

GATA transcription factors are involved in IgE-dependent mast cell degranulation by enhancing the expression of phospholipase C- γ 1

Yasushi Ishijima, Shin'ya Ohmori, Ayano Uenishi and Kinuko Ohneda*

Faculty of Pharmacy, Department of Pharmacy, Takasaki University of Health and Welfare, 60 Nakaorui-machi, Takasaki, Gunma 370-0033, Japan

Mast cell degranulation is a dynamic, highly organized process involving numerous signaling molecules and enzymes. Although the molecular mechanisms underlying antigen-mediated mast cell degranulation have been studied intensively, little is known about the transcriptional control of this process. Here, we show that the hematopoietic transcription factors GATA1 and GATA2 are involved in mast cell degranulation through the control of phospholipase C- γ 1 (PLC- γ 1) expression. Knockdown of GATA1 and/or GATA2 by specific siRNA significantly reduced antigen-induced degranulation and Ca^{2+} mobilization in the rat basophilic leukemia cell line RBL-2H3. RT-PCR analyses showed that PLC- γ 1 expression was significantly decreased by this GATA factor repression. Other GATA factor targets, such as the previously reported α and β subunits of the high-affinity IgE receptor (Fc ϵ RI), were unaffected. Chromatin immunoprecipitation and luciferase reporter assays demonstrated that GATA factors directly activate *PLC- γ 1* gene transcription through a conserved GATA-binding motif that resides in the 5'-upstream sequence. Furthermore, we show evidence that the PLC- γ 1 expression is regulated by GATA2 in mast cells derived from mouse bone marrow. These data indicate that *PLC- γ 1* is a target gene of GATA factors in mast cells and provide evidence that GATA1 and GATA2 control antigen-mediated mast cell degranulation by regulating the expression of PLC- γ 1.

Introduction

Mast cells play central roles in IgE-associated allergic disorders, such as asthma and atopic dermatitis. Antigen-induced signaling in mast cells is mediated through IgE and its high-affinity receptor Fc ϵ RI. Crosslinking of the receptor by multivalent antigen leads to the release of a variety of inflammatory mediators, such as bioactive amines, cytokines, chemokines and proteases, from their cytoplasmic granules. This process is called degranulation. The molecular mechanisms underlying antigen-mediated mast cell degranulation have been widely studied over the past decade (Rivera & Gilfillan 2006; Kalesnikoff & Galli 2008; Metcalfe *et al.* 2009). Antigen-induced Fc ϵ RI aggregation activates a signaling cascade involving numer-

ous kinases and adaptor molecules, which results in an activation of phospholipase C- γ (PLC- γ), a key player in the terminal stage of this pathway. PLC- γ catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), and IP₃ mediates the release of Ca^{2+} from the endoplasmic reticulum (ER), triggering Ca^{2+} influx from the extracellular environment, whereas DAG activates protein kinase C. Both processes are known to be essential for mast cell degranulation.

Despite the identification of numerous signaling molecules involving degranulation, little is known about the transcriptional control of these molecules in mast cells. Given that mast cell degranulation is a complex process involving multiple molecules, a transcription factor that regulates the expression of multiple signaling molecules might be a potential therapeutic target for allergic diseases. Mast cells originate from

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*Correspondence: kohneda@takasaki-u.ac.jp

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hematopoietic stem cells in the bone marrow and express several hematopoietic transcription factors, such as GATA1, GATA2, SCL/Tal1 and PU.1, which are crucial for hematopoietic development (Okayama & Kawakami 2006; Takemoto *et al.* 2008). The zinc finger transcription factors GATA1 and GATA2 likely control mast cell degranulation, as functional GATA-binding sites have been found in the promoter regions of the *FcεRIα* and *β* genes (Nishiyama *et al.* 1999; Maeda *et al.* 2003). GATA1 is expressed in erythroid cells, megakaryocytes, eosinophils, dendritic cells and mast cells of the hematopoietic system (Evans & Felsenfeld 1989; Tsai *et al.* 1989; Martin *et al.* 1990; Romeo *et al.* 1990; Zon *et al.* 1993; Gutiérrez *et al.* 2007). Although the precise roles of GATA1 on mast cell development have not been fully elucidated, previous studies showed that the reduced expression of GATA1 by targeted disruption of an upstream enhancer results in the formation of abnormal progenitors that differentiate into erythroid cells, megakaryocytes and mast cells (Migliaccio *et al.* 2003; Ghinassi *et al.* 2007). GATA2 is expressed in hematopoietic stem cells and multilineage progenitors and plays key roles in the maintenance and proliferation of those cells (Nagai *et al.* 1994; Minegishi *et al.* 1998, 1999). Although the early embryonic lethality observed in *Gata2* null mice (Tsai *et al.* 1994) has precluded studies of embryonic stem (ES) cells have demonstrated that GATA2-deficient ES cells lose the ability to differentiate into mast cells (Tsai & Orkin 1997). These studies demonstrate that both GATA1 and GATA2 are required for proper mast cell development at the progenitor stage. However, the roles of GATA1 and GATA2 in terminally differentiated mast cells, especially in the antigen-induced degranulation process, have not been fully elucidated.

To assess the functions of the GATA factors in mast cell degranulation, we set up knock-down experiments of GATA1 and GATA2 in the rat basophilic leukemia cell line RBL-2H3. We show that reduced GATA1 or GATA2 expression in RBL-2H3 cells impairs antigen-induced degranulation and Ca^{2+} mobilization. Unexpectedly, however, the reduced GATA factor expression did not attenuate the *FcεRI* expression. Instead, the expression of *PLC-γ1* was significantly reduced by the GATA factor repression. We found two conserved GATA-binding motifs that reside in the 5'-upstream sequence of the *PLC-γ1* gene. Chromatin immunoprecipitation and luciferase reporter assays demonstrated that GATA factors directly activate *PLC-γ1* gene transcription through

the distal GATA-binding motif. Finally, we show evidence that the *PLC-γ1* expression is regulated by GATA2 in mast cells derived from mouse bone marrow (BMMCs). These results indicate that *PLC-γ1* is a novel target gene for GATA factors in mast cells and that GATA factors control mast cell degranulation by regulating the expression of *PLC-γ1*.

Results

IgE-dependent degranulation is impaired by GATA factor repression in RBL-2H3 cells

To assess the roles of the GATA factors in mast cells, we introduced GATA1- or GATA2-specific siRNA into the well-characterized rat basophilic leukemia cell line RBL-2H3. Quantitative RT-PCR analyses showed that both siRNAs reduced targeted mRNA levels to less than half of those in control siRNA-transfected cells 24 h after transfection (Fig. 1A). The simultaneous introduction of GATA1 and GATA2 siRNAs reduced GATA1 and GATA2 mRNA levels to 0.22 ± 0.06 and 0.27 ± 0.06 , respectively (Fig. 1A). We measured the release of β -hexosaminidase in these cells to find out if mast cell degranulation is affected by the reduced GATA factor expression (Fig. 1B). IgE-dependent degranulation induced by antigen stimulation was significantly reduced in the single and double GATA1- and GATA2-knockdown cells compared to the control siRNA-transfected cells (Fig. 1B). Notably, simultaneous treatment of GATA1 and GATA2 siRNAs showed a greater effect on the reduction of the IgE-dependent degranulation than the single GATA factor repression, suggesting that GATA1 and GATA2 act in an additive manner. GATA factor repression did not alter the IgE-independent degranulation induced by the calcium ionophore A23187 (Fig. 1B). The β -hexosaminidase mRNA levels were not affected by the GATA factor repression, indicating that the reduced β -hexosaminidase release was not a consequence of direct transcriptional inhibition (Fig. 1C). These results indicate that GATA1 and GATA2 are involved in the IgE-dependent degranulation induced by antigen stimulation in RBL-2H3 cells.

GATA factors are involved in IgE-dependent Ca^{2+} mobilization in RBL-2H3 cells

Ca^{2+} mobilization is a process that is essential for mast cell degranulation (Nishida *et al.* 2005). Therefore, we measured the intracellular Ca^{2+} concentration

