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IgG1 cytoplasmic tail is essential for cell surface expression in Igβ down-regulated cells

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ABSTRACT

It has been shown that cytoplasmic tail of the IgG1 B cell receptors (BCRs) are essential for the induction of T-dependent immune responses. Also it has been revealed that unique tyrosine residue in the cytoplasmic tail of IgG2a has the potential of being phosphorylated at tyrosine and that this phosphorylation modulates BCR signaling. However, it still remains unclear whether such phosphorylation of IgG cytoplasmic tail is involved in the regulation of BCR surface expression. In order to approach the issue, we established and analyzed the cell lines which express wild-type or mutated forms of IgG1 BCR. As the result, we found that IgG1 BCR expressed normally on the surface of A20 B cell line independent of the cytoplasmic tail. In contrast, IgG1 BCR whose cytoplasmic tyrosine was replaced with glutamic acid which mimics phosphorylated tyrosine, was expressed most efficiently on the surface of non-B lineage cells and Igβ-down-regulated B cell lines. These results suggest that tyrosine residue in IgG cytoplasmic tail is playing a essential role for the efficient expression of IgG BCR on the cell surface when BCR associated signaling molecules, including Igβ, are down-regulated.

1. Introduction

It is well known that expression of IgM on the cell surface of B cells is essential for the B cells to survive and respond to the antigen stimulation [1–5]. Utilizing cell lines, it was clearly revealed that concomitant expression of Igα and Igβ is strictly required for IgM to be expressed on the cell surface [6]. Also it has been revealed that Igα/Igβ heterodimer is playing pivotal roles in the expression of IgM in primary B cells [1]. However, due to the small number of IgG^+ B cells *in vivo*, whether IgG B cell receptors (BCRs) also require Igα/Igβ heterodimer for their cell surface expression is yet to be solved.

One of the characteristic structure of IgG BCR compared to IgM BCR is its cytoplasmic tail. IgG BCR has cytoplasmic tail which consists of 28 amino acids in contrast to IgM BCR, which has only 3 amino acids. It has been reported that cytoplasmic tail of IgG1 is playing a pivotal role in both primary and secondary IgG1 responses [7]. However, how the cytoplasmic tail is exerting its function in the immune responses has long been remained to be revealed. Recent study provided a new insight into the function of IgG cytoplasmic tail [8]. Cell line which expresses the IgG2a BCR with Y to F substitution in the immunoglobulin tail tyrosine (ITT) motif within the cytoplasmic tail was mainly analyzed [9]. As the result, they found that tyrosine residue in the ITT motif is phosphorylated following the cross-linking of surface IgG2a and that the adaptor protein Grb2 is recruited to the phosphorylated ITT motif. In addition, BCR-mediated calcium response was attenuated to some extent in YF mutant. These data indicate the role of ITT motif in modulating the IgG BCR-mediated signaling.

On the other hand, consensus sequence for the ITT motif, asparagine and/or methionine residues at the C-terminal positions +2 and +3 relative to the tyrosine residue [9], also fits the Yxxψ motif, YVKM, in CTLA-4 cytoplasmic tail [10]. It has been reported that Yxxψ motif in CTLA-4 regulates the surface expression of this molecule through association with μ2 subunit of AP-2, the clathrin-associated complex [11,12]. These results suggest the possible involvement of ITT motif also in the regulation of surface IgG expression. However, to date, only brief experiments using fibroblasts have been carried out to reveal the machinery which governs the cell surface expression of IgG [13]. Hence, the detailed regulatory mechanism for the cell surface expression of IgG by its cytoplasmic tail is remained to be clarified, including the involvement of ITT motif.

In this study, we found that the cytoplasmic tail of IgG1 is strictly required for the surface expression of IgG1 when the expression of Igα/Igβ heterodimer is down-regulated in B lymphoma, and that ITT motif with phosphotyrosine mimicry

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mutation greatly enhanced the cell surface expression of IgG1, which strongly suggests the involvement of ITT motif in the regulatory mechanism of cell surface IgG1 expression.

