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Transcriptional regulation of SLP-76 family hematopoietic cell adaptor *MIST/Clnk* by STAT5

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Abstract

SLP-76-related adaptor protein MIST (also called Clnk) is expressed in a variety of cytokine-dependent hematopoietic cell lines of myeloid and lymphoid origin as well as some cytokine-independent mast cell lines. To understand the molecular mechanisms underlying the *MIST* gene expression, we have characterized the 5′-flanking region of the mouse *MIST* gene. We have identified an enhancer region (−773 to −709), which is active in P815 mast cells expressing the endogenous *MIST* gene, but not in EL-4 T cells lacking *MIST* expression. Outside of this enhancer region, one STAT element present in the *MIST* promoter (−44 to −36) was found to bind STAT5A when IC-2 mast cells were stimulated with IL-3. Mutation of this STAT element did not affect basal *MIST* promoter activity in P815 mast cells, but was required for STAT5-mediated activation of the *MIST* promoter. Furthermore, endogenous *MIST* gene expression was induced in mast cells by a constitutively activated form of STAT5A, but not by an active mutant of c-Kit receptor. These findings suggest that STAT5 is involved in cytokine-mediated up-regulation of *MIST* gene expression, probably in collaboration with other lineage-specific transcription factors that promote basal *MIST* expression in mast cells.

Keywords: Adaptor; MIST; Promoter; Transcription; Mast cell; STAT5

Immunoreceptor tyrosine-based activation motif-bearing receptors, which include the T cell receptor (TCR), B cell receptor (BCR), and Fc receptors, transduce signals via the sequential activation of protein tyrosine kinases, phosphatases, and adaptor proteins that serve as a molecular link to downstream signaling pathways [1,2]. Adaptor proteins lack enzymatic activity but possess multiple structural modules for protein–protein interaction, by which they function as a scaffold to generate active signaling complexes that are essential for transducing receptor signals to downstream effectors [3,4]. Among these adaptor proteins, the SLP-76 family of adaptor proteins comprises three members, SLP-76 [5], BASH/BLNK/SLP-65 [6–8], and MIST/Clnk [9,10], and possesses similar structural modules for protein–protein interaction, including N-terminal tyrosine phosphorylation sites, central proline-rich regions, and a C-terminal SH2 domain. SLP-76 is expressed in T-lineage cells, myeloid cells, platelets, and mast cells [11], and has been demonstrated to be essential for signal transduction downstream of pre-TCR and TCR in T-lineage cells [12,13], collagen receptor in platelets [14], and the high-affinity IgE receptor (FcεRI) in mast cells [15]. On the other hand, a deficiency of BLNK/BASH/SLP-65, which is expressed mainly in B-lineage cells, causes impaired B cell development, which is due to defective pre-BCR and BCR signaling [16–19]. Thus, this family of adaptor proteins appears to play similar roles in signal transduction from distinct immunoreceptors, which is mainly regulated by their cell lineage-restricted expression patterns.

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MIST has been reported to be constitutively expressed in some mast cell lines as well as cytokine-dependent cell lines of both myeloid and lymphoid origin [9,10]. MIST expression was also induced when T cells and NK cells were activated by cytokines [10]. It has been demonstrated that MIST is capable of restoring BCR signaling in a BASH-deficient DT40 B cell line in collaboration with a membrane adaptor LAT [20], and also TCR signaling in a Jurkat T cell variant lacking SLP-76 [21], implying functional similarity between MIST and the other members. However, physiological roles of MIST in immunoreceptor signaling have not been reported. Because various cytokines produced during immune response are not only a key determinant for the extent and nature of subsequent immune activation, but also are potential inducers for MIST expression in heterogeneous cell populations in a spatio-temporarily distinct manner, MIST may thus play a unique role in immune activation through regulating signaling downstream of various immunoreceptors expressed on each cell type. To understand the precise function of MIST in immune response, it is important to know where and how MIST expression is induced during immune reaction. We therefore intended to examine the mechanism controlling MIST expression by identifying its transcriptional regulatory elements.

In the present study, we have identified the promoter region and several upstream regulatory sequences of the mouse *MIST* gene. Among these sequences, one segment appears to be capable of driving gene expression in a cytokine-independent fashion and the other STAT-binding segment is responsible for cytokine-induced expression.

Materials and methods

Reagents and antibodies. Recombinant mouse IL-3 was purchased from Peprotech (London, UK). Rabbit polyclonal Abs against STAT5A (L-20), STAT5B (C-17), STAT6 (M-20), and c-Kit receptor (G-5) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled anti-phosphotyrosine Ab, PY20, was purchased from Transduction Laboratories (Lexington, KY).