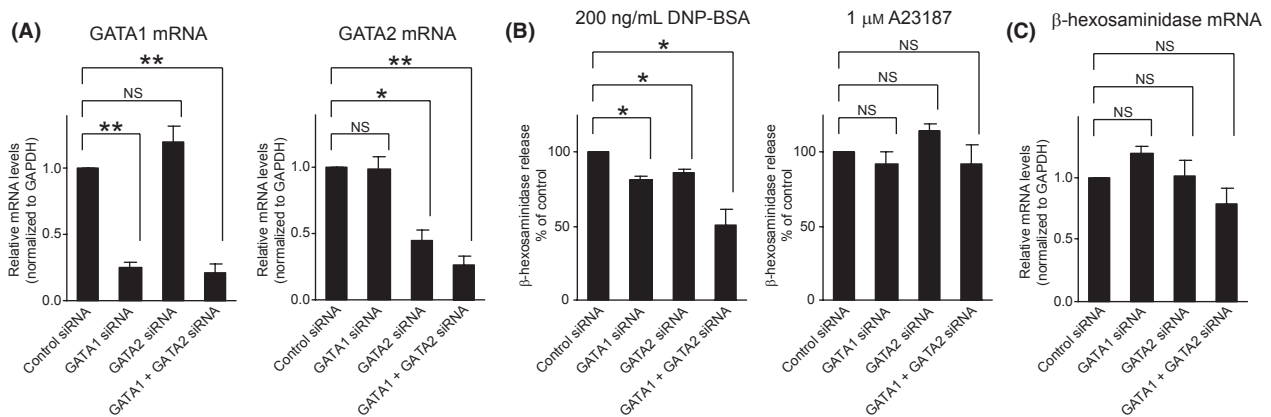


Figure 1 GATA factors are involved in IgE-dependent degranulation in RBL-2H3 cells. (A) The expression of GATA1 (left panel) and GATA2 (right panel) mRNAs in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. The mRNA levels were quantified by real-time RT-PCR 24 h after transfection. (B) The release of β -hexosaminidase in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. Left panel: Cells were sensitized with anti-DNP IgE overnight and stimulated with DNP-BSA for 30 min, followed by measurement of the β -hexosaminidase activity. Right panel: Fc ϵ RI-independent degranulation was measured in cells treated with 1 μ M A23187 for 30 min. The β -hexosaminidase release from control siRNA-transfected cells was set to 100 and relative values are shown. (C) The expression of β -hexosaminidase mRNA in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. The mRNA levels were quantified by real-time RT-PCR 24 h after transfection. In all experiments, data are shown as means $\pm$ SE from three independent experiments, each carried out in triplicate. For the comparisons indicated: * P < 0.05 and ** P < 0.01. NS, not significant.

($[\text{Ca}^{2+}]_i$) in RBL-2H3 cells transfected with GATA1 and GATA2 siRNA. Ca^{2+} mobilization consists of two events: Ca^{2+} release from the ER and Ca^{2+} influx from the extracellular environment. So, we dissected these processes by measuring the $[\text{Ca}^{2+}]_i$ with and without extracellular Ca^{2+} . To measure Ca^{2+} release from the intracellular stores, IgE-sensitized RBL-2H3 cells loaded with fura 2 were resuspended in Ca^{2+} -free Tyrode buffer and stimulated with antigen. Spectrofluorometric analyses showed a rapid and transient Ca^{2+} release from the ER in RBL-2H3 cells transfected with control siRNA (Fig. 2A). Similar $[\text{Ca}^{2+}]_i$ profiles were observed in the GATA-siRNA-transfected cells (Fig. 2A). We then examined Ca^{2+} influx from the extracellular environment by adding 1.8 mM Ca^{2+} to the buffer. Upon stimulation with antigen, a sustained elevation in the $[\text{Ca}^{2+}]_i$ was observed in RBL-2H3 cells transfected with control siRNA (Fig. 2B). Notably, this sustained elevation in extracellular Ca^{2+} influx was suppressed by GATA factor repression (Fig. 2B,C). These findings imply that GATA1 and GATA2 are involved in IgE-dependent Ca^{2+} influx from the extracellular environment without affecting the release of Ca^{2+} from the ER store. The suppressed extracellular Ca^{2+} influx is the presumable cause of the reduced β -hexosaminidase release seen upon GATA factor repression.

Fc ϵ RI expression is not altered by GATA factor repression in RBL-2H3 cells

Previous studies reported that GATA factors bind to the promoter regions of the *Fc ϵ RI α* and *Fc ϵ RI β* genes and regulate their expression in the mouse mast cell line PT18 (Nishiyama *et al.* 1999; Maeda *et al.* 2003). Our mission was to unearth the contribution of GATA factors to Fc ϵ RI expression in RBL-2H3 cells. We examined the cell surface expression of Fc ϵ RI by flow cytometry (Fig. 3A). Unexpectedly, the frequency of FITC-positive cells and the peak fluorescent intensity were similar between control and GATA factor knock-down cells (Fig. 3A). Moreover, quantitative RT-PCR analyses showed that the mRNA levels of the α , β and γ subunits of Fc ϵ RI were unchanged by GATA factor repression (Fig. 3B). Thus, GATA factor repression does not contribute to Fc ϵ RI expression in RBL-2H3 cells.

PLC- γ 1 is directly regulated by GATA factors in RBL-2H3 cells

To identify the GATA target gene that contributes to IgE-dependent degranulation and Ca^{2+} mobilization, the mRNA levels of candidate molecules were examined by quantitative RT-PCR (Fig. 4A). The mRNA

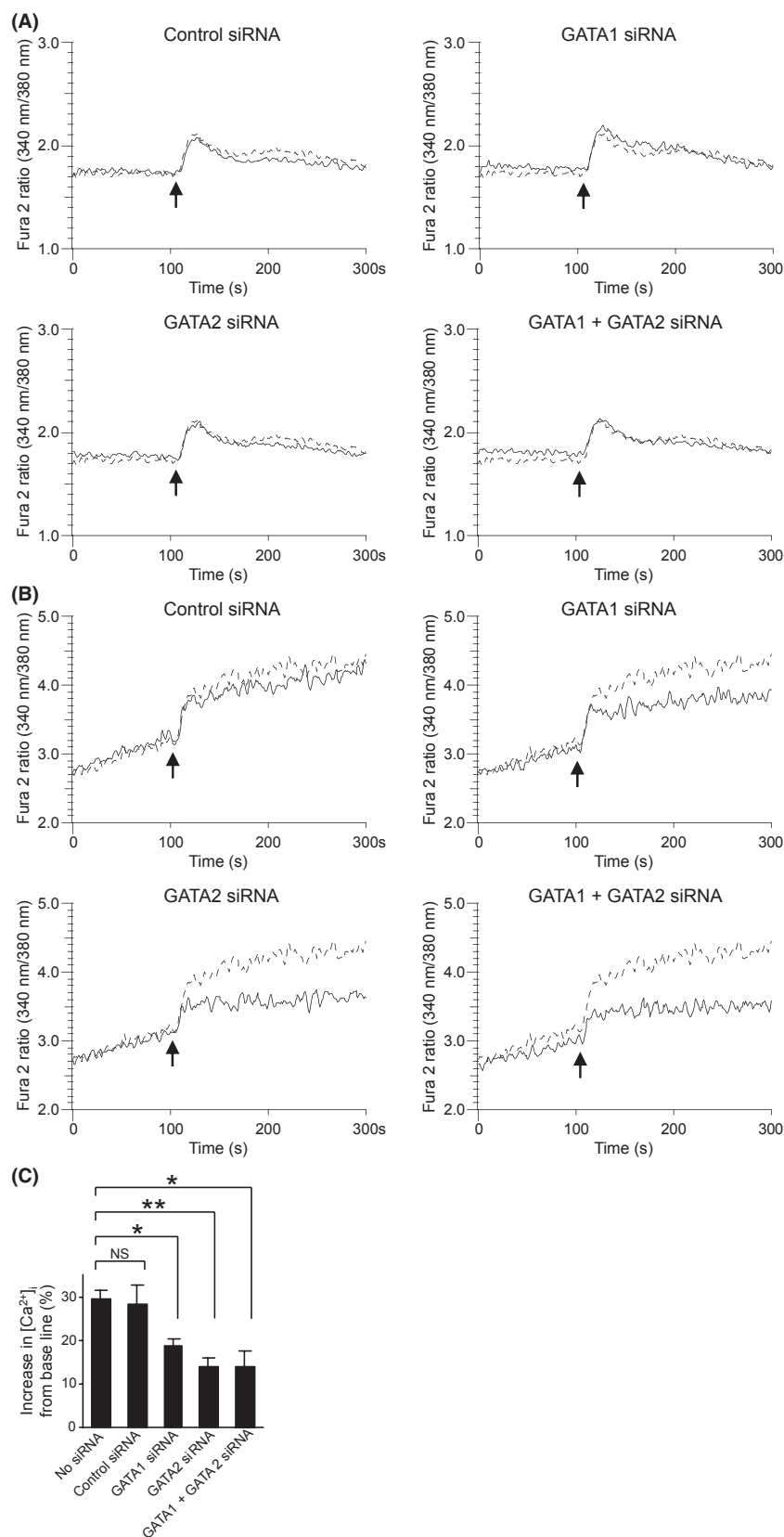


Figure 2 GATA factors regulate IgE-dependent Ca^{2+} mobilization in RBL-2H3 cells. (A,B) The Ca^{2+} release from the intracellular stores (A) and the Ca^{2+} influx from the extracellular environment (B) were measured in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. Sensitized cells were loaded with fura 2, resuspended in Ca^{2+} -free (A) or Ca^{2+} -containing (B) buffer and stimulated with DNP-BSA to activate Fc ϵ RI. The changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were monitored by spectrofluorometry. Fura 2 is excited at 340 and 380 nm of light, and $[\text{Ca}^{2+}]_i$ is represented as the ratio of 340–380 nm fluorescence. The broken line and the solid line represent the changes in $[\text{Ca}^{2+}]_i$ in nontransfected cells and siRNA-transfected cells, respectively. The arrows indicate the time point when DNP-BSA was added. Representative data from three independent experiments are shown. (C) The increase in $[\text{Ca}^{2+}]_i$ following the addition of DNP-BSA. Values from (B) are shown as the % increase at 160 s relative to the $[\text{Ca}^{2+}]_i$ at the time point when DNP-BSA was added (at 100 s). Data are shown as means $\pm$ SE from three independent experiments. For the comparisons indicated: * $P < 0.05$ and ** $P < 0.01$. NS, not significant.

levels of Lyn, Syk, SLP-76 and Vav1 were unaltered by GATA factor repression. Interestingly, we found that the mRNA level of PLC- γ 1, but not PLC- γ 2, was significantly reduced by GATA factor repression (Fig. 4A). The cycle threshold values for PLC- γ 1 and PLC- γ 2 were 26.72 ± 0.61 and 24.51 ± 0.41 , respectively, in control siRNA-transfected cells. We next carried out Western blot analyses to examine whether the PLC- γ 1 protein level was also reduced by GATA factor repression (Fig. 4B). Immunoblot with the respective antibodies demonstrated that both GATA1 and GATA2 protein levels were reduced by the corresponding siRNA treatment. Consistent with the RT-PCR analysis, PLC- γ 1 protein level was reduced by GATA factor repression. Importantly, simultaneous treatment of GATA1 and GATA2 siRNAs showed greater effects on the reduction of PLC- γ 1 mRNA and protein levels, as observed in the IgE-dependent degranulation assays. Collectively, these results indicate that GATA1 and GATA2 control IgE-dependent β -hexosaminidase release by regulating the expression of *PLC- γ 1*.