2. Materials and methods

2.1. Construction of GFP-Fcγ1 expression vectors

GFP-Fcγ1 expression vector was constructed by PCR as follows. First, the leader sequence from Vh186.2 was amplified and connected to EGFP by PCR. On the other hand, cDNA encoding hinge region to the cytoplasmic domain of IgG1 was amplified also by PCR. Resultant fragments were connected by PCR to obtain full length GFP-Fcγ1 cDNA using primer pair IGG1L-F (CACCATGGGATGGAGCTGTATCATGCTCTT)/IGG1WT-R (CTAGGGCGCTTGCCCAATCATGTTCTTGTATTCAGGAACCAGTGT). For generation of TL-IgG1, YE-IgG1 and YA-IgG1, PCRs were carried out using GFP-Fcγ1 cDNA as a template using primer pairs IGG1L-F/IGG1YE-R (CTAGGGCGCTTGCCCAATCATGTTCTTCTCTTCAGGAACCAGTGT) for YE-IgG1, IGG1L-F/IgG1YA-R (CTAGGGCGCTTGCCCAATCATGTTCTTGGCTTCAGGAACCAGTGT) for YA-IgG1 and IGG1L-F/IGG1TL-R (CTAGAAGAGTGTGACAGCAGC) for TL-IgG1, respectively in combination with IGG1L-F and IGG1WT-R.

2.2. Cell lines and transfection of the cell

GP2-293 cells, a HEK293 derived cell line, were purchased from Clontech. A20 cells with a single copy of FRT, Flp recombinase target site, were obtained by transfection of pFRT/LacZeo (Invitrogen) and subsequent isolation of Zeocine resistant clones. The clones which highly express LacZ were selected and further confirmed for the integration of single copy of pFRT/LacZeo by Southern blotting. One of these selected clones was designated as Flp-In A20 and further used. This cell line was co-transfected with 2 μg of pEF5/FRT-based GFP-Fcγ1 expression vector and 18 μg of Flp recombinase expression vector, pOG44 (Invitrogen), by electroporation. Cells were transfected using Nucleofector II (Lonza) according to the manufacturer's instruction. For the suppression of Igβ expression, several siRNA fragments were purchased (Ambion) and checked for their ability to suppress the Igβ expression. RT-PCR analysis was carried out as described elsewhere [14] using 5′-GTGAGCAAGGGCGAGGAGCT-3′ and 5′-CTTGTACAGCTCGTCCATGCCGAGAG-3′ for the amplification of GFP-Fcγ1 cDNA.

2.3. FACS analysis

All cell lines were stained with various combinations of anti-IgG1-APC (BD Biosciences), anti-CD79b-PE (BD Biosciences) and anti-IgM-FITC (BD Biosciences) following manufacturer's instruction and analyzed with FACSAria II or FACSAriaIII (BD Biosciences).

2.4. Immunoprecipitation and Western blot analysis

Cell lysates were obtained by suspending 5×10^6 cells with M-PER reagent according to the manufacturer's instruction. Obtained lysates were immunoprecipitated with anti-IgG1 (BD Biosciences) in combination with Protein G coated magnetic beads (Invitrogen) according to the instruction. Immunoprecipitates were resuspended in M-PER reagent with SDS-sample buffer (Invitrogen) supplemented with 1/10 volume sample reducing agent (Invitrogen). Then the samples were heated at 95 °C for 3 min and subjected to SDS–PAGE. After the electrophoresis, proteins were transferred to the membrane using iBlot device (Invitrogen) according to the instruction. Obtained membrane was blocked with Blocking One (Nacalaitesque), blotted with anti-Grb2 antibody (Cell Signaling Technologies) according to the manufacturer's instruction and incubated with HRP-labeled anti-rabbit IgG (Cell Signaling Technologies) followed by chemiluminescent detection with Pierce Western Blotting Substrate (PIERCE) using LAS4000miniPR (Fujifilm). Intensity of the obtained signals were enumerated by ImageJ 1.43u (NIH).