Cell lines. A mouse IL-3-dependent immature mast cell line, IC-2, obtained from Riken Cell Bank (Tsukuba, Japan), was cultured in RPMI1640 medium supplemented with 10% fetal calf serum in the presence of 5 ng/ml IL-3. A mouse mastocytoma cell line, P815, a T cell line, EL-4, and a B cell line BCL1 were maintained in the same medium without IL-3.

Isolation of the mouse MIST gene promoter. A genomic DNA fragment containing the mouse *MIST* promoter region was amplified from genomic DNA obtained from E14 embryonic stem cells using a Universal Gene-Walker Kit (BD Clontech, Palo Alto, CA) with adaptor primers provided in the kit and primers complementary to the sequence of the 5′-untranslated region of mouse *MIST* mRNA (5′-GTCTTCCTTTAGTCAAGGGCTCAGTGTTGAG-3′). The PCR-amplified fragment was directly cloned into pSK-Script plasmid (Stratagene, La Jolla, CA) and sequenced.

Primer extension analysis. Poly(A)$^+$ RNA (5 μg) from P815 cells was annealed with 8 pmol of a ^{32}P-labeled oligonucleotide primer (5′-TGCTCAGTCTTCCTTTAGTCAAGGGCTCAG-3′) designed to be complementary to the 5′-untranslated region of the mouse *MIST* mRNA. The RNA was reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) for 120 min at 42 °C, and then subjected to phenol–chloroform extraction and ethanol precipitation. The cDNA product was separated by electrophoresis on an 8 M urea/6% polyacrylamide sequencing gel, followed by autoradiography. The sizes of the primer-extended DNA products were determined by comparison with a sequencing reaction of M13 template primed with −40 primer.

Plasmid construction. A *Xba*I/*Nhe*I DNA fragment (−912 to +93) of the *MIST* promoter region was subcloned into the *Nhe*I site of the promoter/enhancer-less pBasic2 luciferase vector (Promega, Madison, WI) in a 5′–3′ orientation. Serial 5′ MIST promoter deletion mutants ending 3′ of −30 bp were generated using exonuclease III digestions. The potential STAT (TTCTCGGAA) and Elf-1 (TTTCCTT)-binding sites of the *MIST* promoter were mutated to TTCTCGAAA and TCGACTT using the site-directed mutagenesis kit (Stratagene), and verified by sequencing. These mutated DNA fragments were also subcloned into the pBasic2 vector. The cDNAs encoding constitutively active forms of mouse STAT5A (STAT5A-1*6) [22] and mouse c-Kit receptor (c-Kit-D814Y) [23], provided by Y. Kitamura, Osaka University, were subcloned into pMX-IRES-EGFP retrovirus vector [24] and pME18S expression vector.

Luciferase assay. All cell lines were transfected by electroporation using a Bio-Rad electroporation apparatus (Bio-Rad) under conditions of 250 V and 975 μF. Cells (5×10^6) were co-transfected with 10 μg of test construct and 1 μg pAct-βGal. The pAct-βGal plasmid was used for normalizing the transfection efficiency. Each experiment included pA-luc containing chicken β-actin promoter and pBasic2-transfected samples. pA-luc was used as an internal positive control to compare different experiments. Data are presented as fold induction beyond the activity of a promoter-less pBasic2 vector. After a 48 h interval, luciferase activity was determined in cell extracts according to the instructions of the luciferase assay kit (Promega). The β-galactosidase assay was carried out using a Galact-light kit (Tropix, Bedford, MA). Light emission was measured in a Lumat LB9501 luminometer (Berthold, Wildbad, Germany). All experiments were repeated three times or more, and the average and standard deviations were calculated for three independent experiments.

Preparation of nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared as described previously [25]. Two types of double-stranded oligonucleotides were used as a probe or a competitor, and their sequences were as follows: MIST–SRE, 5′-GTGGCTGCGTTTCTCGGAAAACC-3′; MIST–SREm, 5′-GTGG CTGCGTTTCTCGAAAAACC-3′. EMSA was performed essentially as previously reported [25]. Briefly, nuclear extract (15 μg of each sample) was incubated in 20 μl binding buffer containing end-labeled probe (10,000 cpm) for 20 min at room temperature. In competition assays, nuclear extracts were incubated with a 200-fold molar excess of unlabeled competitor oligonucleotide prior to the binding reaction with the labeled probe. In supershift assays, the nuclear extracts were pre-incubated with 1 μg of rabbit anti-STAT5A, anti-STAT5B or anti-STAT6 Ab for 30 min at room temperature before the binding reaction. The reaction mixture was electrophoresed, dried, and subjected to autography.