On the basis of these findings, we considered the possibility that the expression of GATA1, GATA2 and PLC- γ 1 might be dynamically regulated by the IgE-dependent antigen stimulation. To test this possibility, mRNA levels of GATA1, GATA2 and PLC- γ 1 were evaluated following the addition of DNP-BSA (Fig. S1A in Supporting Information). Contrary to our expectations, mRNA levels of GATA1, GATA2 and PLC- γ 1 did not increase at 10 min and 30 min following the stimulation. We next examined whether GATA factors control phosphorylation of PLC- γ 1, a fundamental process for Ca^{2+} mobilization (Fig. S1B in Supporting Information). When the band intensity was normalized with that of β -tubulin to adjust for loading differences, the phosphorylated form of PLC- γ 1 was increased upon the antigen stimulation in control siRNA treated cells. Similarly, the induction of phospho-PLC- γ 1 was observed in the presence of GATA1 and GATA2 siRNAs. These data

suggest that GATA factors have an insignificant effect on the activation process of this protein.

To examine if GATA1 and GATA2 directly regulate *PLC- γ 1* gene transcription, we studied the regulatory regions of the mouse, rat and human *PLC- γ 1* genes. Two conserved GATA-binding motifs were found 1.6 and 5.1 kb upstream of the transcriptional initiation site of the rat *PLC- γ 1* gene (Fig. 5A). Hereafter, we refer to these proximal and distal sites as GATA-binding motif A and B, respectively. Chromatin immunoprecipitation (ChIP) assay was used to determine if GATA1 and GATA2 bind to these GATA sites *in vivo* (Fig. 5B). The promoter region of *Fc ϵ RI β* gene containing a GATA-binding motif and the exon 25 coding region of *PLC- γ 1* gene have been examined as positive and negative controls, respectively. We found that a chromatin region containing GATA-binding motif B was occupied by both GATA factors. However, a chromatin region containing GATA-binding motif A was only bound by GATA1. We set up reporter assays using luciferase reporter plasmids containing the GATA-binding motifs (Fig. 5C–F). Reporter activity was significantly reduced in the case of GATA-binding motif B, but not motif A, when the endogenous GATA factor expression was repressed by specific siRNA (Fig. 5C). We then generated mutant reporter constructs for these GATA-binding motifs. Whereas the GATA site mutation significantly attenuated the reporter activity for GATA-binding motif B, the mutant reporter activity was comparable to that of wild type for GATA-binding motif A (Fig. 5D). However, when GATA factors are exogenously overexpressed in QT6 fibroblasts, both GATA-binding motifs were activated by transfection of the GATA1 or GATA2 expression plasmids in a dose-dependent manner (Fig. 5E,F, left panel), and this enhancement was terminated by the GATA site mutation (Fig. 5E,F, right panel). Collectively, these results indicate that GATA1 and GATA2 directly activate *PLC- γ 1* gene transcription mainly through the distal GATA-binding motif B.

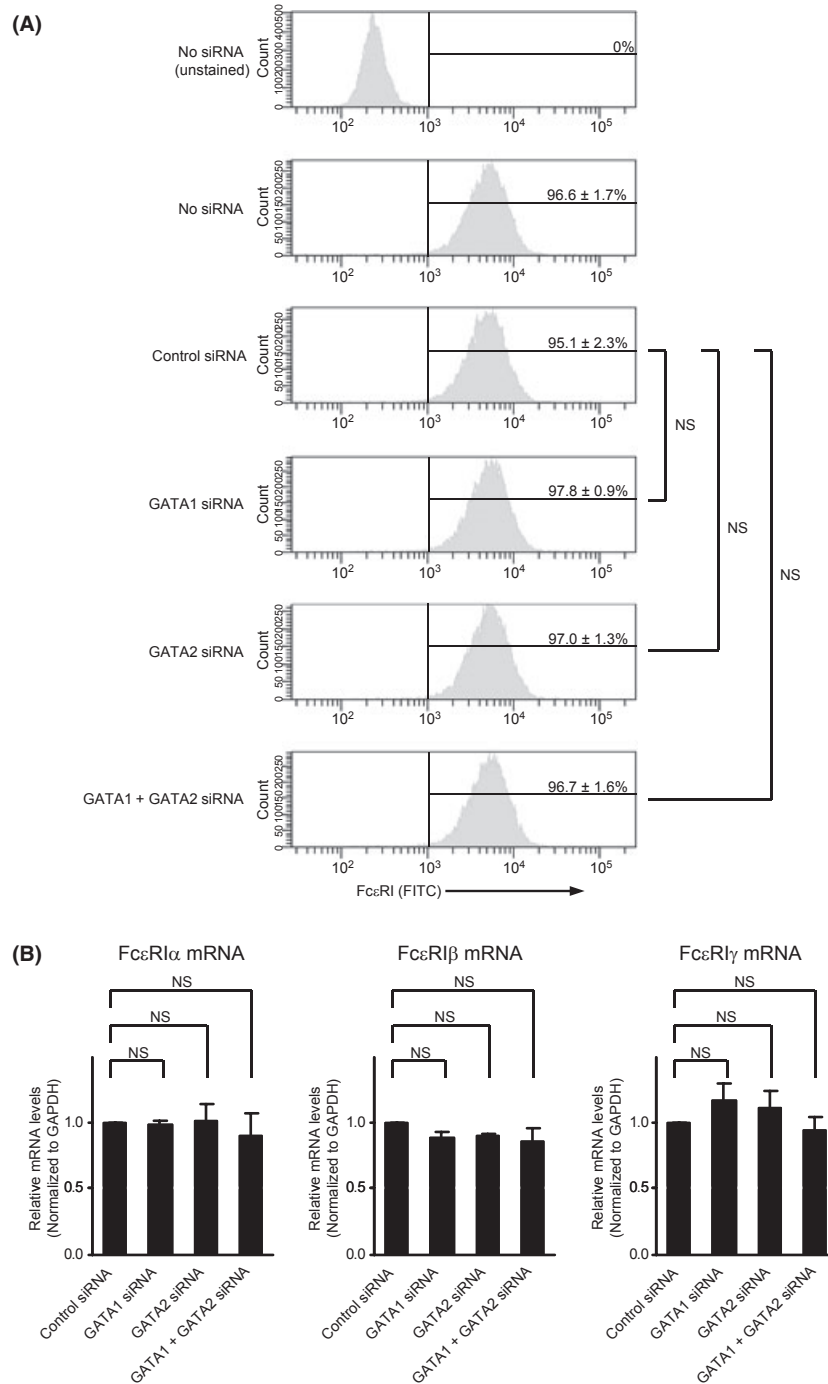


Figure 3 GATA factors are dispensable in FcεRI expression in RBL-2H3 cells. (A) Flow cytometric analysis of FcεRI expression in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. Cells were stained with FITC-conjugated anti-mouse IgE antibody followed by preincubation with mouse IgE. Typical results from three independent experiments are displayed. The percentages of FcεRI-positive cells are shown as means ± SE from three independent experiments. (B) FcεRIα, FcεRIβ and FcεRIγ mRNA levels in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. The mRNA levels were determined by real-time quantitative RT-PCR. Data are shown as means ± SE from three independent experiments, each carried out in triplicate. NS, not significant.