3. Results and discussion

3.1. Expression of cell surface IgG1 in non-B lineage cells and B lymphoma

In order to examine the possible involvement of ITT motif in the cell surface expression of IgG, we decided to construct a GFP-Fcγ1 which can be expressed on the cell surface independent of Ig light chain. Also transfection with these constructs enables to identify the cells which were introduced with the expression vector by monitoring EGFP fluorescence. Utilizing GFP-Fcγ1, we can easily assess the efficiency of surface IgG1 expression by comparing the proportion of surface IgG1 BCR within the $EGFP^+$ population. Thus, we generated GFP-Fcγ1 expression vectors with 4 types of cytoplasmic tail: unmutated (wt-IgG1), cytoplasmic tail with only 3 amino acids, KVK, (TL-IgG1), cytoplasmic tail with tyrosine to glutamic acid substitution, which mimics the phosphorylated ITT mmotif (YE-IgG1), and tyrosine to alanine substituted mutant (YA-IgG1) (Fig. 1A) [15,16].

When these constructs were transfected to A20 B cell line, similar levels of wt-IgG1, TL-IgG1 and YE-IgG1 expression were observed suggesting that cytoplasmic tail is not essential for B cells to express IgG1 on their cell surface when Igα/Igβ heterodimer is expressing normally (Fig. 1B). Previously, it has been shown histologically that IgG1 can be expressed on the cell surface of fibroblast which does not express any B cell related molecules including Igα and Igβ [13]. However, quantitative analysis to reveal the efficiency of IgG to be expressed on the cell surface was yet to be examined. Thus, we decided to examine the surface levels of IgG1 by flow cytometer in GFP-Fcγ1 transfected GP2-293 cells (Fig. 1C). As the result, we found that IgG1 was expressing on the cell surface to some extent (13.5%) in the cells transfected with wt-IgG1. In contrast, although comparable proportion of the cells were EGFP+, we could only detect 2.6% of the cells expressing surface IgG1 in TL-IgG1 transfected cells. When the cells were transfected with YE-IgG1, proportion of surface IgG1+ cells increased to 33.0%, which is significantly higher than the wt-IgG1. These results strongly suggest that ITT motif in the IgG1 cytoplasmic tail not only modulates the BCR-mediated signaling, but also playing a pivotal role in the modulation of IgG surface expression at least in non-B lineage cells.

3.2. Expression of cell surface IgG1 in Igβ down-regulated B lymphoma

It is widely known that concomitant expression of Igα/Igβ heterodimer is required for the expression of IgM on cell surface. Thus, we speculated that the differences in the expression patterns of surface IgG1 between Ramos and GP2-293 might be due to the lack of Igα/Igβ heterodimer in GP2-293 cells.

In order to clarify whether such results can be only observed in non-lymphoid cells or also detected in B cells, we first tried to suppress the expression of Igα/Igβ heterodimer by transfection of Igβ siRNA to mimic the fibroblast environment (Fig. 2A and B). To resemble the IgG-harboring B cells, we decided to use IgG2a+ A20 B cell line.

First we assessed the ability of chosen siRNA to suppress the expression of Igβ in A20 cells. As shown in Fig. 2A, when A20 cells were transfected with Igβ siRNA, we found that both transcription

