Involvement of PU.1 in Mast Cell/Basophil-Specific Function of the Human *IL1RL1/ST2* Promoter

Yosuke Baba1,2, Keiko Maeda1, Takuya Yashiro1, Eisuke Inage1,2, François Niyonsaba1, Mutsuko Hara1, Ryuyo Suzuki2, Yoshikazu Ohtsuka2, Toshiaki Shimizu1,2, Hideoki Ogawa1, Ko Okumura1 and Chiharu Nishiyama1

ABSTRACT

**Background:** The human *IL1RL1/ST2* gene encodes IL33 receptor. Recently, IL33 has been recognized as a key molecule for the development of Th2 response. Although mast cells and basophils are major targets of IL33 and play important roles in IL33-mediated Th2-type immune responses, the expression mechanism of ST2 in mast cells and basophils is largely unknown. In the present study, we analyzed regulation mechanism of the human ST2 promoter in the human mast cell line LAD2 and basophilic cell line KU812.

**Methods:** Promoter activity was determined by reporter assay with plasmids carrying the wild-type ST2 promoter obtained from human genomic DNA and its mutant. The transcription factor binding to the identified cis-element was identified by an electrophoretic mobility shift assay (EMSA). The effect of candidate transcription factor on ST2 expression was confirmed by analyzing ST2 mRNA level in siRNA-introduced cells.

**Results:** Reporter assay demonstrated that a cis-element of typical Ets-family binding sequence was critical for promoter activity in LAD2 and KU812. An Ets-family transcription factor PU.1 bound to this element in an EMSA. When PU.1 expression was suppressed by siRNA, ST2 mRNA level was significantly reduced in KU812.

**Conclusions:** These observations indicated that PU.1 positively regulates the ST2 promoter as a transcription factor that directly transactivates the ST2 promoter via Ets-family-related cis-element in mast cells and basophils.

KEY WORDS
basophils, *IL1RL1/ST2*, IL33 receptor, mast cells, PU.1

INTRODUCTION

IL33, which was identified as a ligand for *IL1RL1/ST2* in 2005,1 is expressed in various cell types including fibroblasts, epithelial cells, and endothelial cells with localization in the nucleus in a steady state.2 IL33 is released upon cell lysis following pro-inflammatory stimulation and causes Th2-type immune responses through binding to *IL1RL1/ST2*. Several studies have demonstrated that not only Th2 but also mast cells and basophils are key target cells of IL33.3–8

The human *IL1RL1/ST1* gene is constitutively transactivated in mast cells/basophils and the transcript is mainly driven from the distal promoter in mast cells/basophils, whereas another proximal promoter at 10.5 kb downstream is activated in fibroblasts.9,10 In previous studies, a transcription factor, GATA1, transactivated the ST2 distal promoter via GATA-motifs in this region in mouse mast cells,10 whereas another GATA-family transcription factor GATA3 transactivated the same promoter in mouse T cells.11 In contrast, another transcription factor PU.1 belonging to the Ets-family regulates mast cell/basophil-specific gene regulation in a synergistic...
manner with GATA1/2. However, the involvement of PU.1 in the human ST2 promoter is largely unknown. These observations prompted us to analyze the role of PU.1 in the regulation of the human ST2 promoter.

METHODS

CELLS
Human mast cell leukemia, LAD2, which was kindly provided by Dr. Arnold Kirshenbaum, and human basophilic leukemia, KU812, were maintained as described previously. This study was approved by the ethics committee of Juntendo University School of Medicine.

PLASMIDS
The human IL1RL1/ST2 gene promoter was amplified by PCR using the following primers and human genomic DNA purified from peripheral blood using a QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany) as template. Synthesized oligonucleotides 5’-CAAAGAAGAATCAGAAACTGCAAGTTAC-3’ (nucleotide replacement and introduced XhoI sites are shown with lower-case letters and italic, respectively) and 5’-ccagcTTCGCCACACTGTTCACAC TCAAGAAGCCA-3’ (nucleotide replacement and introduced HindIII sites are shown with lower-case letters and italic, respectively) were used as forward and reverse primers, respectively, to obtain -100/+84. Amplified DNA was inserted into a reporter plasmid, pGL4-Basic (Promega, Madison, WI, USA) after digestion with appropriate restriction endonucleases. A mutant reporter plasmid lacking Ets-sequence was generated using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with 5’-CAGCTTATCAGTAACCTGGTactCTGTCTCTTAAC-3’, mutated nucleotides are shown with lower-case letters) and mutant Ets competitor (5’-GTAACTTGGTctCTGTTCCTTAAC-3’, mutated nucleotides are shown with lower-case letters) were used in competition assay. Electrophoresis of probe-protein mixture and detection of fluorescence were performed as described previously.

