from our findings of enhanced PLSCR1 mRNA in monocytes, multiple potential mechanisms of thrombosis, such as atherosclerosis, may affect the prothrombotic state in patients with SLE.

Further investigation into the mechanisms and biological significance of impaired PLSCR1 expression will contribute to better management of affected patients.

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Regulatory polymorphisms in *EGR2* are associated with susceptibility to systemic lupus erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune disease induced by the combinations of environmental and genetic factors. Recently, mice in which the early growth response 2 (*EGR2*) gene, a zinc-finger transcription factor, is conditionally knocked out in CD2$^+$ T cells have been shown to develop a lupus-like autoimmune disease. Here, we evaluated if polymorphisms in the *EGR2* gene influence SLE susceptibility in humans. We first analyzed the effect of SNPs in the *EGR2* region on *EGR2* expression, and a significant positive correlation with expression was identified in an SNP located at the 5′ flanking region of *EGR2* (rs10761670, $R = 0.23$, $P = 0.00072$). We then performed a case–control association study using three sets of SLE cohorts by genotyping 14 tag SNPs in the *EGR2* gene region. A peak of association with SLE susceptibility was observed for rs10761670 [Pooled: OR = 1.23 (95% CI 1.10–1.37), $P = 0.00023$). This SNP was also associated with susceptibility to rheumatoid arthritis (RA) [OR = 1.15 (95% CI 1.05–1.26), $P = 0.0019$), suggesting that *EGR2* is a common risk factor for SLE and RA. Among the SNPs in complete linkage disequilibrium with rs10761670 ($r^2 = 1.0$), two SNPs (rs1412554 and rs1509957) affected the binding of transcription factors and transcriptional activity *in vitro*, suggesting that they may be candidates of causal regulatory variants in this region. Therefore, *EGR2* is a genetic risk factor for SLE, in which increased gene expression may contribute to SLE pathogenesis.

INTRODUCTION

Systemic lupus erythematosus (SLE, OMIM # 152700) is an autoimmune disease induced by the combinations of environmental and genetic factors. This disease is characterized by antinuclear autoantibodies, complement activation, hyperproduction of interferon and tissue destruction (1). Genetic studies using a candidate gene approach identified several SLE susceptibility genes, including *HLA-DRB1*, *FCGR2B/3A/3B*, *PTPN22*, *STAT4* and *IRF5* (2–7). In addition, recent genome-wide association studies (GWASs) have uncovered novel SLE susceptibility genes, including *TNFAIP3*, *BANK1*, *ITGAM*, *PXK*, *KIAA1542* and *C8orf13-BLK* (8–11). Among these genes, genes such as *PTPN22*, *STAT4*, *TNFAIP3* and

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IRF5 are also associated with susceptibility to other autoimmune diseases, suggesting that some genetic dispositions are shared between SLE and other autoimmune diseases (7,12–14). These GWASs for SLE have enabled a comprehensive survey of the genome, and the statistical power of individual GWASs has been increasing as growing number of samples have been genotyped. However, a recent large-scale replication study that examined the suggested loci of GWAS demonstrated that the 26 confirmed risk loci only explain an estimated 8% of the total genetic susceptibility to SLE (15). This implicated many genes remained undiscovered probably due to their moderate risk. Therefore, the candidate gene approach is still a complementary tool for the identification of unknown genes with moderate risk that contribute to SLE susceptibility.

The early growth response 2 (*Egr2*) gene in mice is a member of the zinc-finger transcription factor *Egr* family (*Egr1*, *2*, *3* and *4*) that are expressed during thymic T-cell differentiation (16), and plays an essential role in hindbrain development and myelination of the peripheral nervous system (17). Whereas *Egr4* is constitutively expressed in T cells, *Egr1*, *2*, and *3* are up-regulated by T-cell receptor (TCR) engagement. Opposing functions of the *Egr*s have been described, where *Egr1* enhances T-cell function by up-regulating IL-2 and other molecules that stimulate T cells (18). On the other hand, *Egr2* and *Egr3* are considered to negatively regulate T cells, which are demonstrated in a study using *Egr2* and *Egr3*-deficient T cells (19). In that study, it was proposed that *Egr2* and *Egr3*, which are located in the downstream of transcription factor NF-AT signaling, inhibited T-cell function by both up-regulating negative regulators such as *Cbl-b* and inhibiting the expression of T-cell activators such as *Egr1* and *NAB2*. Interestingly, mice whose *Egr2* gene was conditionally knocked out in $CD2^+$ T cells were shown to develop a lupus-like autoimmune disease, characterized by accumulation of interferon-γ and interleukin-17-producing $CD4^+$ T cells, loss of tolerance to nuclear antigens, massive infiltration of T cells into multiple organs and glomerulonephritis (20). More recently, we described a new subset of IL-10-secreting regulatory T cells, termed $CD4^+CD25^-LAG3^+$ regulatory T cells, that characteristically express *Egr2* (21). Because *Egr2*-transduced naïve $CD4^+$ T cells differentiated into IL-10-secreting $CD4^+CD25^-LAG3^+$ regulatory T cells, *Egr2* was considered to be a key transcription factor for the differentiation of these cells. A significant role in intestinal immunity was suggested for these cells by the observation that the frequency of $CD4^+CD25^-LAG3^+$ regulatory T cells in the $CD4^+CD25^-$ T cell population was comparatively higher in Peyer's patch than in the spleen, and that experimentally induced colitis was effectively protected by these regulatory T cells.

In humans, chromosome 10q21, within which *EGR2* is located, has been identified as a candidate locus for Crohn's disease (CD) susceptibility in two independent GWASs (22,23). The landmark SNPs identified in these GWASs are located in the intergenic region between the *ZNF365* and the *EGR2* genes. T cells have been assumed to play a major role in the pathogenesis of CD, as an excess of T cells (both effector and regulatory T cells) were detected in the intestinal mucosa of patients (24,25). Indeed, genetic studies of CD identified several susceptibility genes that are involved in the differentiation of T cells, which further supports the significance of T cells for CD (25). Therefore, given the effects of *Egr2* on murine T cells and the role of $CD4^+CD25^-LAG3^+Egr2^+$ regulatory T cells in the colitis model, *EGR2* is considered to be a strong candidate gene for CD susceptibility at this locus.

The described evidence derived from the murine disease models and from human genetic studies implies the existence of disease causal variations in the *EGR2* gene region, which might be shared between SLE and CD in humans. In the present study, we first searched for functional variants that may affect gene function or gene expression of *EGR2*, and then performed case–control association tests to examine the contribution of *EGR2* to SLE susceptibility.

RESULTS

Correlation between *EGR2* expression and SNP genotypes

To identify variants which potentially increase the risk of disease in the *EGR2* gene region, we first searched for functional variants that might alter the amino acid sequence, or affect the expression level, of *EGR2*. The coding region of *EGR2* was sequenced using DNA from 96 individuals affected with SLE (the power for detecting rare variants with frequency of 0.01 was estimated to be 0.86). In this sequencing analysis, no variation leading to amino acid substitution in *EGR2* was discovered.