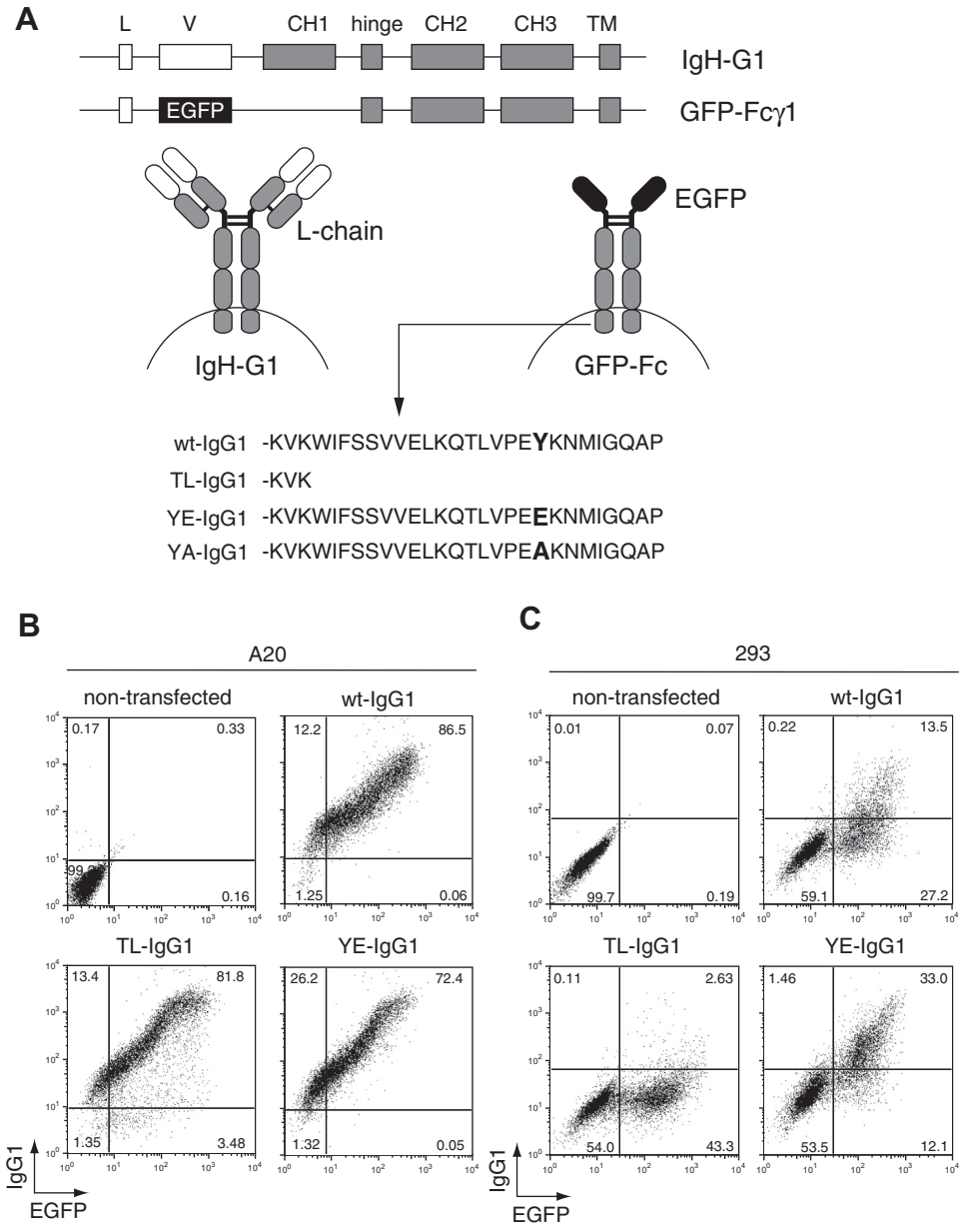

Fig. 1. Expression of cell surface IgG1 in non-B lineage cells and B lymphoma. (A) Schematic diagrams of wild type GFP-Fcγ1 construct, and YA, TL and YE mutants. TL mutant has cytoplasmic tail with only 3 amino acids; KVK, YE mutant has cytoplasmic tail with Y to E mutation and YA mutant with Y to A mutation, which indicated by the bold letters (top). GFP-Fcγ1 can be expressed on cell surface without light chain (middle). GFP-Fcγ1 constructs with three types of cytoplasmic tails were generated as indicated (bottom). (B) Expression of GFP-Fcγ1 in A20 B cells. A20 B cells were transfected with indicated GFP-Fcγ1 expression vectors and the expression levels of EGFP and surface IgG1 were analyzed. (C) Expression of GFP-Fcγ1 in GP2-293 cells. GP2-293 cells were transfected with indicated GFP-Fcγ1 expression vectors and the expression levels of EGFP and surface IgG1 were analyzed.

and surface expression of Igβ were suppressed by almost 5-fold compared with the cells transfected with control siRNA. This result clearly indicates the effectiveness of selected Igβ siRNA in A20 cells. Next, we established the A20 cell lines which express exogenous wt-IgG1 and YE-IgG1 from the unique loci, which made it possible to compare the expression levels of surface IgG1 directly between these cell lines without chromosome effects (see Section 2). When wt-IgG1 and YE-IgG1 expressing A20 cells were transfected with Igβ siRNA, expression level of wt-IgG1 on the cell surface was markedly suppressed. In contrast, surface IgG1 level in YE-IgG1 expressing cells was comparable to those in the cells which received control siRNA (Fig. 2B). This result clearly indicate that YE mutation in the ITT motif affects the expression level of surface IgG1 when surface Igβ level is down-regulated.

