOCS1 and SOCS5 can suppress IL-4-induced STAT6 activation. SOCS1 directly binds to the activation loop of JAK through SH2 domain thereby inhibiting STAT6 activation [21]. SOCS5 protein negatively regulates IL-4-induced STAT6 activation and Th2 differentiation through direct interaction with IL-4R, which is also independent of receptor tyrosine phosphorylation [22]. There is a possibility that interaction of MIP-T3 to IL-13Rα1 may interfere with formation of receptor complex or docking of STAT6 protein to receptor.

We have also examined the effect of cytokine stimulation on MIP-T3-TRAF3 association, which is proven to be in no relation with IL-4/13 (data not shown). As reported by Ling et al., upon CD40 ligand stimulation in 293.CD40 cells, TRAF3 dissociates from MIP-T3 and is recruited to CD40 receptor [14]. But as CD40 receptor binding protein, the role of TRAF3 in CD40 signaling has not been well defined. Association of MIP-T3 to IL-13Rα1 may offer a clue to study the role of TRAF3 in CD40 signaling and it may also help determine the relationship between IL-13 and CD40 signaling pathway.

In summary, we have identified a new IL-13Rα1 binding protein, which is found to have inhibitory effects on IL-13 signals through STAT6. This study would help to elucidate a novel regulation mechanism of IL-13 signaling pathway. Further studies will identify a novel inhibitor of IL-13 signaling, which may be useful in ameliorating various IL-13-dependent conditions including allergies, pulmonary asthma, parasitic infections and cancer in which IL-13 plays a central role.

Acknowledgements: We acknowledge Dr. Shigeno Ohno for providing pSRHisA plasmid. We also thank Ms. Noriko Okamoto, Mr. Makoto Saito and Mrs. Yuhua Ma for technical assistance. This work was supported partly by Grant-in-Aid (for T.M.) from the Ministry of Education, Science, Technology, Sports and Culture of Japan, the Takeda Science Foundation, the Kanagawa Academy of Science and Technology, and KIHARA Memorial Yokohama Foundation for the Advancement of Life Science.

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