We then examined possible correlations between *EGR2* expression and genotype. As *EGR2* has been reported to be expressed in B cells as well as in T cells (26,27), we analyzed a data set of gene expression in Epstein-Barr virus (EBV)-transformed lymphoblastoid B cell lines from HapMap individuals (28). Analysis of the genotype data of Japanese HapMap samples identified three LD blocks in a Japanese population in an 80-kb region that includes the *EGR2* gene on 10q21.3 (Fig. 1A). The correlation between SNP genotype and the expression of *EGR2* in this region was assessed for data sets of three individual HapMap ethnic groups (JPT + CHB, CEU and YRI). The observation of a common peak of correlation in the 5′ flanking region of *EGR2* among these three groups implied common regulatory factors for *EGR2* expression in this chromosomal region (Fig. 1B). We then selected 69 SNPs from this 80-kb region that are common (minor allele frequency; MAF > 0.10) to all three HapMap ethnic groups, and assessed the correlation between their genotypes and the expression of *EGR2* using the pooled data set of all populations ($n = 210$) (Fig. 1B). Rs10761670 (T/A) in the 5′ flanking region of *EGR2* showed the highest correlation ($R = 0.23$). When the expression level of *EGR2* was regressed with the number of alleles of rs10761670, the correlation was statistically significant ($P = 0.00072$, Fig. 1C). This correlation suggested that variations existed in the 5′ flanking region of *EGR2* that affect gene expression, which may potentially increase the risk of disease.

Case–control association study of the *EGR2* gene region

To evaluate the association between *EGR2* polymorphisms and SLE susceptibility, we performed a case–control

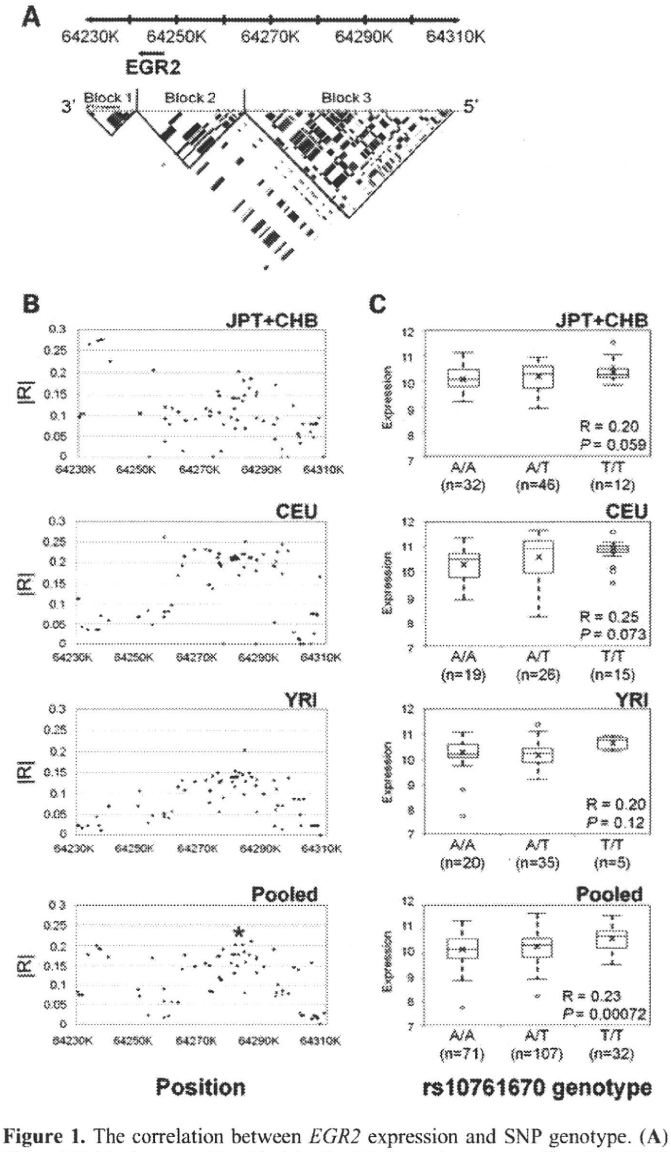

Figure 1. The correlation between *EGR2* expression and SNP genotype. (**A**) Three LD blocks were identified in the 80-kb region surrounding the *EGR2* gene using genotype data of HapMap Japanese (JPT) samples. (**B**) The correlation between *EGR2* expression and SNP genotypes (0, 1, 2) using gene expression data from EBV-transformed lymphoblastoid cell lines of HapMap individuals. The analyses of individual populations [JPT + Han Chinese (CHB), European (CEU) and African (YRI)], and of a pool of all of the populations, are shown. |R|: the absolute value of the correlation coefficient between *EGR2* expression and SNP genotype. The asterisk (*) indicates rs10761670. (**C**) Liner regression analysis of the relationship between the rs10761670 genotype and *EGR2* expression (JPT + CHB: $n = 90$; CEU: $n = 60$; YRI: $n = 60$; pooled: $n = 210$). The x-axis shows the rs10761670 genotypes (AA, AT, TT) and the y-axis represents the $\log_2$-transformed *EGR2* expression level. Top bar of the box-plot represents maximum value and the lower bar represents minimum value. The top of box is third quartile, the bottom of box is first quartile and the middle bar is median value. The x-mark is mean value and the circle is outlier.

association study in the *EGR2* region. Tag SNPs were selected from the HapMap database for association tests. We selected a total of 14 tag SNPs with MAF > 0.10 from the LD blocks described above. Nine of these tag SNPs were from block 3, which spanned the 5′ region of *EGR2* and contained the potential regulatory variants described above, with a threshold value of $r^2 > 0.8$, and the other five SNPs were from blocks 1 and 2 with a threshold value of $r^2 > 0.5$ (Table 1). We initially genotyped 376 SLE cases for the tag SNPs and compared their allele frequencies with those of 940 control individuals. We identified two SNPs (rs10761670 and rs955696) which showed significant association with SLE susceptibility [rs10761670: OR = 1.19 (95% CI 1.00–1.41), $P = 0.049$ and rs955696: OR = 1.23 (95% CI 1.02–1.48), $P = 0.033$, respectively, Table 1]. These SNPs are located in block 3 in the 5′ region of *EGR2*. To validate the case–control association test, we performed a replication study for these two SNPs using additional two sets of SLE cohorts (second set: 293 SLE cases and 881 controls; third set: 223 SLE cases and 658 controls). The association was replicated only for rs10761670 [second set: OR = 1.23 (95% CI 1.02–1.49), $P = 0.029$ and third set: OR = 1.30 (95% CI 1.05–1.62), $P = 0.018$, Table 2], although the possibility remained that the negative results in rs955696 was due to insufficient statistical power of the cohort sets (0.62 and 0.52 for second and third sets, respectively). No heterogeneity was detected among the associations of three cohorts ($P = 0.83$ in a Mantel–Haenszel analysis), and the combined analyses showed a statistically significant association of rs10761670 with SLE susceptibility [OR = 1.23 (95% CI 1.10–1.37), $P = 0.00023$, Table 2]. Both dominant and recessive models showed significant associations in rs10761670 in the pooled set [dominant model: OR = 1.33 (95% CI 1.09–1.62), $P = 0.0045$ and recessive model: OR = 1.32 (95% CI 1.11–1.57), $P = 0.0016$]. After adjustment for gender by a logistic regression analysis, rs10761670 still showed a significant association with SLE susceptibility in the combined set [882 SLE cases and 2467 controls, OR = 1.23 (95% CI 1.09–1.38), $P = 0.00070$].