Since the kinase responsible for the phosphorylation of tyrosine residue in the ITT motif has not been identified, there exists a possibility that intracellular signaling events that may affect the surface expression of IgG might differ in A20 cells and Ramos B cells in which phosphorylation of ITT motif has been detected [8]. Thus, we established a Ramos cell line with down-regulated Igβ level (Fig. 2C). In this IgβLo Ramos cell line, expression of endogenous IgM on the cell surface was markedly diminished in parallel with Igβ down-regulation indicating that Igβ is strictly required for IgM to be expressed on cell surface as expected.

When wt-IgG1 construct was transiently transfected to IgβLo Ramos cells, it was found that more than 90% of transfected cells were $EGFP^+$ and 71% were surface $IgG1^+$ suggesting that IgG1 has the ability to be expressed on cell surface even when Igβ

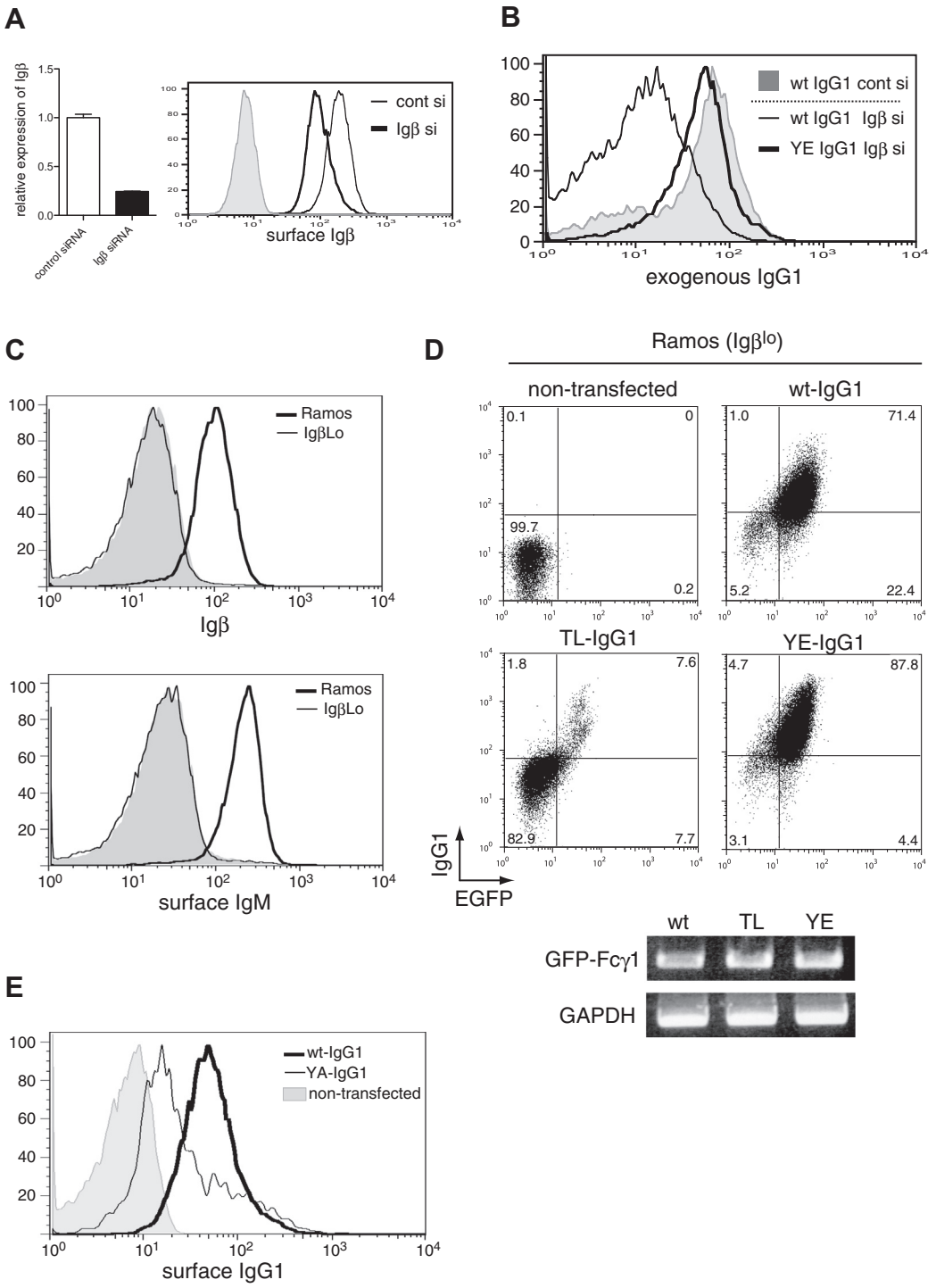

Fig. 2. Expression of cell surface IgG1 in Igβ down-regulated B lymphoma. (A) Analysis of cell surface Igβ expression in siRNA-transfected A20 cells. Total RNA samples were obtained from control siRNA-transfected A20 cells (open bar) and Igβ siRNA-transfected A20 cells (closed bar), and quantitative RT-PCR analysis was performed for each samples (left). A20 cells were transfected with control siRNA (cont si; thin line) and Igβ siRNA (Igβ si; thick line), respectively. The cells were cultured for 48 h and stained with antibodies to Igβ and analyzed by flow cytometer (right). (B) Cell surface expression of IgG1 in A20 cells transfected with siRNA for Igβ. Stable transformants of A20 cells expressing wild type or YE mutant IgG was generated using Flp-In system. A20 cells transfected with Igβ siRNA were analyzed for the surface expression of wild type (wt IgG1 Igβ si; thin line) or YE-mutant (YE IgG1 Igβ si; thick line) IgG1. Shaded histogram indicates the A20 cells expressing wild-type IgG1 transfected with control siRNA (wt IgG1 cont si). These analyses were performed 48 h after the transfection of siRNA. (C) Surface expression of Igβ (top) and IgM (bottom) in the newly established IgβLo Ramos B cell line. Shaded lines indicate isotype matched control. (D) Cell surface expression of IgG1 in IgβLo Ramos B cell line. IgβLo Ramos B cells were transfected with