INTRODUCTION OF SMALL INTERFERING (si) RNA
Ten microliters of 20 μM PU.1 siRNA (Stealth Select RNAi, #HSS144058, Invitrogen, Carlsbad, CA, USA) or control siRNA (Stealth RNAi Negative Universal Control, #46-2000, Invitrogen) was introduced to 2 × 10⁶ cells with a Neon 100 μl kit using an electroporation system, Neon transfection system (Invitrogen), set at program #16. The transfection efficiency of this condition was confirmed to be over 98% by monitoring BLOCK-iT Alexa Fluor Red Fluorescent Oligo (14750-100, Invitrogen) using flow cytometry.

QUANTIFICATION OF mRNA
Total RNA was prepared from LAD2 and KU812 using a RNeasy Micro Kit (QIAGEN) and was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantification of ST2 mRNA and PU.1 mRNA was performed using TaqMan system with TaqMan Gene Expression Assays (Applied Biosystems; #Hs00545033_m1 for IL1RL1/ST2, and #Hs02786711_m1 for Spi1/PU.1) with human GAPDH #4326317E as a housekeeping gene.

RESULTS

AN Ets-MOTIF IN THE HUMAN ST2 PROMOTER IS INVOLVED IN TRANSCRIPTION ACTIVITY
In the previous studies, the essential region of the hematopoietic cell-specific distal promoter was identified to be located approximately 100 bp from the transcription start site, and two GATA-motifs in this re-
Role of PU.1 in the Human IL1RL1/ST2 Promoter

Fig. 1 Transcription activity of the human ST2 promoter in LAD2 and KU812. (A) Nucleotide sequence of the human ST2 gene around the hematopoietic cell-specific distal promoter. The transcription start site determined by 5'-RACE in a previous study is expressed as +1.9 Upper-case and lower-case letters represent the sequences in exon and in the promoter, respectively. A typical Ets-motif in the human ST2 gene is boxed and putative GATA-binding cis-elements previously identified in the mouse ST2 gene are underlined. (B) Relative luciferase activity is displayed as the ratio of luciferase activity versus that in pGL4-Basic transfectant. Data represent means ± SEM of six independent experiments performed with triplicate samples. Open bar, pGL4-Basic; closed bar, wild type ST2 promoter; dotted bar, mutant ST2 promoter lacking an Ets-motif. *p < 0.05, **p < 0.01 in two-tailed paired Student’s t-test.

A

<table>
<thead>
<tr>
<th>human</th>
<th>mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>aatactgcagttacatttgtaacaggtgtcactctgttactcttggttcagcttatcgttaac</td>
<td>aaga-tg-gttacagcttatctgttactcttggttcagcttatcgttaac</td>
</tr>
<tr>
<td>1</td>
<td>-44</td>
</tr>
<tr>
<td>1</td>
<td>-104</td>
</tr>
<tr>
<td>1</td>
<td>-104</td>
</tr>
<tr>
<td>ctgtctctactgtataagaaaaatggaggttttaAAAAGAGGCTGGCT</td>
<td>ctgtctctactgtataagaaaaatggaggttttaAAAAGAGGCTGGCT</td>
</tr>
<tr>
<td>+1</td>
<td>+1</td>
</tr>
</tbody>
</table>