As previous studies have demonstrated that multiple genes, including *PTPN22*, *STAT4*, *TNFAIP3* and *CD244* (29), increase the risk of both SLE and rheumatoid arthritis (RA), we examined the association of *EGR2* with RA susceptibility using two sets of RA cohorts (first set, 1112 RA cases and 940 controls; second set, 830 RA cases and 881 controls). A significant association was observed in the first set [OR = 1.21 (95% CI 1.07–1.37), $P = 0.0020$], but we did not identify a significant association in the second set [OR = 1.09 (95% CI 0.95–1.24), $P = 0.22$]. The combined analyses showed a statistically significant association with RA susceptibility [OR = 1.15 (95% CI 1.05–1.26), $P = 0.0019$] (Table 3). These results suggested that the *EGR2* polymorphism increased the risk of RA as well as of SLE.

Search for regulatory SNPs

To identify causal variants, we examined whether nuclear transcription factors bind to genomic sequences surrounding the SNPs and compared the binding ability of susceptible alleles with that of non-susceptible alleles. We selected 15 SNPs that are in complete linkage disequilibrium (LD) with rs10761670 ($r^2 = 1.0$) using the genotype data of Japanese HapMap samples. These SNPs were examined by electrophoretic mobility shift assay (EMSA) using nuclear extracts from an EBV-transformed lymphoblastoid cell line (PSC cells). We found allelic differences in the binding ability of two

Table 1. Association analysis of *EGR2* with SLE susceptibility

dbSNP ID	Allele (1/2)	LD blocks	Number of subjects		Frequency of allele 1		Odds ratio (95%CI)[b]	*P*-value (allele)[a]
			Case	Control	Case	Control		
rs10995312	C/T	Block 1	376	940	0.54	0.51	1.12 (0.94–1.33)	0.20
rs224278	A/G	Block 2	375	933	0.64	0.63	1.03 (0.86–1.22)	0.77
rs224285	A/C		374	934	0.23	0.22	1.09 (0.89–1.34)	0.40
rs224292	G/A		376	934	0.80	0.77	1.16 (0.94–1.43)	0.16
rs11817939	A/G		374	934	0.89	0.87	1.18 (0.91–1.53)	0.22
rs2136613	A/G	Block 3	376	934	0.36	0.35	1.01 (0.85–1.21)	0.88
rs7078554	A/T		374	939	0.24	0.23	1.07 (0.87–1.30)	0.52
rs224307	G/A		375	934	0.66	0.63	1.14 (0.95–1.36)	0.16
rs10761670	T/A		375	939	0.55	0.51	1.19 (1.00–1.41)	0.049
rs10995335	G/A		375	940	0.90	0.88	1.16 (0.88–1.53)	0.30
rs10995337	C/T		374	939	0.91	0.89	1.25 (0.93–1.68)	0.13
rs3939306	T/C		376	940	0.27	0.26	1.06 (0.88–1.28)	0.55
rs949566	C/G		376	940	0.81	0.78	1.19 (0.97–1.48)	0.10
rs955696	C/T		376	938	0.30	0.26	1.23 (1.02–1.48)	0.033

[a]*P*-value (allele) was calculated using an allele frequency comparison test.
[b]95% CI = 95% confidence interval.

Table 2. Replication study and combined analysis of *EGR2* with SLE susceptibility

dbSNP ID	Allele (1/2)	Case–control cohorts	Number of subjects		Frequency of allele 1		Odds ratio (95%CI)[b]	*P*-value (allele)[a]
			Case	Control	Case	Control		
rs10761670	T/A	2nd	287	876	0.56	0.51	1.23 (1.02–1.49)	0.029
		3rd	220	652	0.57	0.51	1.30 (1.05–1.62)	0.018
		Combined analysis[c]	882	2467	0.56	0.51	1.23 (1.10–1.37)	0.00023
rs955696	C/T	2nd	290	873	0.28	0.26	1.12 (0.91–1.38)	0.30
		3rd	223	654	0.31	0.27	1.24 (0.98–1.57)	0.073
		Combined analysis[c]	889	2465	0.30	0.26	1.19 (1.06–1.34)	0.0040

[a]*P*-value (allele) was calculated using an allele frequency comparison test.
[b]95% CI = 95% confidence interval.
[c]The Mantel–Haenszel method was used for the combined analysis of first, second and third sets.

Table 3. Association analysis of *EGR2* with RA susceptibility

dbSNP ID	Allele (1/2)	Case–control cohorts	Number of subjects		Frequency of allele 1		Odds ratio (95%CI)[b]	*P*-value (allele)[a]
			Case	Control	Case	Control		
rs10761670	T/A	1st	1105	939	0.56	0.51	1.21 (1.07–1.37)	0.0020
		2nd	827	877	0.53	0.51	1.09 (0.95–1.24)	0.22
		Combined analysis[c]	1932	1816	0.55	0.51	1.15 (1.05–1.26)	0.0019

[a]*P*-value (allele) was calculated using an allele frequency comparison test.
[b]95% CI = 95% confidence interval.
[c]The Mantel–Haenszel method was used for the combined analysis of first and second sets.

SNPs, rs1412554 and rs1509957. For rs1412554, the intensity of the shifted band was higher for the susceptible allele than for the non-susceptible allele. In contrast, a particular band was seen specifically to the non-susceptible allele for rs1509957 (Fig. 2A). Competition assays with unlabeled oligonucleotides showed that these complexes were specific for each oligonucleotide. Similar results were obtained when nuclear extracts of Jurkat cells were tested (Fig. 2A).

The transcriptional activity of rs1412554 and rs1509957 was then analyzed using luciferase assays. Allele specific constructs containing the SNPs and surrounding genomic sequences were constructed and transfected into Jurkat cells. Cells transfected with the susceptible allele of rs1412554 displayed a 1.2-fold greater enhancement of transcriptional activity than cells transfected with the non-susceptible allele (Fig. 2B). In contrast, only cells transfected with the