indicated GFP-Fcγ1 expression vectors and the expression levels of EGFP and surface IgG1 were analyzed (top). Total RNA was extracted from IgβLo Ramos B cell lines expressing wt-IgG1, TL-IgG1 and YE-IgG1, respectively. Obtained RNA was subjected to RT-PCR to detect expression levels of GFP-Fcγ1 (bottom). (E) Cell surface expression of YA-IgG1 in IgβLo Ramos B cell line. IgβLo Ramos B cells were transfected with either wt-GFP-Fcγ1 or YA-GFP-Fcγ1 expression vectors and the expression levels of surface IgG1 in EGFP+ cells were analyzed. Mean fluorescence intensities for each histogram were 6.7 for non-transfected cells, 64.9 for wt-IgG1 expressing cells and 36.5 for YA-IgG1 expressing cells.

expression was diminished (Fig. 2D, top). In contrast, only 7.6% of TL-IgG1 transfected cells were surface IgG1$^+$ and 15.3% were EGFP positive (Fig. 2D, top), although the expression level of GFP-Fcγ1 mRNA was comparable to that of wt-IgG1 (Fig. 2D, bottom). These results suggest that the cytoplasmic tail is required for the stable expression of IgG1 on the cell surface and that TL-IgG1 molecule

might be easily endocytosed as in the case of CTLA4, which also possesses YxxM motif [11], and/or accumulated in the endoplasmic reticulum, and degraded promptly at least in Ramos cells. In case of YE-IgG1, proportion of surface IgG1+ cells increased to almost 90%, strongly suggesting that phosphorylated tyrosine residue in the ITT motif may support the efficient expression of cell surface IgG1 when expression of Igβ is insufficient. In order to further examine the importance of tyrosine residue, we expressed YA-IgG1 in IgβLo Ramos cells. As the result, we found that the mean fluorescence intensity decreased from 64.9 of wt-IgG1 to 36.5 (Fig 2E). Since it has been reported that tyrosine residue in the ITT motif can be phosphorylated by BCR cross-linking in Ramos B cells [8], this result supports the possibility that phosphorylated tyrosine in the ITT motif may play a pivotal role in the regulation of surface IgG expression. However, since we have not directly examined the phosphorylation status of the tyrosine residue in the ITT motif, we cannot rule out the possibility that the tyrosine residue itself is essential for the cell surface expression of IgG BCR independent of its phosphorylation status.