Generation of stable transfectants using retrovirus vectors. The IC-2 mast cell line was used to generate stable transfectants expressing a constitutively active form of STAT5A or c-Kit receptor, using the pMX-IRES-EGFP retrovirus expression system with the PLAT-E packaging cell line [26], as described previously [24]. After the infection, cells were cultured without IL-3, and more than 95% of the cells growing continuously in the absence of IL-3 expressed STAT5A-1*6 or c-Kit-D814Y, as evaluated using EGFP fluorescence.

Immunoprecipitation and immunoblotting. Cells were lysed with 1% NP40 lysis buffer containing protease and phosphatase inhibitors, and

immunoprecipitated with appropriate antibodies. The immunoprecipitates and aliquots of total cell lysate were resolved by SDS–PAGE and transferred to PVDF membranes. The membranes were immunoblotted with the antibodies described above and the secondary antibodies conjugated with HRP, and then developed with the enhanced chemiluminescence (ECL) system (Amersham Bioscience, Piscataway, NJ).

Northern blot analysis. Total RNA (10 μg) was separated in a denaturing agarose gel, as described previously [7]. After blotting, hybridization was performed with either the mouse *MIST* or β-actin cDNA probe.

Results

Identification of the transcription initiation site and potential transcription factor binding sites in the 5′-flanking region of the MIST gene

To gain insights into the transcriptional regulation of the *MIST* gene, a 3 kb genomic fragment containing the 5′-flanking region of the *MIST* gene was isolated using an adaptor-mediated PCR method and a ~1 kb fragment proximal to the 5′-untranslated region was sequenced. The transcription start site was initially determined by the use of a primer extension technique employing an oligonucleotide complementary to the 5′-untranslated region of the *MIST* mRNA. When the resultant reaction products were compared with the size markers run on the same polyacrylamide gel, three major extended products differing in abundance were detected at positions 175, 171, and 153 bp upstream from the initiation ATG (Fig. 1A). The most upstream start site corresponds to the cytosine residue located 175 bp upstream of the translation initiation codon and was assigned as the +1 position. A 1 kb DNA sequence from the 5′ end to the transcription start site was then searched for consensus binding sites for transcription factors (Fig. 1B). The 5′-flanking region of the *MIST* gene lacks a TATA box typical of eukaryotic type II promoters, but contains several putative binding sites for transcription factors, including two consensus binding sites for the GATA transcription factor (positions −827 to −821 and −645 to −637) and AP-1 (−885 to −877 and −194 to −186). A binding motif for the Ets family transcription factor Elf-1 was also identified (−670 to −664), and the region downstream of these elements contains a potential binding site for STAT (−44 to −36).

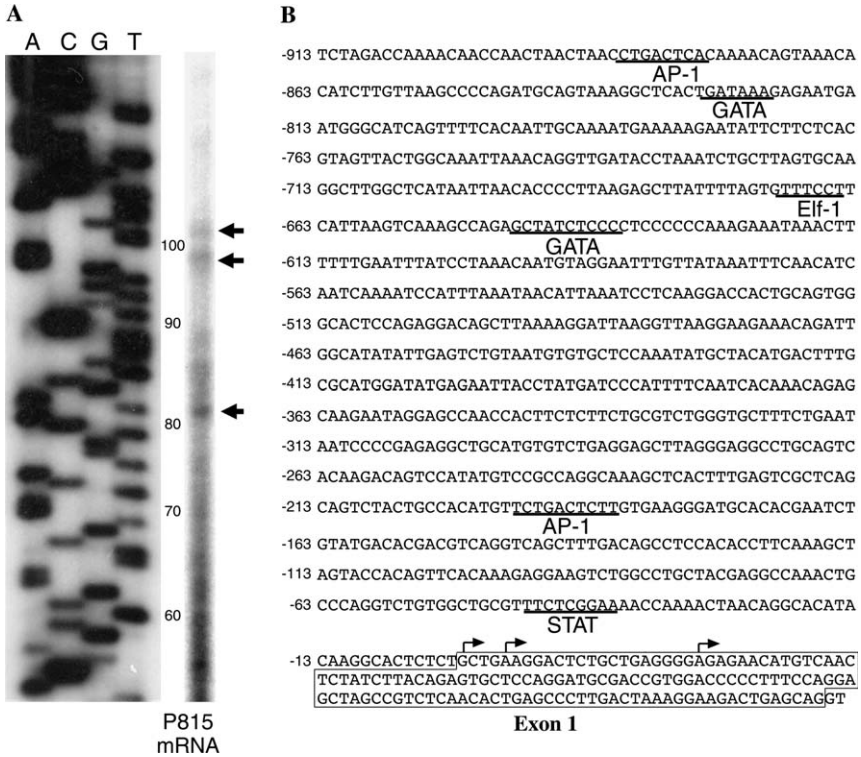

Fig. 1. Transcription start sites and potential transcription factor binding sites of the *MIST* gene. (A) Transcription start sites were determined by primer extension analysis. The nucleotide sequence ladder on the left is for calculating the size of the extension products. The numbers of nucleotides from the 5′ end of the primer are shown at the right of the ladder. Three major extension products are indicated by arrows. (B) The transcription start sites are indicated by arrows and the most extreme 5′ start site is designated as +1. Positions of the nucleotide sequence are indicated on the left. The nucleotide sequence corresponding to exon 1 of the *MIST* gene encoding the 5′-untranslated region is boxed. Potential binding sites (underlined) for transcription factors were determined using the TFSEARCH program. The sequence has been deposited to DDBJ/GenBank/EMBL database with an Accession No. (AB160955).