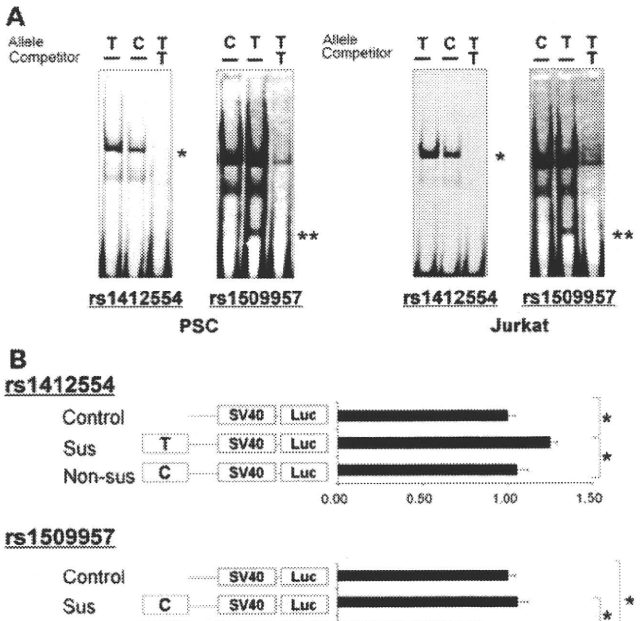

Figure 2. Transcriptional activity is affected by rs1412554 and rs1509957. (**A**) EMSAs were performed with nuclear extracts from PSC and Jurkat cells. Biotin-labeled 25-bp oligonucleotides corresponding to each SNP (rs1412554 and rs1509957) served as probes for the susceptible (Sus), and the non-susceptible (Non-sus), allele, respectively, and the abilities of nuclear factors to bind to these oligonucleotides were evaluated (for rs1412554: Sus = T, Non-sus = C; for rs1509957: Sus = C, Non-sus = T). Unlabeled oligonucleotides were used for competition assays. A single asterisk (*) and double asterisks (**) indicate bands showing allelic differences in rs1412554 and rs1509957, respectively. PSC cells (Left) and Jurkat cells (Right). (**B**) Transcriptional activities of the 31-bp genomic sequences around SNPs [rs1412554 (T/C) and rs1509957 (C/T)] were evaluated by luciferase assays. Each 31-bp oligonucleotide corresponding to these genomic sequences was inserted into the pGL3-promoter vector upstream of the SV 40 promoter and allele-specific constructs were transfected into Jurkat cells. Relative promoter activity was expressed as the ratio of the luciferase activity of each allele specific construct to the luciferase activity of the pGL3-promoter control vector. Data are presented as the mean ± SEM. Each experiment was independently repeated three times and each sample was studied in sextuplicate. *$P < 0.05$ by Student's *t*-test. Rs1412554 (top) and rs1509957 (bottom).

non-susceptible allele of rs1509957 displayed a repression of transcriptional activity (Fig. 2B). Therefore, rs1412554 and rs1509957 may contribute to transcriptional modulation of the *EGR2* gene, suggesting that these two SNPs could be candidates of causal variants in this chromosomal region.

DISCUSSION

In the present study, we demonstrated that polymorphisms in the *EGR2* gene are associated with SLE susceptibility, presumably through up-regulating the gene expression. An SNP in the 5′ flanking region of *EGR2*, termed rs10761670, showed the highest correlation between *EGR2* expression and its genotype. Furthermore, in case–control association tests, the peak association with SLE susceptibility was observed for rs10761670. This correspondence between the two independent associations (gene expression and disease susceptibility) strongly indicates the presence of disease-causing regulatory variants in this region. Two SNPs, rs1412554 and rs1509957, which are in complete LD with rs10761670, and were shown to affect the binding of transcription factors and to modulate gene expression in *in vitro* assays, are candidate SNPs for the causal variants, although other undiscovered variants may also contribute to the regulation of gene expression.

When the effective size of the *EGR2* polymorphism that contributes to disease risk is measured as OR, this size is relatively small compared with that of other loci described in recent GWASs of SLE, suggesting that *EGR2* polymorphism has a moderate risk for an individual. However, the risk allele of rs10761670 is common in the population (the allele frequency = 0.51). This indicates that the *EGR2* polymorphism contributes to the onset of SLE in a wide population in contrast to rare but high penetrant variants. We calculated the population attributable risk proportion (PARP) to assess the reduction in susceptibility to SLE, if these risk alleles were removed from the population, and the PARP for rs10761670 in *EGR2* was 0.088.

In GWASs of CD in Caucasian populations, two landmark SNPs (rs224136 and rs10761659) were located in the intergenic region between *ZNF365* and *EGR2* (at the 3′ side of *EGR2*). Because T cells are considered to play a significant role in the pathogenesis of CD, *EGR2*, which is a potential regulator of TCR signaling, is a strong candidate for CD susceptibility at this locus. Therefore, genetic variations with disease-causing potential in this region could potentially be shared genetic risk factors for CD and SLE. However, the landmark SNP identified in the present study (rs10761670) is located in the 5′ flanking region of *EGR2* and is not in strong LD ($r^2 < 0.20$) with SNPs of CD (rs224136 and rs10761659) in either Japanese or Caucasian populations. This finding implies that causal variants may be different between SLE and CD or may differ between ethnic populations. Therefore, comparative analyses of both diseases between populations are needed to clarify this problem.

In mice, *Egr2* conditional knockout in $CD2^+$ T cells results in the development of a lupus-like autoimmune disease, where complete loss of *Egr2* function may lead to the loss of regulation of effector T cells and self-reactive T cells (20). In addition, transduction of *Egr2* into naïve $CD4^+$ T cells differentiates them to IL-10-secreting $CD4^+CD25^-LAG3^+$ regulatory T cells that would suppress self-reactive cells (21). These protective roles of *Egr2* against autoimmunity in mice contradict our findings that enhanced expression of *EGR2* may increase SLE susceptibility in humans. Similar observations were reported for the *PTPN22* gene, another established susceptibility gene for both SLE and RA (4,30). *PTPN22* encodes a tyrosine phosphatase, LYP that has an inhibitory effect on TCR signaling. For LYP, a non-synonymous SNP (rs2276601, R620W) was associated with disease susceptibility, and increased inhibition of TCR signaling was shown with the disease risk allele W620 (31). Furthermore, knockout of *Pep*, the murine ortholog of *PTPN22*, leads to various immune abnormalities, including expansion of effector T cells and increased antibody production (32). Taken together, enhanced negative regulation of T cells by inhibitory components such as *EGR2* and *PTPN22* may lead to tolerance

breakdown in human diseases, while complete loss of function of these components may result in a hyperactive or autoimmune response in mice. Moreover, another plausible explanation for the role of *EGR2* in human autoimmunity has been recently raised by an analysis on *Egr2* deficient thymocytes in mice, which concluded that *Egr2* plays a central role in the up-regulation of the survival molecule *Bcl-2* during the positive selection of thymocytes (33). As overexpression of *Bcl-2* has been shown to result in the increased self-reactive thymocytes (34), up-regulated expression of *Egr2* may also lead to increased selection of self-reactive thymocytes.

In conclusion, *EGR2* is a genetic risk factor for SLE as well as for RA, for which increased gene expression may contribute to disease pathogenesis. Analyses of *EGR2* in other autoimmune disorders, including CD, are needed to elucidate its precise role in autoimmunity.

MATERIALS AND METHODS

Subjects

Three independent sets of SLE ($n = 376$, 293 and 223) and two independent sets of RA ($n = 1112$ and 830) patients, and of control subjects ($n = 940$, 881 and 658), were enrolled in the study through medical institutes in Japan. The control subjects in the first set were the members of Midousuji Rotary Club and those in third set was recruited through Pharma SNP Consortium (35). All subjects were self-identified Japanese. SLE subjects met the revised American College of Rheumatology (ACR) criteria for SLE (1) and RA subjects met the revised ACR criteria for RA (36). All control subjects were confirmed to be not affected with autoimmune diseases including SLE and RA by reviewing their medical records. All subjects provided informed consent to their participation in the study, as approved by the Ethics Committee of the Center for Genomic Medicine, RIKEN. DNA was extracted from peripheral blood cells using a standard protocol.