Ets

GATA

Relative luciferase activity

0 5 10 15 20 25 30 35

Relative luciferase activity

0 5 10 15

pGL4-Basic

ST2 promoter

Luc

Ets

Luc

TTCC

X

Luc

LAD2

pGL4-ST2 WT

pGL4-ST2 ΔEts

pGL4-ST2 +Ets

KU812

B

The binding of an Ets-family transcription factor PU.1 to the Ets-motif

To determine the transcription factor(s) binding to the cis-element at -39/-36, EMSA was performed using double-stranded DNA at -48/-25 of the ST2 promoter as a probe. When nuclear proteins extracted from KU812 cells were added, several shift bands with lower mobility than that of free probe appeared (Fig. 2A, lane 3). The density of a major band was reduced in the presence of anti-PU.1 Ab (Fig. 2A, lane 1, shown with an arrowhead), whereas Ab against Spi-B, which is another Ets-family protein, did not affect this band intensity (Fig. 2A, lane 2). The most pronounced band in the mixture of the probe and PU.1 protein that was synthesized with the in vitro transcription/translation system completely disappeared upon addition of anti-PU.1 Ab but not by anti-Spi-B Ab (Fig. 2A, lanes 4, 5, and 6). The mobility of the complex with the probe and in vitro translated PU.1 was slightly lower than that of the complex with nuclear PU.1. The difference of the mobility is likely to have been due to the fusion of Flag-tag at the N-terminus of PU.1, resulting in the higher molecular weight of in vitro translated PU.1. Regardless of the slight difference of mobility shift, these results suggest that PU.1 binds to the ST2 promoter region around the cis-element in vitro. EMSAs were performed with competitive oligonucleotides to further confirm whether PU.1 specifically binds to the probe DNA via a core sequence at -39/-36 (Fig. 2B). The specific band shift, which disappeared upon addition of anti-PU.1 Ab (Fig. 2B, lane 2), was retained by addition of the mutant competitor lacking Ets-motif, mEts (Fig. 2B, lane 4), whereas the specific band dis-
Fig. 2  PU.1 binds to -48/-25 of the human ST2 promoter in vitro. (A) EMSA profile with a probe of -48/-25 and proteins in the presence or absence of Ab. Nuclear extract, nuclear proteins from KU812 cells; in vitro PU.1, in vitro transcription/translation reaction mixture with PU.1 expression plasmid; -, without Ab; PU.1, with anti-PU.1 Ab; Spi-B, with anti-Spi-B Ab. The specific band of complex with PU.1 and a probe is marked with an arrowhead. Competition assay using in vitro translated PU.1 (B) or KU812 nuclear extract (C). mEts, oligonucleotide containing mutated Ets site (nucleotide sequence is shown in Methods); self, competitive oligonucleotide with wild-type sequence.

appeared in the presence of wild-type competitor (Fig. 2B, lane 3). When the mutant probe with same sequence as mEts was used instead of wild-type probe, the specific band was not detected (Fig. 2B, lane 5). Similarly, when competition assay was performed with KU812 nuclear proteins, the specific band with PU.1 was still detected in the presence of mEts competitor (Fig. 2C, lane 3), whereas this band and all other bands disappeared in the presence of wild-type competitor (Fig. 2C, lane 2). The band (shown with an asterisk) showing lower mobility than that of the band containing PU.1 (shown with an arrowhead) was also detected when mEts was used as competitor (Fig. 2C, lane 3), suggesting that another protein may bind the core sequence as well as PU.1. Taken together, these results demonstrate that PU.1 binds to the ST2 promoter region via a core sequence at -39/-36.

ST2 PROMOTER TRANSACTIVATION ACTIVITY OF PU.1
In order to evaluate the effect of PU.1 on ST2 promoter activity, co-expression reporter analysis was performed using KU812. The presence of exogenous PU.1 increased ST2 promoter activity, whereas pGL4-Basic was not affected by PU.1 co-expression (Fig. 3).

The presence of both binding elements for PU.1 and GATA has been found in mast cell-specific enhancer regions.12,22 PU.1 and GATA1/2 cooperate in mast cells,12,13 in contrast, PU.1 and GATA1/2 inhibit each other in other hematopoietic lineages.23-26 Then, the effects of PU.1 and GATA2 on the human ST2 promoter were analyzed to confirm whether the ST2 promoter is one of the models of this cooperation system. As shown in Figure 3, co-expression of PU.1 and GATA2 caused significantly higher transcription activity of the ST2 promoter compared with co-expression of PU.1 alone.
Role of PU.1 in the Human \textit{IL1RL1}/\textit{ST2} Promoter

**Fig. 3** PU.1 and GATA2 cooperatively transactivates the human \textit{ST2} promoter. Relative luciferase activity is displayed as the ratio of luciferase activity versus that in pGL4-Basic and mock transfectant. Data represent means ± SD of triplicate samples. The total amount of co-expression plasmid was adjusted to 6 μg as follows: m, 6 μg mock vector (pCR3.1); P, 3 μg pCR-PU.1 and 3 μg pCR3.1; P + G, 3 μg pCR-PU.1 and 3 μg pCR-GATA2. ns, not significant. *p < 0.05.