Functional mapping of the MIST promoter and localization of enhancer elements

To determine the region responsible for the expression of the *MIST* gene, the 5′ 1.0 kb DNA fragment (−912 to +93) was cloned upstream of a luciferase reporter gene and transfected into the P815 cell line and EL-4 T cell line. The entire 1.0 kb 5′-flanking region of the *MIST* reporter showed luciferase activity to a level 10-fold above the background when transfected into the P815 cells that express the endogenous *MIST* gene (Fig. 2). The same construct yielded no detectable luciferase activity when transfected into the EL-4 T cell line that does not express endogenous *MIST*. Thus, the promoter elements responsible for the expression of *MIST* appear to reside in this 1.0 kb 5′-flanking region.

To further analyze the regulatory region for *MIST* promoter activity, we constructed a series of 5′-end deletion mutants of the *MIST* gene (Fig. 2). Each mutant was fused to a luciferase reporter gene and transfected into the same cell lines used in the experiments described above. Deletion of the 5′-flanking sequences up to position −773 (Del 1) had no significant effect on promoter activity. However, further deletion of the sequence up to position −667 (Del 2) profoundly reduced promoter activity, thereby suggesting the presence of positive regulatory elements in this region. Additional truncation of the sequence from −430 to −375 restored promoter activity, and the construct retaining 375 bp of the *MIST* promoter region (Del 5) exhibited a similar level of reporter gene activity to Del 1, suggesting that a 56 bp region between positions −430 and −375 contains a negative element for promoter activity. However, further sequence removal up to position −152 (Del 9) gradually reduced promoter activity. Taken together, the transcription factors interacting with a region between positions −773 and −667 in the *MIST* promoter are likely to be specifically expressed in P815 mast cells and to be important for mast cell-specific promoter activity of the *MIST* gene.

Identification of a functional STAT-binding site in the MIST promoter

Because cytokines induce tyrosine phosphorylation and activation of STAT proteins [27], we anticipated that cytokine-mediated up-regulation of the *MIST* gene expression is mediated by the interaction of STAT with the putative STAT-responsive element (−44 to −36) present in the *MIST* promoter (hereafter designated as MIST–SRE). We therefore examined the specific binding of STAT to MIST–SRE by EMSA using nuclear extracts prepared from IC-2 mast cells before and after stimulation with IL-3. As shown in Fig. 3A, the IL-3-treated nuclear extracts, but not the extracts from non-stimulated cells, possessed activity to bind the MIST–SRE oligonucleotide (lanes 1 and 4). The binding completely disappeared on the addition of an excess amount of unlabeled MIST–SRE competitor (lane 5), but not excess amounts of the unlabeled MIST–SREm oligonucleotide which contains one nucleotide mutation in the consensus STAT-binding sequence (Fig. 3A, lane 6). This suggests that the IL-3-induced DNA-binding complex is formed in a sequence-specific manner. Furthermore, this DNA-binding complex was entirely supershifted by pre-incubation with anti-STAT5A or anti-STAT5A/B Abs (Fig. 3B, lanes 2 and 3), but not with anti-STAT6 Ab (Fig. 3B, lane 4), indicating that