SNP discovery

Unknown SNPs were revealed by direct sequencing of the DNA of 96 individuals affected with SLE. DNA fragments were amplified for sequencing with the appropriate primers and were purified using a MultiScreen PCR filter plate (Millipore, Billerica, MA, USA). The amplified DNAs were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and signals were detected using an Applied Biosystems ABI 3700 DNA Analyzer.

Genotyping

We selected 14 tag SNPs from an 80-kb region on 10q21.3, which comprised three LD blocks and included the *EGR2* gene, using the HapMap data of the Japanese population and Haploview software, v.4.0. These SNPs were genotyped using TaqMan SNP genotyping assays (Applied Biosystems) as indicated by the manufacturer. Fluorescence was detected using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). All SNPs were successfully genotyped with call rates >0.99 and were in Hardy–Weinberg equilibrium in control subjects ($P > 0.01$).

Electrophoretic mobility shift assays

PSC (an EBV-transformed lymphoblastoid cell line) and Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Nuclear extracts were prepared as previously described (37). In brief, following stimulation with 50 ng/ml phorbol myristate acetate (Sigma, St Louis, MO, USA) for 2 h, centrifuged cells were collected and resuspended in Buffer A (20 mM HEPES pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8.0, 1 mM DTT, 0.1% NP-40 and Protease inhibitor cocktail). The cells were then incubated on ice for 10 min, centrifuged and the pellets were resuspended in Buffer B (20 mM HEPES pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA pH 8.0, 1 mM DTT, 0.1% NP-40 and Protease inhibitor cocktail). Following incubation on ice for 30 min and then centrifugation to remove cellular debris, the supernatant fraction containing nuclear proteins was collected. Oligonucleotides (25 bp) were designed that corresponded to genomic sequences surrounding the SNPs. Single-stranded 25-bp oligonucleotide probes were labeled using a Biotin 3′ End DNA Labeling Kit (Pierce Biotechnology, Rockford, IL, USA) and sense and antisense oligonucleotides were then annealed. The LightShift Chemiluminescent EMSA kit (Pierce Biotechnology) was used for the detection of DNA–protein interactions. Biotin-labeled probes were incubated with nuclear extracts in a binding reaction mixture (10× binding buffer, 50 ng/μl poly dI-dC, 50% glycerol, 5 mM $MgCl_2$ and 0.05% NP-40) for 20 min at 25°C. In competition studies, unlabeled oligonucleotides (100-fold excess) were preincubated with the nuclear extract and binding reaction mixture for 5 min before addition of the biotin-labeled probes. The DNA–protein complexes were separated on a non-denaturing 5% polyacrylamide gel in 1× TBE (Tris-borate-EDTA) running buffer for 70 min at 150 V. Gel protein–DNA complexes were transferred to a nitrocellulose membrane for 30 min at 380 mA and the transferred complexes were cross-linked to the membrane by exposure to UV light of 120 mJ/cm^2 for 1 min. DNA–protein complexes on the membrane were detected using the Chemiluminescent Nucleic Acid Detection Module (Pierce) and a LAS-3000 mini lumino-image analyzer (Fujifilm, Tokyo, Japan). Allelic differences were analyzed using MultiGauge software (Fujifilm) by measuring the intensity of the bands.

Luciferase assay

Oligonucleotides (31 bp) were designed that corresponded to genomic sequences that included susceptible or non-susceptible alleles. Complementary sense and antisense oligonucleotides were then annealed. To construct luciferase reporter plasmids, the pGL3-Promoter vector (Promega, Madison, WI, USA) was digested with *Mul*I and *Bgl*II and a single copy of a 31-bp oligonucleotide was ligated into the vector upstream of the SV 40 promoter using the TaKaRa Ligation kit ver. 2.1 (TaKaRa, Shiga, Japan). After confirmation of the sequence, these plasmids were purified using

a HiSpeed Plasmid Midi kit (Qiagen, Valencia, CA, USA). Jurkat cells (5×10^5), grown as described above, were transfected with 2.5 μg of the constructs and 0.5 μg of the pRL-TK vector (an internal control for transfection efficiency) using the Lipofectamin2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). After 24 h, the cells were collected and luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega) and an Ultra Sensitive Tube Luminometer, Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). Each experiment was independently repeated three times and sextuplicate samples were assayed each time.

Statistical analysis

We used χ^2 contingency table tests to evaluate the significance of differences in allele frequency in the case–control subjects. We defined haplotype blocks using the Solid spine of LD definition of the Haploview v4.0. We performed a Mantel–Haenszel analysis to calculate the pooled *P*-value and odds ratio of two independent association studies. We calculated the power of each cohort for testing association by Quanto Software (http://hydra.usc.edu/gxe/). To adjust for the confounding effects such as gender, we performed a logistic regression analysis using the STATISTICA software (StatSoft). We calculated PARP using the following formula; $\text{PARP} = f(\text{OR}-1)/(1 + f(\text{OR}-1)) \times 100$, where f is the allele frequency in the control subjects and OR is the odds ratio. PARP is defined as the reduction of disease incidence that would be achieved if the population had been entirely unexposed. Luciferase assay data were analyzed by Student's *t*-test.

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Conflicts of Interest statement. None declared.

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Plasma gelsolin facilitates interaction between β_2 glycoprotein I and $\alpha_5\beta_1$ integrin

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Abstract

Antiphospholipid syndrome (APS) is characterized by thrombosis and the presence of antiphospholipid antibodies (aPL) that directly recognizes plasma β_2-glycoprotein I (β_2GPI). Tissue factor (TF), the major initiator of the extrinsic coagulation system, is induced on monocytes by aPL *in vitro*, explaining in part the pathophysiology in APS. We previously reported that the mitogen-activated protein kinase (MAPK) pathway plays an important role in aPL-induced TF expression on monocytes. In this study, we identified plasma gelsolin as a protein associated with β_2GPI by using immunoaffinity chromatography and mass spectrometric analysis. An *in vivo* binding assay showed that endogenous β_2GPI interacts with plasma gelsolin, which binds to integrin $\alpha_5\beta_1$ through fibronectin. The tethering of β_2GPI to monoclonal anti-β_2GPI autoantibody on the cell surface was enhanced in the presence of plasma gelsolin. Immunoblot analysis demonstrated that p38 MAPK protein was phosphorylated by monoclonal anti-β_2GPI antibody treatment, and its phosphorylation was attenuated in the presence of anti-integrin $\alpha_5\beta_1$ antibody. Furthermore, focal adhesion kinase, a downstream molecule of the fibronectin-integrin signalling pathway, was phosphorylated by anti-β_2GPI antibody treatment. These results indicate that molecules including gelsolin and integrin are involved in the anti-β_2GPI antibody-induced MAPK pathway on monocytes and that integrin is a possible therapeutic target to modify a prothrombotic state in patients with APS.