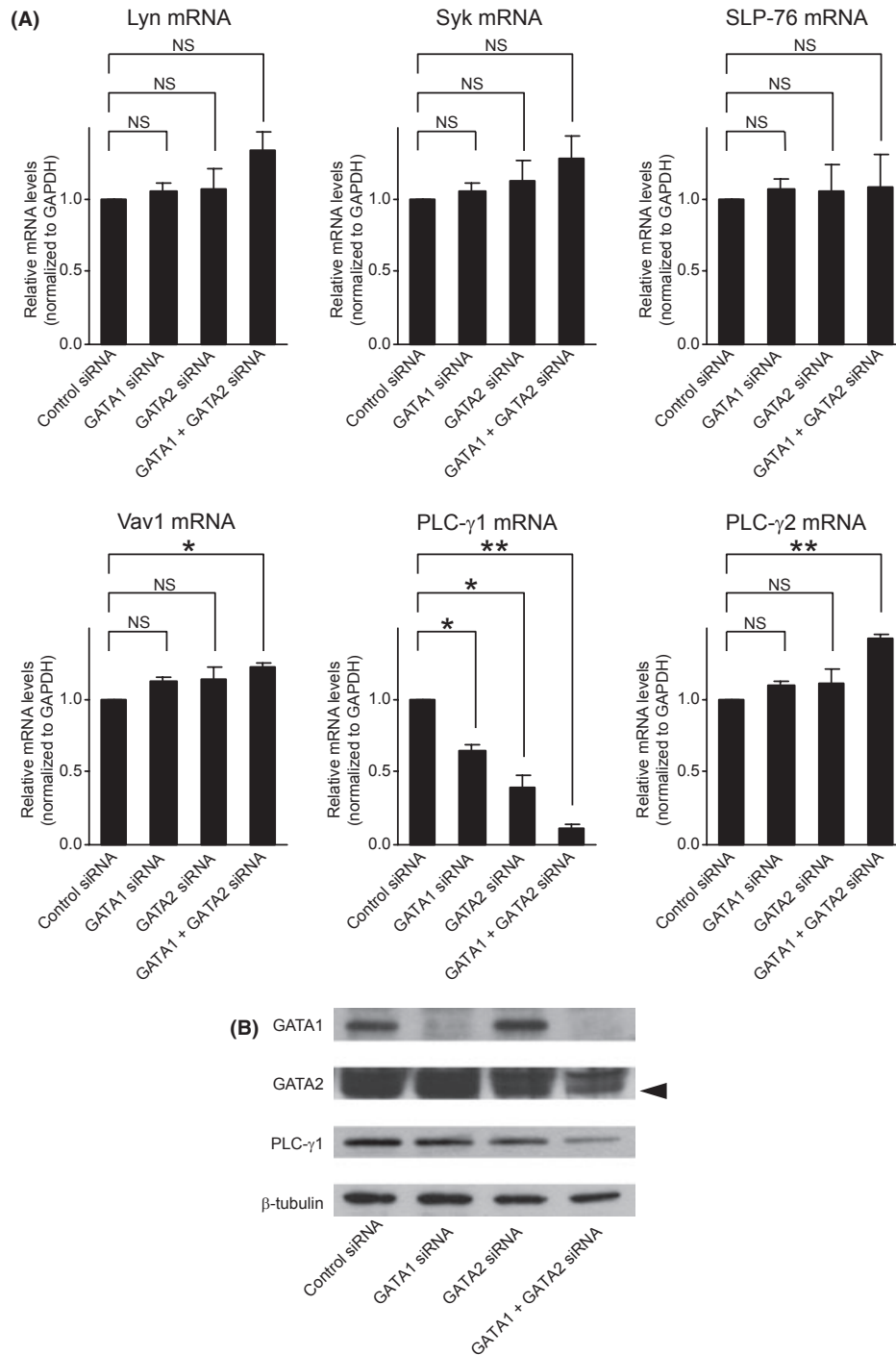


Figure 4 A reduction in the expression levels of the GATA factors alters the PLC- γ 1 mRNA and protein levels in RBL-2H3 cells. (A) The mRNA levels of Lyn, Syk, SLP-76, Vav1, PLC- γ 1 and PLC- γ 2 in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. Values were determined by real-time quantitative RT-PCR and shown as means $\pm$ SE from three independent experiments, each carried out in triplicate. * P < 0.05 and ** P < 0.01 for the comparisons indicated. NS, not significant. (B) The protein levels of GATA1, GATA2 (arrowhead) and PLC- γ 1 in RBL-2H3 cells transfected with GATA1 and/or GATA2 siRNA. Representative results from three independent experiments are shown. Immunoblot with anti- β -tubulin antibody was used as a loading control.

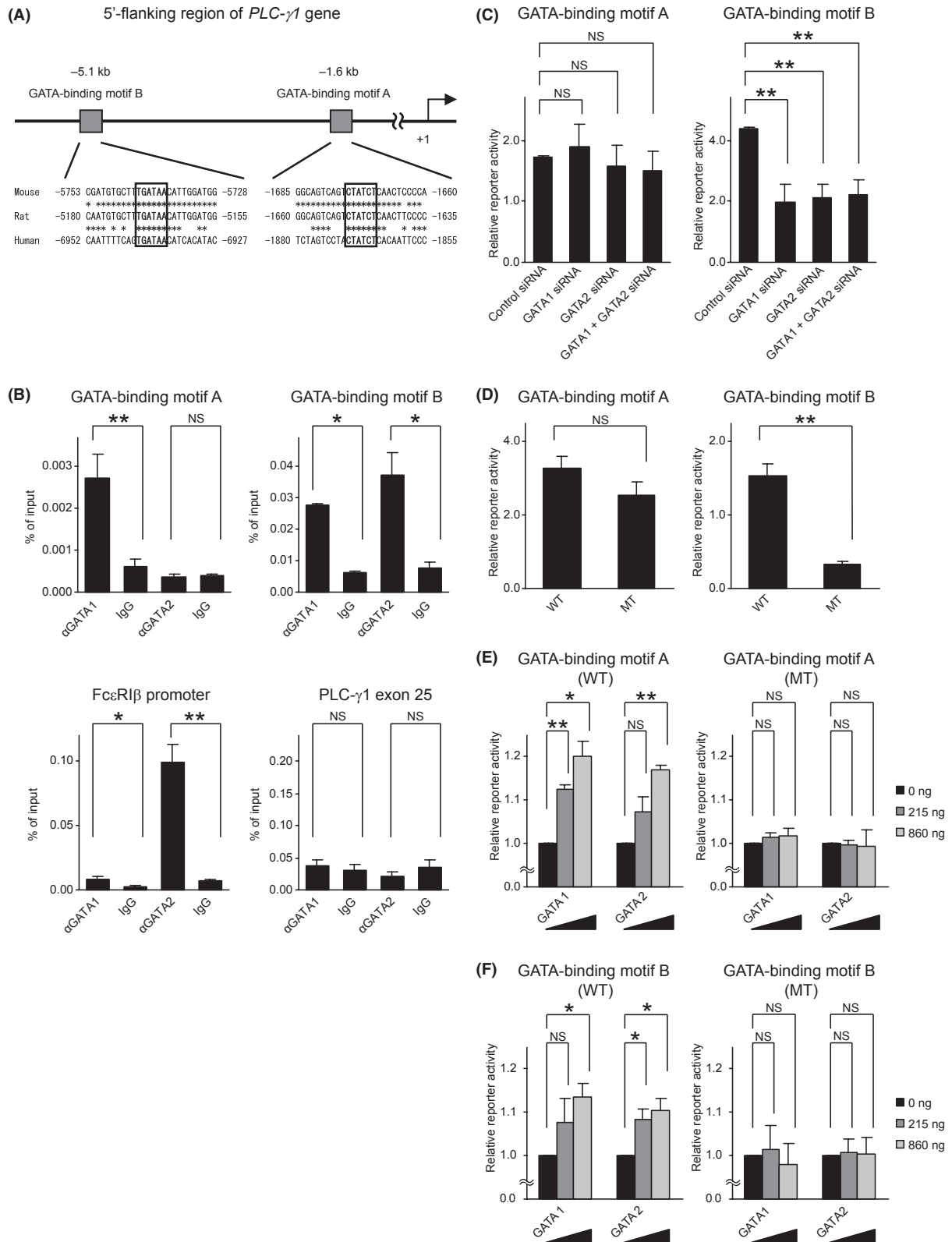


Figure 5 GATA factors directly activate *PLC- γ 1* gene transcription in RBL-2H3 cells. (A) Schematic diagram of the 5'-upstream sequence of the *PLC- γ 1* gene including the two GATA-binding motifs referred to as A and B that are conserved in mouse, rat and human. In the rat *PLC- γ 1* gene, GATA-binding motifs A and B are located 1.6 and 5.1 kbp, respectively, upstream of the transcriptional initiation site. (B) ChIP analysis of GATA-binding motifs A and B, *Fc ϵ RI β* promoter region (positive control) and exon 25 coding region of *PLC- γ 1* (negative control) using either anti-GATA1 antibody or anti-GATA2 antibody in RBL-2H3 cells. Purified DNA from the immunoprecipitated complexes was quantified by real-time PCR using primers specific for GATA-binding motif A or B. The values obtained from IP were normalized with those from input DNA without IP. The results are shown in comparison with those from negative control using normal IgG. Data are shown as means $\pm$ SE from three independent experiments, each carried out in triplicate. (C) RBL-2H3 cells were co-transfected with GATA1 or GATA2 siRNA and a luciferase reporter containing either GATA-binding motif A (left) or B (right). A reporter plasmid expressing *renilla* luciferase was used as a transfection control. The results are presented as the activities relative to that of control siRNA-transfected cells. (D) A luciferase reporter containing either GATA-binding motif A or B (WT) or those with GATA site mutation (MT) was transfected into RBL-2H3 cells. A reporter plasmid expressing *renilla* luciferase was used as a transfection control. The relative reporter activities are shown. (E,F) QT6 cells were co-transfected with GATA1 or GATA2 expression plasmid and a luciferase reporter containing GATA-binding motif A (E) or B (F). The amounts of GATA expression plasmids used were 0, 215 and 860 ng and are depicted by black, dark gray and light gray columns, respectively. The same experiments were repeated using WT or MT luciferase reporter vectors. A reporter plasmid expressing *renilla* luciferase was used as a transfection control. The reporter activities without GATA1 or GATA2 cDNA were set as 1.0 and the relative values are shown. In (C–F), data are shown as means $\pm$ SE from five independent experiments. For all experiments, * P < 0.05 and ** P < 0.01 for the comparisons indicated. NS, not significant.