3.3. Constitutive recruitment of Grb2 to the YE cytoplasmic tail

As described above, it has been reported that Grb2 associates with the ITT motif when tyrosine is phosphorylated by BCR cross-linking [8]. Thus, in order to strengthen our assumption that YE mutation in the ITT motif resembles ITT motif with phosphorylated tyrosine, we examined the association of Grb2 to the cytoplasmic tail of YE-IgG1. Following the cross-linking of surface IgG1, wt-IgG1 and YE-IgG1 were immunoprecipitated from transfected Ramos cells and examined for the co-precipitation of Grb2. As the result, we found that Grb2 is more efficiently co-precipitated in wt-IgG1-expressing cells when surface IgG1 was cross-linked as expected. In contrast, amount of co-precipitated Grb2 before cross-linking was comparable to that in cross-linked YE-IgGl-expressing cells (Fig. 3). Although, we cannot completely rule out the possibility that Grb2 is indirectly associating, these results are consistent with the previous report [8] and further support our assumption that YE mutation mimics phosphorylated tyrosine in ITT motif, at least in our experimental condition.

Although the physiological relevance of IgG cytoplasmic tail *in vivo* has been clearly shown [7], the molecular mechanism by which the cytoplasmic tail exerts its function in the immune responses has not been shown. As described above, recent report shed a light on the modulation of BCR signal by the phosphorylation of ITT motif [8]. However, it still remains to be revealed whether the role of ITT motif other than the signal modulation might exist or not. We have approached this issue by employing newly designed GFP-Fcγ1 constructs, which made it possible to assess the total expression efficiency and the proportion of surface IgG1+ cells, concomitantly. As the result, we found the possible involvement of tyrosine phosphorylated ITT motif in the stable expression of cell surface IgG1 in non-B lineage cells and B lymphoma especially when expression of Igβ is down-regulated. Although our results and the previous experiments in the fibroblasts [13] are consistent, it remains to be revealed whether the environment where expression of Igβ and/or Igα is suppressed in B cells really exists *in vivo*. One potential candidate might be the germinal center B cells in which the class-switch recombination and somatic hypermutation take place. Germinal center B cell subset, which is extensively proliferating, is known to down-regulate the expression of surface BCR [17]. Although it has not been examined in detail, it might be possible that BCR signaling molecule, including Igβ, might be also down-regulated in these cells. Indeed, expression of Igβ is shown to be markedly down-regulated, different from Igα, in active human germinal center [18].

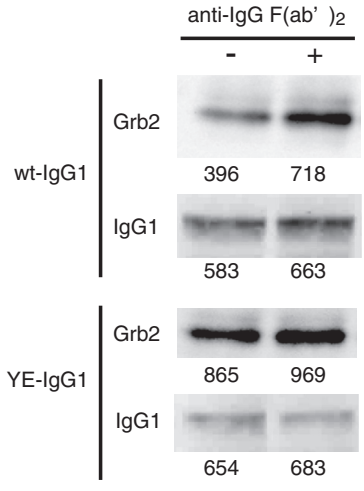

Fig. 3. Constitutive recruitment of Grb2 to the YE cytoplasmic tail. Cell surface IgG1 receptors were stimulated with anti-IgG F(ab')$_2$ for 5 min and the cells were lysed. Total cells lysates were immunoprecipitated with anti-IgG1 from each cell line and the resultant immune complex was subjected to Western blot analysis to detect the associated Grb2. Amounts of IgG1 were analyzed as the control. Intensities of each band were enumerated and indicated below each band.

Declaration of competing financial interests

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