Keywords: β_2GPI • gelsolin • integrin • TF • APS

Introduction

Antiphospholipid syndrome (APS) is a clinical condition characterized by recurrent thrombotic events and/or pregnancy morbidity associated with the persistence of antiphospholipid antibodies (aPL). Although the original concept of aPL considers that those antibodies were directed against anionic phospholipids, evidence shows that phospholipid-binding plasma proteins such as β_2-glycoprotein I (β_2GPI) [1–3] and prothrombin [4] are the dominant antigenic targets recognized by aPL in patients with APS.

Among the aPL found in patients with APS, antibodies directing to cardiolipin-β_2GPI complex (aCL/β_2GPI), also called anticardiolipin antibodies or anti-β_2GPI antibodies, have been the best studied in their clinical or biological properties in the last two decades [5]. β_2GPI is a single-chain glycoprotein containing 326 amino acids and contains a high proportion of proline and cysteine residues and is heavily glycosylated [6]. β_2GPI is a member of the complement control protein repeat or short consensus repeat (SCR) superfamily and is composed of five homologous motifs of approximately 60 amino acids designated as SCR or as sushi domains. Each motif contains four conserved half cysteine residues, related to the formation of two internal disulphide bridges. While the first four domains are typical, the fifth domain of β_2GPI is a modified form containing 82 amino acid residues and six half cysteines. The tertiary structure of β_2GPI revealed a highly glycosylated protein with an elongated fishhook-like arrangement

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of the globular SCR domains [7, 8]. β_2GPI binds to solid phase phospholipids through a major phospholipid binding site located in the fifth domain, C281KNKEKKC288 close to the hydrophobic loop [9].

The aCL/β_2GPI recognize the epitopes that appear on β_2GPI only when β_2GPI interacts with anionic phospholipids [10]. The location of the exact epitopic sites for aCL/β_2GPI on β_2GPI molecule has been focus of intensive debate. ACL/β_2GPI have been shown to recognize different epitopes located in all five domains of β_2GPI. Domain IV or I were reported as candidates for major epitopic location by using a series of deletion mutant proteins of β_2GPI [11]. Recently, de Laat *et al.* showed that pathogenic aCL/β_2GPI bind a cryptic epitope on domain I of β_2GPI, which is accessible for aCL/β_2GPI only after conformational change, and is induced by the binding of β_2GPI to a negatively charged surface *via* a positive-charge patch in domain V [12, 13]. Moreover, our group demonstrated that epitopic structures recognized by aCL/β_2GPI are cryptic and that three electrostatic interactions between domain IV and V (D^{193}-K^{246}, D^{222}-K^{317} and E^{228}-K^{308}) are involved in their exposure [14]. This hypothesis is also supported by our previous data showing that replacement of one single amino acid at position 247 of β_2GPI, which is important for the interaction between domain IV and V, can alter the antigenicity of β_2GPI for pathogenic autoantibodies [14, 15].

Recently, great interest has arisen on the binding of aCL/β_2GPI to endothelial cells or other procoagulant cells and how this binding mediates cell dysfunctions that potentially induce the clinical manifestations of the APS. A number of *in vitro* studies have shown that procoagulant cells, treated with aCL/β_2GPI, are activated and express procoagulant molecules such as tissue factor (TF) [16, 17]. Further research has focused on the signal transduction mechanisms implicated in the increased expression of pro-coagulants substances in response to aPL. The adapter molecule myeloid differentiation protein (MyD88)-dependent signalling pathway and the nuclear factor κB (NF-κB) have been involved in endothelial cell activation by aPL [18–21]. We [22] and others [23–26] showed clear evidence that the p38 mitogen-activated protein kinase (MAPK) pathway of cell activation plays an important role in aPL-mediated cell activation. Such cell activation by aCL/β2GPI might require an interaction between β_2GPI and a specific cell surface receptor. The Toll-like receptor (TLR) family may mediate a role in the interaction of the β_2GPI-aCL/β_2GPI complex on the endothelial cell surface [18]. Annexin II, also known as Annexin A2, is an endothelial cell receptor for tPA and plasminogen, and suggested to interact with the β_2GPI-aCL/β_2GPI complex on the endothelial cell surface mediating cell activation [27, 28]. Some members of low-density lipoprotein receptor family, such as LDL-R related protein, megalin, the very-low density lipoprotein receptor, were shown to bind to β_2GPI [29]. However, no evidence has shown a direct interaction between β_2GPI and TLRs. Annexin II does not span the cell membrane thus cannot induce cell activation unless the presence of an unknown 'adaptor' is present. β_2GPI was required to be chemically dimerized to bind to any of LDL receptors [29]. In addition, no information has been available regarding β_2GPI on monocytes. In fact, monocytes are more potent to produce TF compared with endothelium, therefore the investigation of β_2GPI-aCL/β_2GPI interaction on monocytes are essential to explore the pathophysilogy of APS.

In this study, we identified a plasma gelsolin as a novel protein associated with β_2GPI by using affinity purification and liquid chromatography with mass spectrometry (LC-MS) analysis, and we showed functional interaction of plasma gelsolin with β_2GPI.

Materials and methods

Cell culture

RAW264.7 and HEK293T cell lines were cultured under an atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal bovine serum (Gibco BRL, Paisley, UK). To remove β_2GPI, the culture medium was changed to serum-free DMEM for 16 hrs before the assay.

Cloning of cDNAs and plasmid construction

The signal sequence region and other region of human β_2GPI cDNA lacking the signal sequence (β_2GPI[ss-]) were amplified by PCR from human B cell cDNA (CLONTECH Laboratories, Inc., Mountain View, CA, USA). The resulting fragment containing β_2GPI(ss-) was ligated into the EcoR I and Sal I sites of p3xFLAG CMV7.1 vector (Sigma Chemical Co.). The fragment containing the human β_2GPI signal sequence was ligated into the BamH I and Pst I sites of pBluescript II SK^+ vector (pBS-Sig) (Stratagene, La Jolla, CA, USA). The β_2GPI(ss-) cDNA fragment with 3xFLAG was ligated into the Pst I and Sal I sites of pBS-Sig. The Sig-3xFLAG-β_2GPI fragment was then ligated into pcDNA3 (Invitrogen, Carlsbad, CA) (pcDNA3-Sig-3xFLAG-β_2GPI) or into pCAG-puro vector which contains a puromycin-resistant gene in pCAGGS vector provided by Dr. J. Miyazaki (Osaka University). The plasma gelsolin cDNA was obtained from ATCC (#MGC-39262, Manassas, VA, USA) and ligated into pcDNA3.