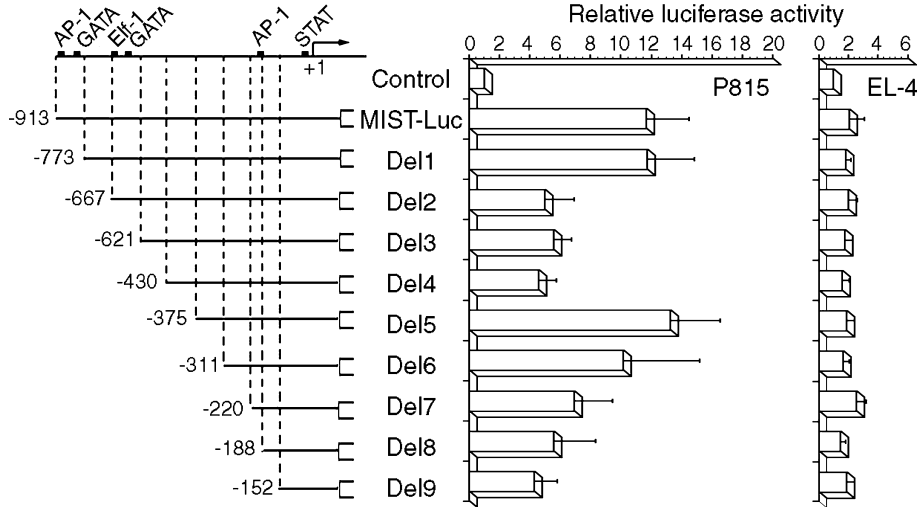

Fig. 2. Activity of *MIST* promoter constructs in P815 and EL-4 cell lines. Luciferase constructs, shown in the left panel, were transiently co-transfected with a pAct-βGal plasmid in the cell lines by electroporation. Two days after transfection, cells were harvested and lysed, and lysates were analyzed for luciferase and β-gal activity. Each histogram represents the mean and SEM (error bar) of the fold induction of the normalized value over the luciferase activity of a promoter-less pBasic2 vector in each transfected cell.

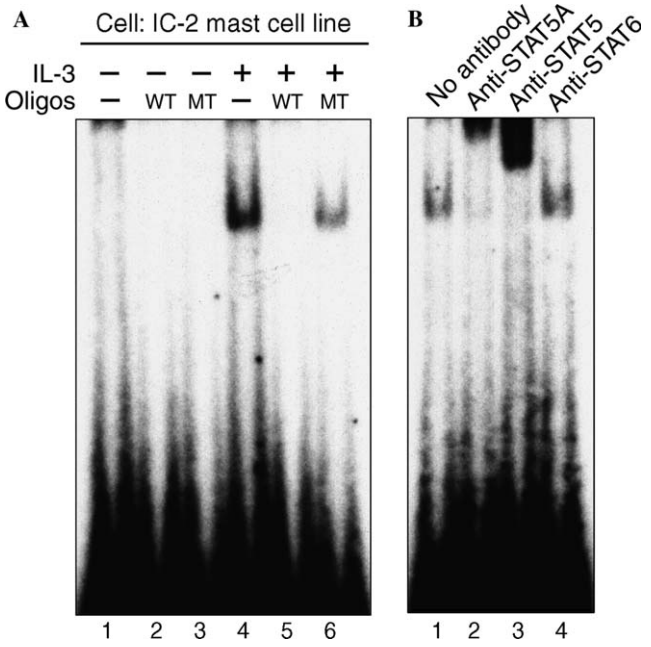

Fig. 3. Binding of activated STAT5 to the STAT element of the *MIST* promoter. (A) A total of 5μg of nuclear extract from IC-2 cells unstimulated (−) or stimulated with IL-3 (+) was incubated with a radiolabeled MIST probe (5′-GTGGCTGCGTTTCTCGGAAAACC -3′) corresponding to nucleotides −114 to −100 before running on a 5% polyacrylamide gel. Competition for the binding was tested with the same unlabeled probe (WT) or a probe containing a one-nucleotide mutation in a potential STAT-binding element (MT: 5′-GTGGCTGCGTTTCTCGAAAAACC-3′). (B) Supershift analysis was done using nuclear extracts from IL-3-stimulated IC-2 cells by adding anti-STAT5A, STAT5 or STAT6 Ab.

the MIST–SRE-binding complex is composed of at least STAT5A. No MIST–SRE-binding complex was detectable in nuclear extracts obtained from P815 cells using the same assay (data not shown).

Having demonstrated that STAT5A binds to a specific sequence of the *MIST* promoter, we evaluated the functional relevance of that observation by luciferase reporter assay using a constitutively active STAT5A (STAT5A-1*6) as an effector. STAT5A-1*6, which harbors two mutations of H298 to R and S710 to F, has been shown to be constitutively tyrosine phosphorylated, translocated into the nucleus, and has DNA-binding activity in unstimulated cells [22]. The mutation in MIST–SRE as well as in a putative Elf-1 binding site did not affect the basal *MIST* promoter activity in P815 cells (Fig. 4), suggesting that basal *MIST* promoter activity in P815 cells is STAT-independent. However, co-transfection with the STAT5A-1*6 expression vector in P815 cells strongly augmented the wild-type *MIST* promoter activity, as compared with that with control vector (Fig. 4). This enhancement of *MIST* promoter activity was completely abolished when the mutation was introduced in MIST–SRE, suggesting the involvement of STAT5 in IL-3-mediated up-regulation of *MIST* gene transcription through the STAT-binding site in the *MIST* promoter in mast cells. It also should be noted that, in contrast to the transactivation of *MIST* promoter by STAT5A-1*6 in P815 cells, no such effect was observed in EL-4 cells (Fig. 4).