Overexpression of PLC- γ 1 restores the suppressed degranulation by GATA factor repression

The present data do not exclude the possibility that other factors are involved in the reduced IgE-dependent degranulation caused by GATA factor repression. We therefore examined if overexpression of PLC- γ 1 is sufficient to restore the reduced IgE-dependent degranulation in RBL-2H3 cells transfected with GATA1 or GATA2 siRNA (Fig. 6A). We found that β -hexosaminidase release was restored when the PLC- γ 1 expression plasmid was co-transfected with GATA1 or GATA2 siRNA. This indicates that a loss of PLC- γ 1 expression plays a major role in reduced degranulation in GATA factor knock-down cells. We ascertained if direct repression of PLC- γ 1 results in effects similar to those of GATA factor repression on degranulation and Ca^{2+} mobilization. Direct inhibition of PLC- γ 1 by specific siRNA resulted in a reduction of PLC- γ 1 mRNA level (0.34 ± 0.02 of control; Fig. 6B). Accordingly, a significant reduction in IgE-dependent degranulation was observed in PLC- γ 1 siRNA-transfected cells (Fig. 6C). Interestingly, no notable change was observed in the transient Ca^{2+} release from the ER stores (Fig. 6D), whereas the subsequent extracellular Ca^{2+} influx was clearly attenuated by PLC- γ 1 siRNA (Fig. 6E). These data are similar to the results for the Ca^{2+} mobilization observed in cells with GATA factor repression. We conclude that GATA factors directly regulate *PLC- γ 1* gene expression, thereby participating in IgE-dependent degranulation in RBL-2H3 cells.

GATA2 controls *PLC- γ 1* gene expression in mouse bone marrow-derived mast cells

Finally, we examined whether our findings in RBL-2H3 cells could also be applied to BMMCs as well (Fig. 7). ChIP assays demonstrated that both GATA factors bound to the two chromatin regions containing the conserved GATA-binding motifs (Fig. 7A). Notably, GATA2 binding to the GATA-binding motif A was observed in BMMCs, but not in RBL-2H3 cells (Figs 7A and 5B). We then introduced GATA1- or GATA2-specific siRNA into BMMCs and ascertained the reduction of mRNA (Fig. 7B) and protein (data not shown) levels by the corresponding siRNA treatment. Interestingly, the repression of PLC- γ 1 was observed in GATA2, but not GATA1 knockdown cells (Fig. 7C). Consistent with the observation in RBL-2H3 cells, the decrease of IgE-dependent degranulation by GATA factor repression seemed smaller than the PLC- γ 1 repression (as discussed below). Nonetheless, the β -hexosaminidase release stimulated by antigen was significantly reduced by GATA2, but not GATA1 repression (Fig. 7D). Collectively, these results indicate that PLC- γ 1 expression is regulated by GATA2 in BMMCs.

Discussion

Mast cell degranulation is a dynamic, highly organized process involving numerous signaling molecules and enzymes. It is widely accepted that this process is regulated mainly by post-transcriptional modifications such as receptor tyrosine phosphorylation. However,

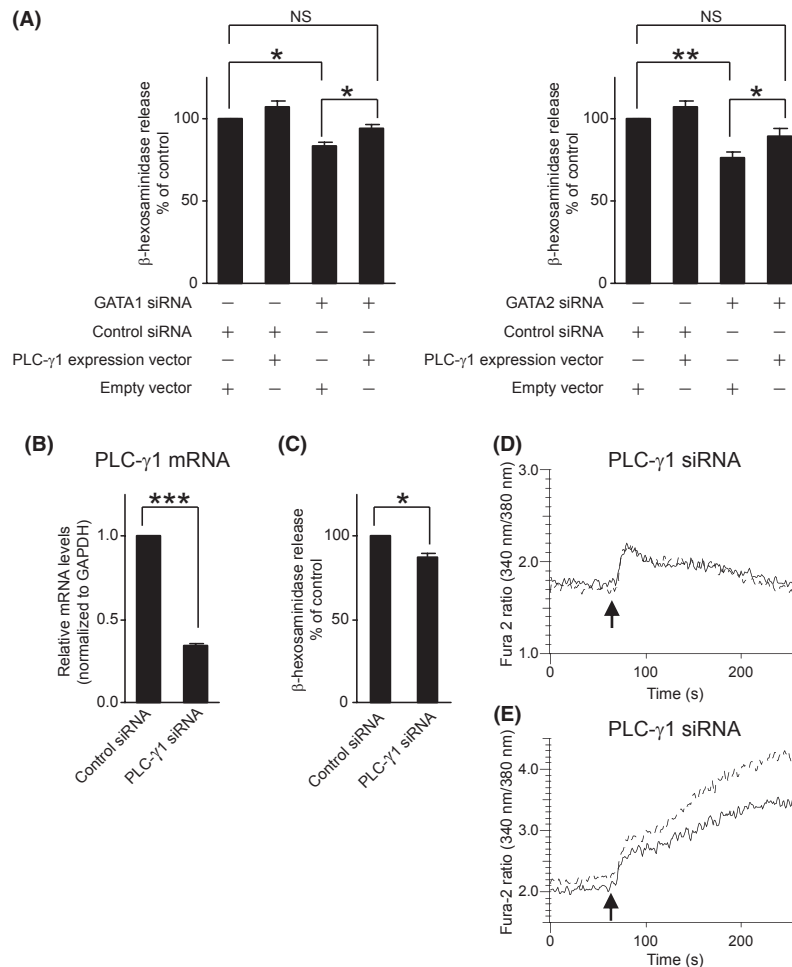


Figure 6 Exogenous PLC- γ 1 expression restores the suppressed IgE-dependent degranulation resulting from GATA factor repression. (A) The release of β -hexosaminidase in RBL-2H3 cells transfected with a PLC- γ 1 expression plasmid and either GATA1 or GATA2 siRNA. The IgE-dependent β -hexosaminidase release was evaluated as described in Fig. 1B. The β -hexosaminidase activities from cells co-transfected with control siRNA and empty vector were set to 100 and the relative values are shown. Data are shown as means $\pm$ SE from five independent experiments. * P < 0.05 and ** P < 0.01 for the comparisons indicated. NS, not significant. (B) The expression of PLC- γ 1 mRNA in RBL-2H3 cells transfected with PLC- γ 1 siRNA. The mRNA level was quantified by real-time RT-PCR 24 h after transfection. Data are shown as means $\pm$ SE from three independent experiments, each carried out in triplicate. *** P < 0.001 for the comparisons indicated. (C) The IgE-dependent release of β -hexosaminidase in RBL-2H3 cells transfected with PLC- γ 1 siRNA. The release of β -hexosaminidase was measured as described in Fig. 1B. The β -hexosaminidase release from control siRNA-transfected cells was set to 100 and the relative values are shown. Data are shown as means $\pm$ SE from three independent experiments, each carried out in triplicate. * P < 0.05 for the comparisons indicated. (D, E) Ca^{2+} release from the intracellular stores (D) and the Ca^{2+} influx from the extracellular environment (E) in RBL-2H3 cells transfected with PLC- γ 1 siRNA. The changes in $[\text{Ca}^{2+}]_i$ were monitored by spectrofluorometry, as described in Fig. 2A,B. The broken and solid lines represent the changes in the $[\text{Ca}^{2+}]_i$ in cells transfected with control siRNA and PLC- γ 1 siRNA, respectively. The arrows denote the time point at which DNP-BSA was added. Representative data are shown from three independent experiments.

it should be noted that transcription factors expressed in mast cells underpin the production of molecules required for the entire process of degranulation. Whereas post-transcriptional modification enables rapid and transient changes, regulation by transcrip-

tion factors might cause persistent and significant changes by altering multiple target gene expression. Thus, the transcriptional control of mast cell degranulation might provide a potential therapeutic target for allergic diseases. The present study demonstrates a