Proteins and antibodies

Recombinant human β_2GPI was purified as described previously [11]. FLAG-β_2GPI was collected from the culture supernatant of HEK293T cells transiently transfected with pcDNA3-Sig-3xFLAG-β_2GPI using FuGENE6 (Roche, Branchburg, NJ). Expression of all constructs was performed in conditioned serum-free Opti-MEM (Gibco BRL). Furthermore, a stable cell line expressing FLAG-β_2GPI was generated by transfection with pCAG-I-puro vector encoding FLAG-tagged-β_2GPI cDNA. The culture supernatant of FLAG-β_2GPI-expressing cells was collected after 4 days of culture and then filtered (0.22 μm). The antibodies used in this study were as follows: mouse monoclonal anti-β_2GPI antibody (WBCAL1; aCL/β_2GPI [30], and MAB1066, Chemicon International Inc., Temecula, CA, USA), mouse monoclonal anti-gelsolin antibody (clone 2, BD Transduction Laboratories, San Jose, CA, USA), rat monoclonal anti-integrin $\alpha_5\beta_1$ antibody (MAB1984, Chemicon International Inc.), mouse monoclonal anti-integrin β_1 antibody (Ha2/5, BD), rabbit polyclonal anti-p38 MAP kinase antibody (#9212, Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit polyclonal anti-phospho-p38

MAP kinase antibody (#9211, Cell Signaling Technology, Inc.), mouse monoclonal anti-focal adhesion kinase (FAK) antibody (clone 77, BD), mouse monoclonal anti-FAK(pY397)-phospho-specific antibody (clone 18, BD), mouse anti-β-actin (AC15, Sigma Chemical Co.) and mouse monoclonal anti-FLAG (M2) (Sigma Chemical Co.). EZ-Link Sulfo-NHS-Biotin Reagent was used as a biotinylation reagent. IgG with aPL activity was purified from sera of six patients with APS diagnosed by Sapporo criteria. Control IgG was isolated from normal human serum. Protein concentrations were determined by Bradford method. Consent forms for this study were signed by all of the patients and healthy donors.

Purification of the β_2GPI-related proteome

NHS-activated Sepharose 4 Fast Flow (0.5 ml) (Amersham Biosciences AB, Sweden) washed with 100 mM HCl was mixed with 250 μg of anti-FLAG (M2) mAb (250 μg) in coupling buffer (0.2 M $NaHCO_3$-NaCl, pH 8.3), and the mixture was rotated for 2 hrs at room temperature for conjugation. The unconjugated antibody was removed from the resin by washing with 500 mM ethanolamine (pH 8.3) and 0.1 M CH_3COOH (pH 4.0), and the resin was then equilibrated with PBS. RAW264.7 cells were cultured under an atmosphere of 5% CO_2 at 37°C in serum-free DMEM for 16 hrs. After incubation, 5×10^7 cells were collected, suspended with 1 ml of PBS, and incubated for 2 hrs at 4°C with 0.5 ml of culture supernatant (FLAG-β_2GPI) after transfection with pcDNA3-Sig-3xFLAG-β_2GPI. The cells were then washed twice with 1 ml of PBS, suspended with 1 ml of PBS, and incubated for 2 hrs at 4°C after addition of the membrane-impermeable cross-linker 3,3′-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce) was added to a final concentration of 1 mM. Then Tris-HCl (pH 7.5) was added as a stop solution to a final concentration of 10 mM. The cells were then harvested, washed with PBS, lysed in 10 ml of a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, aprotinin (10 mg/ml), leupeptin (10 mg/ml), 1 mM PMSF, 400 mM Na_3VO_4, 400 mM EDTA, 10 mM NaF and 10 mM sodium pyrophosphate (buffer A), and centrifuged at 16,000 × *g* for 10 min. at 4°C. The resulting supernatant was applied to the M2 column of 100 μl bed volume and the column was then washed with buffer A. HA-peptide (Roche) was loaded to the column to remove non-specific binding and then bound proteins were eluted with two volumes of FLAG peptide (Sigma Chemical Co.). The eluents from the column were concentrated by precipitation with TCA and subjected to in-solution digestion for LC-MS/MS analysis.

In-solution digestion of purified proteins

Proteins were precipitated with 10% TFA and washed with acetone twice. Precipitated proteins were dissolved in 100 mM Tris-HCl and 7 M guanidium hydroxide (pH 8.0), diluted with 100 mM Tris-HCl (pH 8.0) to 1 M guanidium hydroxide, and then digested with Lys-C endopeptidase (500 ng) for 16 hrs at 37°C. The resulting peptides were desalted with a C18 disc settled microtip, dried, and dissolved in 0.1% TFA/2% acetonitrile.

MS and database searching

Peptides were analysed by using a quadrupole time of flight hybrid mass spectometer (Q-tof2, Waters) equipped with an Agilent HP1100 nanoflow pump with a laboratory-made nano-spray stage and ESI column. C18 beads (L-column, 3 μm) were packed in the spray tip and used as a nano-ESI column (5 cm in length, 100 μm in id). The sample was loaded to the ESI column at a flow rate of 800 nl/min with mobile phase A (0.1% formic acid/2% acetonitrile) and eluted with a linear gradient of 5 to 35% B (0.1% formic acid/90% acetonitrile) at a flow rate of 200 nl/min. CID spectra were acquired automatically in the data-dependent scan mode in which the two highest peaks were selected for precursor ions. All MS/MS spectra were processed by a MASCOT distiller for generation of peak list files and were subjected to a database search by the MASCOT algorithm (Matrix Science, London) against the non-redundant National Center for Biotechnology Information (nrNCBI) database. Search parameters were set as follows: Lys-C/P was selected as an enzyme allowing one miscleavage, oxidized methionine and pyroglutamine derived from the amino terminus of glutamine were selected as variable modifications, and the mass tolerance was 0.3 Da for precursor ions and 0.3 Da for MS/MS ions.

Transfection, immunoprecipitation and immunoblot analysis

HEK293T cells were transfected by the calcium phosphate method or lipofection method. After 48 hrs, the cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were centrifuged at 16,000 × *g* for 10 min. at 4°C, and the resulting supernatant was incubated with antibodies for 2 hrs at 4°C. Protein G-Sepharose (Amersham Biosciences AB) that had been equilibrated with the same solution was added to the mixture, which was then rotated for 1 hr at 4°C. The resin was separated by centrifugation, washed four times with ice-cold lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with the primary antibodies with horseradish peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin G (1:10,000 dilution, Promega Corporation, Madison, WI, USA) and an enhanced chemiluminescence system (ECL, Amersham Biosciences, Little Chalfont, UK).

Binding assay

The binding between phospholipid-β_2GPI complex and plasma gelsolin was confirmed by an ELISA. Non-irradiated microtitre plates (Sumilon type S, Sumitomo Bakelite, Tokyo, Japan) were coated with 30 μl of 50 μg/ml cardiolipin (Sigma Chemical Co.) and dried overnight at 4°C. To avoid nonspecific binding of proteins, wells were blocked with 150 μl of Tris-buffered saline (TBS) containing 1% fatty acid-free bovine serum albumin (BSA, A-6003, Sigma Chemical Co.) and $CaCl_2$ (BSA-Ca). After 3 washes in TBS containing 0.05% Tween 20 (Sigma Chemical Co.) and $CaCl_2$ (TBS-Tween-Ca), 50 μl of 10 μg/ml β_2GPI in BSA-Ca was added to half of the wells in the plates and the same volume of BSA-Ca alone (as a sample blank) was added to the other half of the wells. After 1-hr incubation at 37°C, plates were washed and 50 μl of plasma gelsolin (Sigma Chemical Co.) (0–10 μg/ml) in BSA-Ca was added in duplicate. Plates were incubated for 1 hr at room temperature, followed by incubation with mouse monoclonal anti-gelsolin antibody, alkaline phosphatase conjugated goat antimouse IgG and substrate. The optical density of wells coated with cardiolipin alone was subtracted from that of wells containing cardiolipin-β_2GPI complex. All procedures were done in the presence of 0, 1 or 2 mM $CaCl_2$.