The active-form of STAT5A, but not active-form of c-Kit, induces endogenous MIST gene expression in mast cells

Most of the factor-independent mast cell lines, including P-815, express a constitutively activated mutant of the c-Kit receptor [28,29]. The constitutive activation of c-Kit in P815 cells is due to a point mutation in the cytoplasmic kinase domain resulting in the substitution of tyrosine for aspartic acid at codon 814 (c-Kit-D814Y) [23]. To address whether the constitutively active c-Kit is responsible for *MIST* gene expression in factor-independent mast cell lines such as P815, we took advantage of the IL-3-dependent immature mast cell line IC-2. IC-2 cells do not express c-Kit, and they become factor-independent by stable introduction with active mutants of the *c-Kit* gene [29]. This IC-2 cell system also can be used to prove

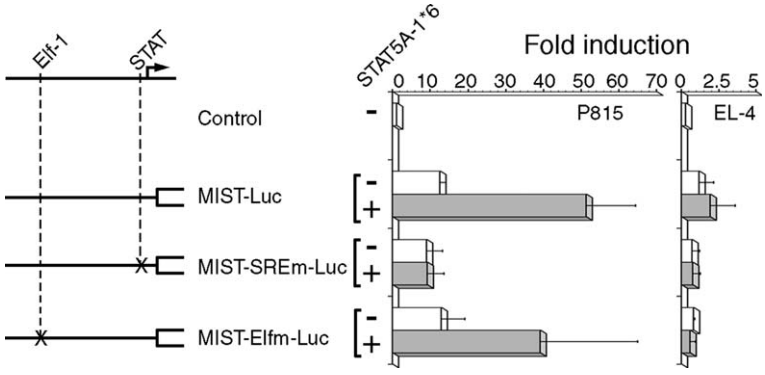

Fig. 4. The STAT element is essential for STAT5A-mediated transactivation of the *MIST* promoter activity. MIST promoter constructs containing a mutation (represented by crosses) at the STAT site or at the Elf-1 site were transfected into the indicated cell lines, along with an expression vector encoding a constitutively active form of STAT5A (STAT5A-1*6) (+) or an empty vector (−). Each histogram represents the mean and SEM (error bar) of the fold induction of the normalized value over the luciferase activity of a promoter-less pBasic2 vector (control) in each transfected cell.

whether STAT5 can directly activate the endogenous MIST gene by transfecting STAT5A-1*6.

The pMX-IRES-EGFP retrovirus vector containing either STAT5A-1*6 or c-Kit-D814Y cDNA was transfected into the PLAT-E packaging cell line, and the culture supernatants containing infectious retrovirus were used to infect IC-2 cells. After the infection, cells were cultured without IL-3. More than 95% of cells expressing STAT5A-1*6 or c-Kit-D814Y grew continuously in the absence of IL-3, as verified based on EGFP fluorescence (Fig. 5A).

Strong tyrosine phosphorylation of the transduced STAT5A and c-Kit was detected in IC-2 cells expressing STAT5A-1*6 and c-Kit-D814Y, respectively (Fig. 5B). Nuclear extracts obtained from transfectants expressing STAT5A-1*6 formed a MIST–SRE-binding complex, whereas those from the transfectant expressing c-Kit-D814Y as well as IL-3-starved parental IC-2 cells did not (Fig. 5C). No enhancement of *MIST* promoter activity was observed in the IC-2 transfectant expressing c-Kit-D814Y, in contrast to that expressing STAT5A-1*6. (Fig. 5D). Furthermore, a significant increase in endogenous