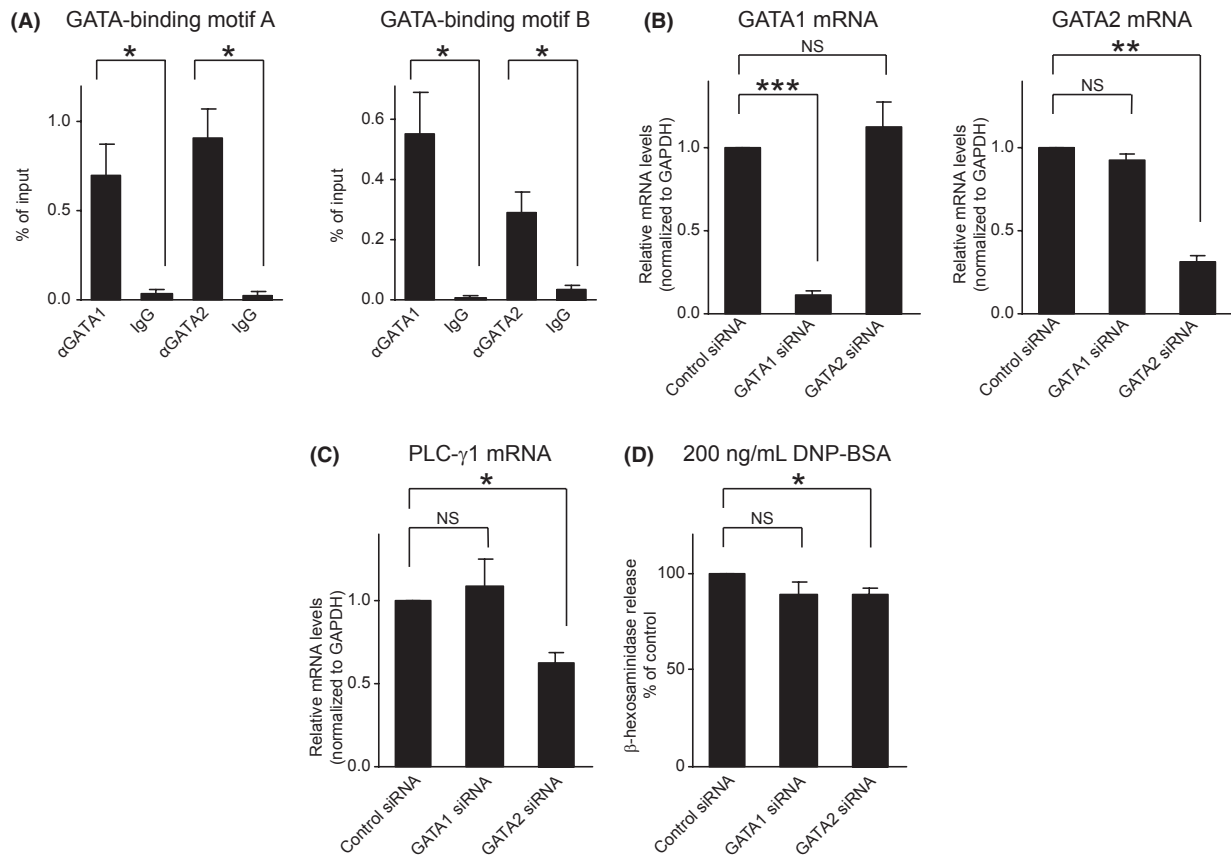


Figure 7 GATA2 controls PLC- γ 1 expression in BMMCs. (A) ChIP analysis of GATA-binding motifs A and B using BMMCs. Purified DNA from the immunoprecipitated complexes was quantified by real-time PCR using primers specific for GATA-binding motif A or B. The values obtained from IP were normalized with those from input DNA without IP. The results are shown in comparison with those from negative control using normal IgG. (B) The mRNA levels of GATA1 and GATA2 in BMMCs transfected with GATA1 or GATA2 siRNA. Values of mRNAs were determined by real-time quantitative RT-PCR and shown as means $\pm$ SE from three independent experiments, each carried out in triplicate. (C) The expression of PLC- γ 1 mRNA in BMMCs transfected with GATA1 or GATA2 siRNA. The mRNA level was quantified by real-time RT-PCR 24 h after transfection. (D) The release of β -hexosaminidase in BMMCs transfected with GATA1 or GATA2 siRNA. Cells were sensitized with anti-DNP IgE for 3 h and stimulated with DNP-BSA for 30 min, followed by measurement of the β -hexosaminidase activity. The β -hexosaminidase release from control siRNA-transfected cells was set to 100 and relative values are shown. In all experiments, data are shown as means $\pm$ SE from three independent experiments. For the comparisons indicated: * P < 0.05, ** P < 0.01 and *** P < 0.001. NS, not significant.

prototype of the transcriptional regulation of mast cell degranulation by GATA1 and GATA2. Consistent with our observation, Masuda *et al.* (2007) reported inhibition of IgE-dependent degranulation by GATA factor repression in mast cells derived from mouse bone marrow. Our present study shows that PLC- γ 1 is a novel GATA factor target that is responsible for the GATA factor-mediated control of degranulation.

GATA-binding sites have been found in the regulatory regions of several genes expressed in mast cells. These include mast cell carboxypeptidase A (Zon *et al.*

1991), the α and β subunits of Fc ϵ RI (Nishiyama *et al.* 1999; Maeda *et al.* 2003), IL-4 (Kwan *et al.* 2005) and IL-13 (Masuda *et al.* 2004). We initially assumed that the reduced degranulation by GATA factor repression might be owing to the down-regulation of Fc ϵ RI α and β . However, the cell surface expression of Fc ϵ RI, as well as the mRNA levels of Fc ϵ RI α and β , was unaffected by GATA factor repression. Interestingly, we showed by ChIP assay that GATA1 and GATA2 bind to the Fc ϵ RI β promoter region in RBL-2H3 cells (Fig. 5B). Collectively, these results suggest that GATA factors might contribute to Fc ϵ RI β (and

presumably *FcεRIα*) gene transcription in part, but are dispensable in maintaining the physiological expression level of *FcεRIβ* in RBL-2H3 cells. Thus, the *in vivo* requirement for GATA factors in the expression of previously reported target genes should be assessed carefully in different cellular contexts.

Transcriptional regulation of the *PLC-γ1* gene remains largely unexplored. A previous study demonstrated that a positive regulatory region containing at least three protein binding sites resides approximately 500 bp upstream from the transcription start site of the rat *PLC-γ1* gene (Lee *et al.* 1995). However, this region does not contain a GATA consensus motif. The present data illustrate that GATA factors activate *PLC-γ1* gene transcription through conserved GATA-binding motifs A and B in the 5'-upstream sequence. Transient transfection assays demonstrated that the repression of endogenous GATA factors declined reporter activity for the distal GATA-binding motif B in RBL-2H3 cells, whereas the reporter activity for the proximal GATA-binding motif A was activated only when GATA factors are exogenously overexpressed in QT6 fibroblasts (Fig. 5C,E). Consistent with these findings, mutations in the GATA-binding motif B, but not motif A, significantly attenuated the basal reporter activity (Fig. 5D). Furthermore, ChIP assays demonstrated that both GATA factors bind to the GATA-binding motif B chromatin region, whereas only GATA1 binds to the GATA-binding motif A (Fig. 5B), despite that both GATA factors contribute to the *PLC-γ1* gene expression in knockdown experiments (Fig. 4A,B). Collectively, these findings indicate that the GATA-binding motif B plays a major role for the GATA factor-mediated *PLC-γ1* gene transcription in RBL-2H3 cells. Notably, the sequence of distal GATA-binding motif B closely resembles the extended GATA consensus sequence (C/G)(A/T)GATAA(G/A/C)(G/A/C) that was most frequently occupied by GATA1 in a genome-wide ChIP-sequence analysis (Fujiwara *et al.* 2009).

We found that the *PLC-γ1* expression is regulated by GATA factors not only in RBL-2H3 cells but also in BMMCs. Interestingly, whereas GATA1 and GATA2 contributed almost equally to *PLC-γ1* gene expression in RBL-2H3 cells, GATA2 dominantly acts over GATA1 in BMMCs. In a separate study, we observed that the mRNA levels of GATA2 and several mast cell-specific genes such as mouse mast cell carboxypeptidase, mouse mast cell protease 5 and c-kit were increased during the culture of BMMC, whereas the GATA1 mRNA level remained

unchanged during the culture period (S. Ohmori, Y. Ishijima, K. Ohneda, unpublished observation). Taken together with the results from the present study, these findings raise the possibility that GATA2 might have a major role for maintaining mast cell-specific function in BMMCs. Further studies need to be carried out to fully understand the precise roles of GATA1 and GATA2 in the regulation of mast cell-specific genes.

Although GATA factors have not been implicated in *PLC-γ1* gene regulation, there are striking overlaps in the phenotypes of *Gata1*, *Gata2* and *PLC-γ1* knockout mice. The *PLC-γ1*^{-/-} genotype is embryonically lethal around day 9.0 owing to growth retardation (Ji *et al.* 1997; Shirane *et al.* 2001). There was an absence of *PLC-γ1*^{-/-} cells in the hematopoietic tissues of chimeric mice developed from *PLC-γ1*^{-/-} ES cells (Shirane *et al.* 2001). These results suggest that *PLC-γ1* plays an essential role in the early stage of hematopoietic development, overlapping with the role of GATA2 in embryonic development (Tsai *et al.* 1994; Tsai & Orkin 1997). Furthermore, in the absence of *PLC-γ1*, erythroid cell differentiation was severely impaired, whereas granulocyte/monocyte differentiation was not completely abrogated, as observed in colony-forming assays of ES cells, a yolk sac and an embryonic body *in vitro* (Shirane *et al.* 2001; Liao *et al.* 2002). A similar phenotype was observed in experiments using *Gata1*-null embryonic stem cells (Weiss *et al.* 1994; Weiss & Orkin 1995). Thus, it is most likely that *PLC-γ1* is a critical target for GATA factors in all hematopoietic lineages and plays vital roles in hematopoietic development.