**EFFECT OF PU.1 siRNA ON ST2 EXPRESSION**
To examine the involvement of PU.1 in ST2 expression, the expression of PU.1 was knocked down by siRNA. By the detection of red fluorescent oligonucleotides, which were introduced by electroporation using a Neon system, the transfection efficiency in KU812 was confirmed to be over 98% (data not shown). When PU.1 siRNA was introduced in KU812 under the same conditions, PU.1 mRNA level was reduced by 90% at 24 h after transfection and the reduction was continued until at least 72 h (Fig. 4, top). In these conditions, the amount of ST2 mRNA was significantly lower than that in control siRNA transfectants (Fig. 4, bottom). These results indicate that PU.1 participates in expression of ST2.

**DISCUSSION**
IL33 receptor is encoded by the human \textit{IL1RL1}/\textit{ST2} gene that possesses two promoters, the distal promoter for hematopoietic lineages and the proximal promoter for non-hematopoietic lineages.\textsuperscript{9,10} Among hematopoietic lineages known as the target of IL33, mast cells and basophils play important roles in IL33-mediated Th2-type immune responses. In previous studies regarding the transcriptional regulation mechanism of the mouse \textit{ST2} promoter, two GATA-motifs in the distal promoter were identified to be critical \textit{cis}-elements, which were recognized with GATA1 in mouse mast cells\textsuperscript{10} and with GATA3 in mouse T cells.\textsuperscript{11} However, when nucleotide sequences of the \textit{ST2} promoters were compared between human and mouse, transcription start site of the human \textit{ST2} gene was shown to be located approximately 60 bp upstream compared with that of mouse and the sequence around a GATA-motif at 5'-
site was highly conserved containing a typical Ets-motif, but another GATA-motif was absent (Fig. 1A). In the present study, the involvement of this Ets-motif in promoter function was examined, because we have previously found that the Ets-motif close to the GATA-motif exhibited mast cell/basophil-specific enhancing activity mediated by PU.1.12

A mutant promoter carrying nucleotide replacement in the Ets-motif at -39/-36 showed significantly reduced transcriptional activity compared with the wild-type ST2 promoter in LAD2 and KU812 (Fig. 1). Among several bands in EMSA using nuclear extract from KU812, a major band was identified to represent a complex of probe and PU.1 (Fig. 2). In addition, exogenous expression of PU.1 transactivated the ST2 promoter (Fig. 3). These results suggest that an Ets-motif at -39/-36 is critical for the ST2 promoter and that PU.1 is one of the candidates transactivating the ST2 promoter via this Ets-motif. Finally, PU.1 knockdown by siRNA resulted in suppression of ST2 expression in KU812 (Fig. 4). From these results, it is concluded that PU.1 transactivates the human ST2 promoter via binding to an Ets-motif at -39/-36 in these lineages.

Although we have tried to identify other transcription factor(s) that bind to a critical element at -39/-36 in EMSA using Abs against various transcription factors, no additional specific disappearance of bands has been observed so far. Further experiments to identify the transcription factor(s) that bind to the element at -39/-36 will be required to reveal the mechanism of ST2 expression in mast cells and basophils. Regardless, we demonstrated that a cis-element at -39/-36 is critical for ST2 promoter activity and that PU.1 is involved in ST2 expression in the present study.

We have previously reported that PU.1 is involved in transcription of the human FcεRI α gene.12 Recently, we introduced PU.1 siRNA into LAD2 to confirm our previous findings by using the up-to-date experimental technologies, and we found that PU.1 knockdown reduced the cell expression of FcεRI (Image, E., unpublished data). Therefore, PU.1 knockdown may exhibit anti-allergic effects by suppressing multiple pathways in mast cells/basophils including IL33- and IgE-mediated activation signaling.

ACKNOWLEDGEMENTS

We are grateful to members of the Atopy (Allergy) Research Center, the Department of Pediatrics, and the Department of Immunology of Juntendo University School of Medicine for helpful discussions. We thank Drs. Nobuhiro Nakano, Nao Kitamura, Maya Kamijo, Kentarou Ishiyama, and Ryusaku Matsuda for useful suggestions, and Ms. Michiyo Matsumoto for secretarial assistance. This work was supported by the Funding Program for Next Generation World-Leading Researchers from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to C. N.).

REFERENCES

1. Schmitz J, Owyang A, Oldham E et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005; 23:479-90.
7. Pecaric-Petkovic T, Didichenko SA, Spiegel N, Dahinden CA. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 2009; 113:1526-34.