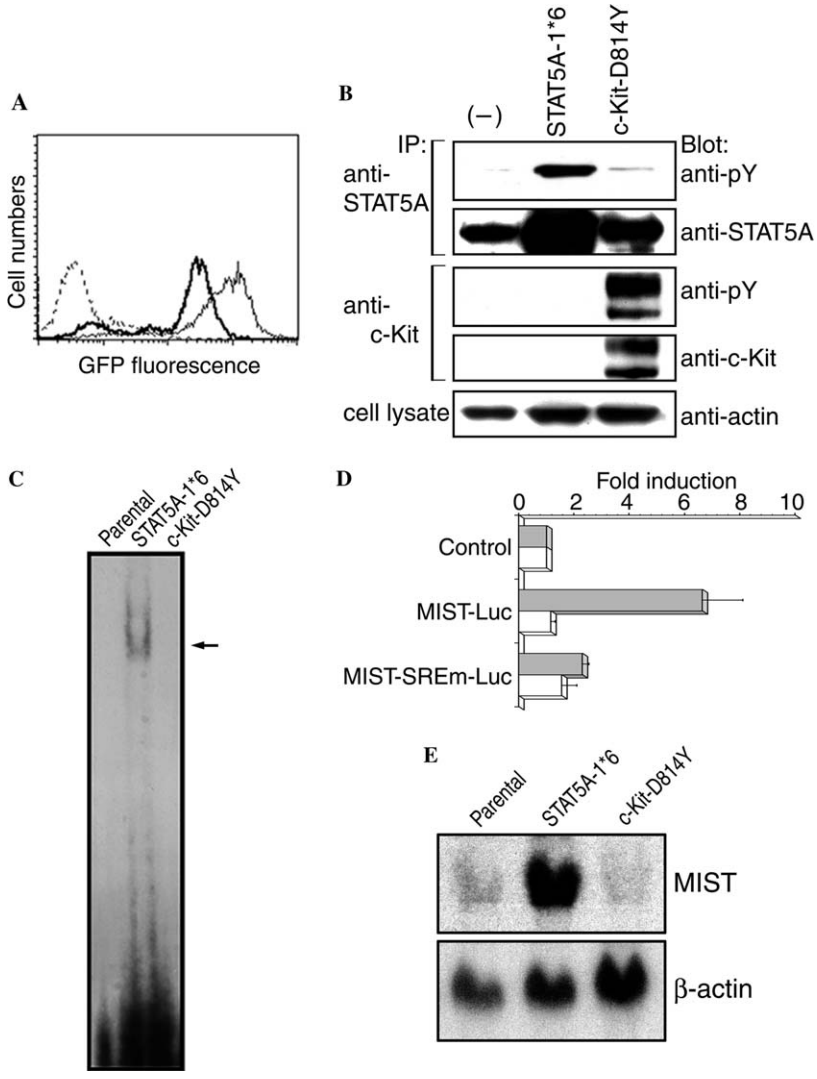

Fig. 5. Up-regulation of endogenous *MIST* gene expression by stable expression of a constitutively active form of STAT5A but not of c-Kit in IC-2 cells. (A) GFP fluorescence of IC-2 cells infected with pMX-STAT5A-1*6-IRES-GFP (a solid line) or pMX-c-Kit-D814Y-IRES-GFP (a bold line), growing in the absence of IL-3. The GFP fluorescence of IL-3-starved parental IC-2 cells is also shown by a dotted line. (B) Tyrosine phosphorylation of STAT5 and c-Kit in IL-3-starved parental IC-2 cells and the cells immortalized by STAT5A-1*6 or c-Kit-D814Y. Cell lysates were immunoprecipitated with anti-STAT5A or anti-c-Kit Ab and blotted with anti-phosphotyrosine (pY) mAb. Filters were reprobed with anti-STAT5A and c-Kit Abs, and whole cell lysates were also blotted with anti-β-actin Ab. (C) Binding of activated STAT5A to the STAT binding element of the *MIST* promoter. Nuclear extracts from the indicated cells were incubated with a radiolabeled oligonucleotide corresponding to the STAT-binding site of the *MIST* promoter. A band corresponding to the specific DNA–protein complex is shown by an arrow. (D) MIST promoter activity in IC-2 cells immortalized by STAT5A-1*6 (shaded bars) or c-Kit-D814Y (open bars). Reporter constructs containing the wild-type *MIST* promoter or its STAT-binding site mutant used also in Fig. 4 were transiently transfected into the cells, and 48 h after transfection, the luciferase activity was determined as in Fig. 2. (E) Northern blot analysis of endogenous *MIST* mRNA. Total RNA obtained from indicated cells was hybridized with the *MIST* cDNA (upper panel) or β-actin cDNA probe (lower panel).

MIST mRNA expression was observed in the IC-2 transfectant expressing STAT5A-1*6, whereas no such enhancement of *MIST* mRNA expression was detectable in the transfectant expressing c-Kit-D814Y (Fig. 5E). These results demonstrated that the c-Kit-mediated signal can replace the IL-3-mediated signal in driving cell growth but not in inducing *MIST* gene transcription. Therefore, it seems unlikely that basal *MIST* gene transcription in cytokine-independent c-Kit-dependent mast cell lines, such as P815, is induced by the c-Kit-mediated signal. In addition, since stable expression of the active form of STAT5A also did not induce endogenous *MIST* mRNA expression in the IL-5-responsive BCL1 B cell line (data not shown), STAT5A appears to be involved in *MIST* gene transcription in the context of mast cell-specific transcription machinery.

Discussion

We have demonstrated here that transcription of the *MIST* gene is regulated by at least two different mechanisms: one is a STAT-independent mechanism for basal transcription in P815 cells and the other is a STAT5-dependent mechanism for enhanced *MIST* gene transcription in the presence of cytokines.