Our study has demonstrated that the reduced *PLC-γ1* expression resulting from GATA factor repression attenuates the sustained extracellular Ca²⁺ influx, whereas the preceding intracellular Ca²⁺ release from the ER remains the same. Similar results were observed when *PLC-γ1* expression was reduced directly by specific siRNA. This might be explained by the differential contributions of *PLC-γ1* to the two phases of Ca²⁺ mobilization. The release of Ca²⁺ from the ER is triggered by IP₃, a product of *PLC-γ1*-mediated PIP₂ hydrolysis. So, the lipase activity of *PLC-γ1* is essential for controlling the first phase. The ensuing sustained Ca²⁺ influx is primarily induced by depletion of the ER Ca²⁺ store, a process termed 'store-operated Ca²⁺ entry (SOCE)'. However, recent studies have shown that the second phase is modulated by additional Ca²⁺ entry routes that are not necessarily dependent on exhaustion of the Ca²⁺ store (Kalesnikoff & Galli 2008). Notably, a lipase-inactive mutant of

PLC- γ 1 can induce Ca^{2+} entry in response to an agonist in neuronal cell lines (Patterson *et al.* 2002). Moreover, PLC- γ 1 enhances Ca^{2+} entry by interacting directly with transient receptor potential channel 3 (TRPC3), which is a member of the cationic channel family (van Rossum *et al.* 2005). These data suggest that PLC- γ 1 controls extracellular Ca^{2+} influx in a variety of ways. Thus, it is likely that the reduced level of PLC- γ 1 is sufficient for Ca^{2+} release from the ER, whereas a higher level of PLC- γ 1 might be required for a sustained extracellular Ca^{2+} influx. In addition, it is possible that PLC- γ 2, which is also involved in Fc ϵ RI-mediated mast cell degranulation (Wang *et al.* 2000), might compensate partly for PLC- γ 1. In fact, the decrease of β -hexosaminidase release by repression of GATA factors was smaller than the reduction of PLC- γ 1 expression in RBL-2H3 cells (Figs 1B and 4A,B) as well as in BMMCs (Fig. 7C,D). This discrepancy can partly be explained by the substitution of PLC- γ 2. Alternatively, the phosphorylated form of PLC- γ 1 that is induced by antigen stimulation in the GATA factor knockdown cells (Fig. S1B in Supporting Information) might be sufficient to support the Ca^{2+} mobilization in these cells.

Besides the GATA factors, the hematopoietic transcription factors SCL/Tal1 and PU.1 have been shown to play essential roles in mast cell differentiation (Walsh *et al.* 2002; Salmon *et al.* 2007). The regulatory networks of multiple factors to control mast cell-specific genes might be different from those in other cell lineages. For instance, although GATA1 and PU.1 act reciprocally in the lineage determination of hematopoietic stem/progenitor cells (Arinobu *et al.* 2007), a co-operative interplay between PU.1 and the GATA factors has been reported in mast cells (Henkel & Brown 1994; Walsh *et al.* 2002; Takemoto *et al.* 2010). The unveiling of multiple transcription factor circuits controlling the expression of degranulation-related genes should provide fruitful insights into mast cell biology as well as advancing therapy for allergic diseases.

Experimental procedures

Cell culture

RBL-2H3 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in minimum essential medium supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. Cells were passaged at 60–70% confluence with trypsin in phosphate-buffered saline (PBS). Quail fibrosarcoma cells (QT6 cells) were cultured in Dulbecco's modified Eagle's

medium with 1.0 g/L glucose supplemented with 10% FBS, penicillin and streptomycin. BMMCs were obtained from bone marrow harvested from 6- to 8-week-old C57BL/6 mice. The cells were cultured for 2 weeks in RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin in the presence of 10 ng/mL recombinant murine IL-3 (PeproTech), and subsequently cultured for 2 weeks with IL-3 and 10 ng/mL recombinant murine stem cell factor (PeproTech). Maturation of mast cells was verified by the expression of c-Kit and Fc ϵ RI on a flow cytometer (FACSCantoII; BD Biosciences).

Plasmids

The expression plasmids for GATA1 and GATA2 were generated previously (Shimizu *et al.* 2001) using a pEF-BOS vector (Mizushima & Nagata 1990). For construction of the PLC- γ 1 expression plasmid, PLC- γ 1 cDNA was synthesized from RBL-2H3 cells by RT-PCR using Superscript III First-Strand Synthesis Supermix (Invitrogen) and *Pfu* DNA polymerase (Stratagene) and inserted into a pBAT12 vector (Ohneda *et al.* 2000). For making the luciferase reporter plasmids, the DNA fragments containing GATA-binding motifs A and B of the 5'-upstream sequences of the rat *PLC- γ 1* gene were amplified by PCR with the following primers: 5'-GAG AGA GGT ACC ACA CGG ATT CAG GTC CCC AC-3' (forward) and 5'-GAG AGA GAG CTC GTT CAG ACA ACG CAC CCA GC-3' (reverse) for GATA-binding motif A; 5'-GAG AGA GGT ACC TTC ATA TGT GAG ATG GGT GC-3' (forward) and 5'-GAG AGA GAG CTC AAC CAC ATG GAG AAC CAT CC-3' (reverse) for GATA-binding motif B. The PCR products were then cloned into the *KpnI/SacI* site of the pGL3-promoter vector (Promega) for a firefly luciferase reporter. The luciferase reporter vectors harboring the mutation in the GATA-binding motifs were constructed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. The PCR primers used for site-directed mutagenesis were as follows: 5'-GGC AGT CAG TCG CTC TCA ACT TCC-3' (forward) and 5'-GGA AGT TGA GAG CGA CTG ACT GCC-3' (reverse) for GATA-binding motif A; 5'-CAA TGT GCT TTA GCC ACA TTG GAT GG-3' (forward) and 5'-CCA TCC AAT GTG GCT AAA GCA CAT TG-3' (reverse) for GATA-binding motif B. The sequences of the constructed vectors were all confirmed by DNA sequencing.

Transfection of small interfering RNA (siRNA)

The siRNA duplexes for rat and mouse, GATA1, GATA2 and PLC- γ 1, were purchased from Thermo Scientific (rat) and Invitrogen (rat and mouse). The control siRNA was purchased from Sigma. RBL-2H3 cells or BMMCs (2.0×10^6 cells) were transfected with 200 pmol of siRNA by electroporation using an Amaxa Nucleofector (Lonza). Cells were harvested 24 h after transfection and used for the analyses. In some experiments,

siRNA was co-transfected with 5 µg of the expression plasmids containing PLC-γ1 cDNA.

Real-time quantitative RT-PCR analysis

Total RNA was extracted from RBL-2H3 cells and BMMCs with Isogen (Nippon Gene) and NucleoSpin RNA II (TaKaRa), respectively. Reverse transcription reactions were carried out using Superscript III First-Strand Synthesis Supermix as outlined by the manufacturer. Synthesized cDNAs were analyzed in triplicate by an Mx3000P real-time PCR system (Stratagene) with GoTaq qPCR Master Mix (Promega). PCR conditions were one cycle of 95 °C for 2 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Results were normalized to the GAPDH levels. The PCR primers used for RT-PCR analysis were as follows: 5'-AGC GGA GCC CCT TAA CTT TG-3' (forward) and 5'-CTC TCA CCA ACA GCG CTT GC-3' (reverse) for β-hexosaminidase; 5'-AGA GCA AAC CTG TGT ACT TG-3' (forward) and 5'-GTG GAC TTT CCA TTT CTT CC-3' (reverse) for FcεRIα; 5'-AGG CTA CCC ATT CTG GGG TG-3' (forward) and 5'-GGC TGC CTC TCA CCA GAT AC-3' (reverse) for FcεRIβ; 5'-CTC CTT TTG GTG GAA GAA GC-3' (forward) and 5'-TGA GTC GAC AGT AGA GCA GG-3' (reverse) for FcεRIγ; 5'-GCC AGT TCC TGA ATC TCA AC-3' (forward) and 5'-TCG TCT GGG TGG ATG CCA TC-3' (reverse) for Lyn; 5'-TGG CAA CAT CTC CAG AGA CG-3' (forward) and 5'-GTG CAA AGG AGC CGT TGT TG-3' (reverse) for Syk; 5'-TAC CAC ATT GAT GGC GCT CG-3' (forward) and 5'-CTT ACT GAG AAT CGG CAT CC-3' (reverse) for SLP-76; 5'-AAC TGC TTA ACA ACC TGC TC-3' (forward) and 5'-GCA AGT AGA CAG GAA GGT TC-3' (reverse) for Vav1; 5'-CGG ACA AAA TCG AGG GGT CC-3' (forward) and 5'-GCA GGG TCT TCT TGG TAG CG-3' (reverse) for PLC-γ1; 5'-TAC GAC CCC TTC CGT CTG TG-3' (forward) and 5'-TCC GCC CAT TGA GCG AAA AC-3' (reverse) for PLC-γ2. For each, a single PCR product was confirmed by 2% agarose gel electrophoresis and melting-curve analysis.