Both STAT5A and B have been demonstrated to be activated by a wide range of cytokines, including growth hormone, IL-3, IL-5, GM-CSF, G-CSF, thrombopoietin, erythropoietin, IL-2, IL-4, IL-7, IL-13, and IL-15 [27]. Since *MIST* expression was observed in several cytokine-dependent hematopoietic cell lines [10], the identification of a functional STAT5-binding element in the mouse *MIST* promoter has now provided a molecular basis for *MIST* gene expression in those cell lines. Although IL-3 activates STAT3 in addition to STAT5 in mast cells, only STAT5 was found to bind to the STAT-binding motif in the *MIST* promoter. Thus, the STAT-binding motif in the *MIST* promoter appears to be a preferable binding site for STAT5 and may not be accessible for other STAT proteins. In this regard, the activation of c-Kit by stem cell factor also has been reported to result in the activation of STATs, leading to the formation of heterodimers containing STAT5A or STAT5B together with STAT1 [30–32]. These STAT5/STAT1 heterodimers specifically recognize a STAT-binding motif in the β-casein promoter, but not in the c-Fos promoter [32], indicating sequence selectivity for the binding of each STAT heterodimer [33]. This is in line with our findings that the constitutively active form of c-Kit failed to induce DNA–protein complex formation at the STAT-binding site in the *MIST* promoter and to up-regulate endogenous *MIST* mRNA expression in IC-2 mast cells.

One unexpected finding is that a constitutively active form of STAT5A failed to activate MIST promoter activity in EL-4 T cells and BCL1 B cells (Fig. 4 and data not shown). One possible explanation for this finding is that some other transcription factors missing in these cell lines are required for STAT5-mediated *MIST* gene expression. To support this, *MIST* mRNA was very weakly induced in CD4 or CD8-positive T cells upon stimulation with TCR and IL-2 (our unpublished data). Furthermore, despite that all the cytokines have the potential to activate STAT5, GM-CSF-induced bone marrow dendritic cells as well as spleen B cells stimulated with anti-CD40 antibody plus cytokines, including IL-4, IL-2 or IL-5, did not express *MIST* mRNA (unpublished data). In contrast, a high level of *MIST* expression was readily detectable in IL-2- or IL-15-induced NK cells and NKT cells as well as IL-3-induced bone marrow mast cells, supporting the idea that some lineage-restricted transcription factors regulate STAT5-mediated *MIST* gene expression. Therefore, some unknown lineage-specific transcription factors appear to be necessary for the STAT5-mediated transcription of the *MIST* gene. It is also possible that STAT5 may not be accessible to the *MIST* gene locus in the absence of such a lineage-specific transcription factor. The enhancer element that is involved in the basal *MIST* gene promoter activity in the cytokine-independent mast cell line P815 would be candidates for binding sites of mast cell-specific transcription factors that positively regulate *MIST* gene expression, in collaboration with STAT5. Alternative possibility is that some repressor proteins that suppress STAT5-mediated *MIST* gene transcription are present in hematopoietic lineage cells in which *MIST* expression is not induced by an active form of STAT5A or by cytokines.

The development of hematopoietic cell lineages that have a capacity to express the *MIST* gene appears to depend on STAT5. For example, mast cells and NK cells are absent in animals deficient in both STAT5A and B [34,35], and NKT as well as NK cell development is compromised in transgenic mice over-expressing CIS, an inhibitor for STAT5 activation [36]. Thus, in these cell lineages, a constitutively active STAT5 signaling pathway appears to be required for their development, and transcription factors shared in these cell lineages may collaborate with STAT5 to induce *MIST* gene expression.

The c-Kit signal is also essential for mast cell development and survival [37,38], and the mutation of c-Kit is a common cause of mast cell malignancy [39,40]. The activated c-Kit receptor is known to transduce signals through several signaling cascades, including Ras-MAP kinases, phosphatidylinositol 3′-kinase, and STAT [41]. Although we failed to detect a positive effect of c-Kit-mediated signals on the *MIST* gene expression, we could not exclude the possibility that c-Kit-mediated signals collaborate with STAT5 to activate *MIST* gene expression.

In summary, this study demonstrates the mechanisms that lead to the expression of the SLP-76 family adaptor

MIST gene in hematopoietic cells. It indicates that the induction of the *MIST* gene by cytokine signals involves at least two distinct mechanisms. One of these mechanisms is dependent on the STAT element that can be targeted by STAT5 and the other modulates STAT5-mediated transcription of the gene, in addition to regulating lineage-specific basal expression. Cytokines regulate a variety of immune responses, and the spatio-temporal expression at the site of immune reactions determines the extent and nature of the immune response. Since MIST can function as a signaling scaffold downstream of multiple immune receptors [9,20,21], our findings on the transcriptional regulation of the *MIST* gene would thus help to understand how lineage-specific immunoreceptor signaling pathways are established and regulated during the activation and differentiation of various hematopoietic cells.

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