Degranulation assay

Degranulation was determined by β-hexosaminidase release. RBL-2H3 cells or BMMCs were plated at a density of 1.0×10^5 cells in a 24-well plate 24 h after transfection. Cells were sensitized in medium supplemented with 0.5 µg/mL monoclonal anti-dinitrophenyl (DNP) murine IgE antibody (clone SPE-7; Sigma) overnight, washed with PBS twice and stimulated with 200 ng/mL DNP-BSA (Calbiochem) in Tyrode buffer (25 mM HEPES, 140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 0.49 mM MgCl₂, 5.6 mM D-glucose, 12 mM NaHCO₃, 0.37 mM NaH₂PO₄ and 0.1% BSA) at 37 °C for 30 min. For FcεRI-independent stimulation, cells were stimulated with 1 µM A23187 in Tyrode buffer at 37 °C for 30 min. After incubation, supernatants were collected and cells were solubilized with Tyrode buffer containing 0.5% Triton

X-100. The supernatants and cell lysates were incubated with 2 mM N-acetyl-β-D-glucosaminide (Sigma) in 0.1 M citrate buffer, pH 4.5, at 37 °C for 30 min. The reaction was terminated with 0.2 M glycine-NaOH buffer, pH 10.7, and the absorbance at 405 nm was measured. Degranulation was calculated as β-hexosaminidase release (%) = supernatant activity/(supernatant + cell lysate) activity × 100.

Intracellular Ca²⁺ measurement

IgE-sensitized RBL-2H3 cells were resuspended in culture medium containing 1 µM fura 2-AM (Nacalai Tesque) and incubated at 37 °C for 30 min. For the measurement of Ca²⁺ release from intracellular stores, the cells were washed with Tyrode buffer, resuspended in Ca²⁺-free Tyrode buffer containing 1 mM EGTA and stimulated with 200 ng/mL DNP-BSA. Fluorescence was monitored by an F-2500 Fluorescence Spectrophotometer (Hitachi). The measurement was carried out at 37 °C with constant stirring and the excitation wavelengths of fura 2 were 340 and 380 nm. For the measurement of Ca²⁺ influx from the extracellular environment, cells loaded with fura 2 were resuspended in 1.8 mM Ca²⁺-containing Tyrode buffer and stimulated with 200 ng/mL DNP-BSA and fluorescence was measured by spectrofluorometry.

Flow cytometry

When 24 h had passed following transfection, RBL-2H3 cells were incubated with monoclonal anti-DNP murine IgE antibody in PBS containing 2% FBS at 4 °C for 30 min. Cells were stained with the secondary antibody FITC-conjugated anti-mouse IgE (BD Biosciences) in PBS containing 2% FBS at 4 °C for 30 min. After washing with PBS, cell fluorescence was analyzed by FACSCanto II (BD Biosciences).

Western blot analysis

Whole cell extracts were prepared from RBL-2H3 cells 24 h after the siRNA transfection. The cell extracts were separated on 8% SDS-polyacrylamide gel electrophoresis and electroblotted to a polyvinylidene difluoride membrane. The membrane was incubated overnight at 4 °C with a primary antibody, followed by an incubation for 1 h at room temperature with the corresponding peroxidase-conjugated anti-IgG as a secondary antibody. Primary antibodies used were as follows: anti-GATA1 (N6; Santa Cruz), anti-GATA2 (H116; Santa Cruz), anti-PLC-γ1 (#2822; Cell Signaling), anti-phospho-PLC-γ1 (#2821; Cell Signaling) and anti-β-tubulin (clone TUB 2.1; Sigma). The detection was carried out with an Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Chromatin immunoprecipitation assay

A total of 2.0×10^6 RBL-2H3 cells per immunoprecipitation (IP) were fixed with 0.4% formaldehyde at room temperature

for 10 min and the fixation was stopped with 125 mM glycine for 5 min. Cross-linked cells were washed twice with cold PBS, resuspended in SDS cell lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% SDS, 1 mM EDTA and protease inhibitor cocktail) and sonicated four times for 15 s using a TOMY UD-201 sonicator (Output: 3; Duty: 50) to generate chromatin fragments. Lysates were centrifuged at 21,500 *g* for 5 min and the supernatants were collected and five times diluted with ChIP dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail). The diluted samples were used for IP with a rat monoclonal antibody for GATA1 (N6; Santa Cruz) or a rabbit polyclonal antibody for GATA2 (H116; Santa Cruz). To prepare antibody-bead complexes, 15 μ L of Dynabeads-Protein A (Invitrogen) was equilibrated with PBS for 30 min at 4 °C. For the GATA1 ChIP assay, the beads were resuspended in 675 μ L of PBS containing 50 μ g/mL BSA and 5 μ g of anti-rat IgG rabbit antibody (Jackson ImmunoResearch) and incubated on a rotator at 4 °C for 2 h. The beads were then washed twice with PBS, resuspended in 675 μ L of PBS containing 50 μ g/mL BSA and 1 μ g of anti-GATA1 antibody and incubated on a rotator at 4 °C for 2 h. After incubation, the antibody-bead complexes were washed twice with ChIP dilution buffer. For the GATA2 ChIP assay, 2 μ g of anti-GATA2 antibody was conjugated directly to Dynabeads-Protein A without a secondary antibody. The prepared antibody-bead complexes were mixed with the diluted sample and incubated on a rotator at 4 °C overnight. The immune complexes were washed twice with ChIP dilution buffer, once with wash buffer I (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail), once with wash buffer II (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 0.25 M LiCl, 0.5% Deoxycholate, 0.5% NP-40 and protease inhibitor cocktail) and twice with TE buffer. After washing, the immune complexes were resuspended in 300 μ L of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% SDS). Reverse cross-linking was carried out by incubating the samples at 65 °C for 3 h with Proteinase K. The DNA fragments were extracted with phenol-chloroform, precipitated with ethanol and resuspended in TE buffer. Purified DNA from IP was analyzed in triplicate using an Mx3000P real-time PCR system with GoTaq qPCR Master Mix. PCR conditions were one cycle of 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The results obtained from IP using anti-GATA1 antibody or anti-GATA2 antibody were normalized with the results from input DNA without IP. The PCR primers used for the ChIP assays were as follows: 5'-TGT CAC TAG GTG GCA GTC AG-3' (forward) and 5'-GCT GTT TCC CTT GAG GGT GG-3' (reverse) for GATA-binding motif A; 5'-CAG GCA AAG CAC TCA TAC AC-3' (forward) and 5'-CAC TGC TTC TGA AAG CCC AG-3' (reverse) for GATA-binding motif B; 5'-ACT GAT ATC CAT CAG CCT GGA GAC-3' (forward) and 5'-GGC AGT TAA GAT GGG TTG ACT C-3' (reverse) for Fc ϵ RI β promoter; 5'-GTT CCT CCA GTA CAA CCG GC-3' (forward)

and 5'-GCA GAT CCA CAT GGG CAG AG-3' (reverse) for PLC- γ 1 exon 25. These primer pairs amplified a single product that was confirmed by 2% agarose gel electrophoresis and melting-curve analysis.

Luciferase reporter assay

QT6 cells were plated at a density of 2.0×10^5 cells per transfection in a 12-well plate 24 h before transfection. The cells were co-transfected with various combinations of a firefly luciferase reporter vector (wild type or mutant), a *renilla* luciferase control reporter vector (pRL-TK; Promega) and the expression vector for either GATA1 or GATA2 using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. In some experiments, 2.0×10^6 RBL-2H3 cells were co-transfected by electroporation with a firefly luciferase reporter vector, a *renilla* luciferase control reporter vector and siRNA for GATA1, GATA2 or control and seeded in a 12-well plate. RBL-2H3 and QT6 cells were solubilized 24 and 48 h, respectively, following transfection. Cell lysis and measurement of the luciferase activity were carried out by the Dual-Luciferase Reporter Assay System (Promega) according to the supplied protocol.

Statistical analysis

Comparison between two groups was carried out using the Student's *t*-test. Data are presented as means $\pm$ SE. For all analyses, statistical significance is defined as a *P*-value of <0.05 .

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Supporting Information/Supplementary material

The following Supporting Information can be found in the online version of the article:

Figure S1 The expression of GATA1, GATA2 and PLC- γ 1 in RBL-2H3 cells with and without IgE-dependent antigen stimulation.

Additional Supporting Information may be found in the online version of this article.

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Supporting Information

Figure. S1.

The expression of GATA1, GATA2 and PLC- γ 1 in RBL-2H3 cells with and without IgE-dependent antigen stimulation. (A) The mRNA levels of GATA1, GATA2 and PLC- γ 1 were determined by real-time quantitative RT-PCR in RBL-2H3 cells stimulated with antigen. Cells were sensitized with anti-DNP IgE for 3 h and total RNA was isolated at 0, 10 and 30 min following the antigen stimulation. Values are shown as means $\pm$ SE from 3 independent experiments. (B) The protein levels of total and phosphorylated PLC- γ 1 in RBL-2H3 cells treated with and without IgE-dependent antigen stimulation. Cells were transfected either control or GATA1 and GATA2 siRNAs 24 h prior to the 3 h sensitization with anti-DNP IgE. Whole cell extract was isolated from cells stimulated with DNP-BSA for 30 min (+), or from cells without the antigen stimulation (-). Immunoblot with anti- β -tubulin antibody was used as a loading control.

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