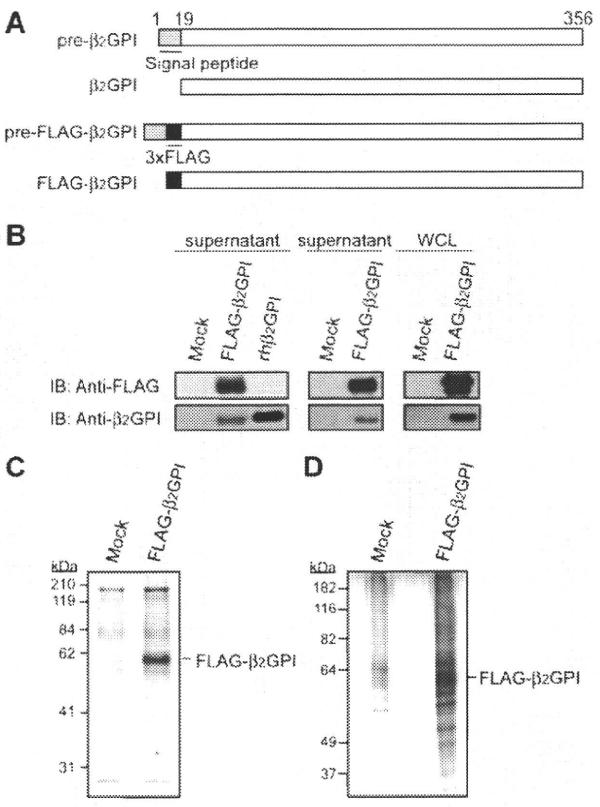


Fig. 1 Immunoaffinity purification of the β_2GPI-related proteome. (**A**) Schematic representation of β_2GPI. Grey box: signal peptide, black box: FLAG-tag. (**B**) Expression of secretory β_2GPI and intracellular β_2GPI. HEK293T cells were transfected with expression plasmids encoding FLAG-tagged β_2GPI. Cells were lysed and subjected to immunoblot analysis with anti-FLAG or anti-β_2GPI antibody. (**C**) Pull-down analysis of biotinylated cell surface proteins binding to FLAG-β_2GPI. RAW264.7 cell surface proteins were biotinylated using EZ-Link Sulfo-NHS-Biotin Reagent and then the cells were cross-linked with FLAG-β_2GPI using 3,3′-Dithiobis(sulfosuccinimidylpropionate). The cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. The resulting precipitates were subjected to SDS-PAGE and visualized with HRP-conjugated streptavidin. (**D**) Silver staining of β_2GPI-associated proteins. The β_2GPI-associated proteins purified by the procedure indicated in (**C**) were detected by silver staining.

Flowcytometric analysis

Surface aCL/β_2GPI and gelsolin binding on RAW264.7 cells was analysed using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with the CellQuest program. RAW264.7 cells were cultured with serum-free DMEM for 16 hrs. The cultured cells were washed with PBS including 2% BSA and 0.1% NaN_3 and treated with 50 μg/ml of β_2GPI at room temperature for 10 min., followed by exposure to primary antibodies for 30 min. on ice. After washing twice, cells were stained with Alexa488-labeled goat antimouse IgG antibody (Invitrogen) for 30 min. on ice. After washing twice, cells were analysed using FACSCalibur.

Luciferase assay

Stable κB luciferase reporter-expressing RAW264.7 cells were inoculated into a 24-well dish at 1×10^6 cells/500 μl of cell culture medium and stimulated as indicated [31]. After stimulation at 37°C for 4 hrs, the cells were harvested and lysed in 50 μl of cell culture lysis reagent (Promega Corporation), and then luciferase activity was measured using 20 μl of lysate and 100 μl of luciferase assay substrate (Promega Corporation). The luminescence was quantified with a luminometer (Berthold Japan, Tokyo, Japan).

Results

Immunoaffinity purification of β_2GPI-associated proteins

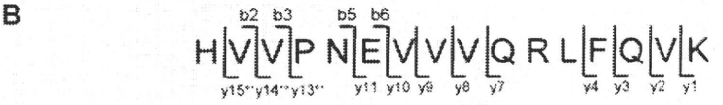
To isolate β_2GPI-binding proteins, we constructed FLAG-tagged human β_2GPI (Fig. 1A). Since β_2GPI binds to anionic phospholipids *via* a lysine-rich motif on domain V at the carboxy-terminus, we decided to preserve the intact structure at the carboxy-terminus. β_2GPI is a secretory apolipoprotein that is mainly produced in the liver and secreted to plasma. β_2GPI cDNA encodes a protein of 345 amino acids including a hydrophobic amino-terminal signal sequence (19 amino acid residues) that is lacking in the mature form of β_2GPI. Thus, the FLAG-tag sequence was placed between the signal peptide sequence and mature protein (Fig. 1A). An expression vector encoding FLAG-β_2GPI was transfected into HEK293T cells, and the culture supernatant and whole cell lysate were analysed by immunoblotting using anti-FLAG or anti-β_2GPI antibody. Immunoblot analysis showed that FLAG-β_2GPI was detected by anti-FLAG and anti-β_2GPI antibody from both the cell lysate and culture supernatant (Fig. 1B).

To confirm that β_2GPI binding proteins exist on the cell surface of RAW264.7 cells, we performed a pull-down assay using biotinylated cell surface proteins from RAW264.7 cells and FLAG-tagged β_2GPI. The cell surface proteins of RAW264.7 cells were biotinylated, incubated with FLAG-β_2GPI, and then biotinylated cell surface membrane proteins were chemically cross-linked with FLAG-β_2GPI. Cells were lysed, and the lysates were immunoprecipitated with anti-FLAG antibody to purify biotinylated proteins cross-linked to FLAG-β_2GPI and visualized with HRP-conjugated streptavidin. Smeared proteins other than FLAG-β_2GPI were reproducibly found (Fig. 1C). Next, we performed large-scale immunoaffinity chromatography with an anti-FLAG pull down assay. RAW264.7 cells were cultured with FLAG-β_2GPI and then the lysate of RAW264.7 cells was used for affinity chromatography with anti-FLAG antibody-conjugated Sepharose beads. The purified fraction eluted using FLAG-peptides was subject to SDS-PAGE and detected with silver staining. Silver staining indicated that a large number of smeared proteins interact with β_2GPI (Fig. 1D).

A

Start-stop	Charge	Sequence	Delta mass	Mascot score
2-10	2	VVEHPEFLK	-0.05	26
11-24	3	AGKEPGLQIWRVEK	-0.08	22
127 - 142	3	HVVPNEVVVQRLFQVK	-0.10	55
534 - 546	2	SGALNSNDAFVLK.	-0.06	58
547 - 564	2	TPSAAYLWVGAGASEAEK	-0.06	20

B



C

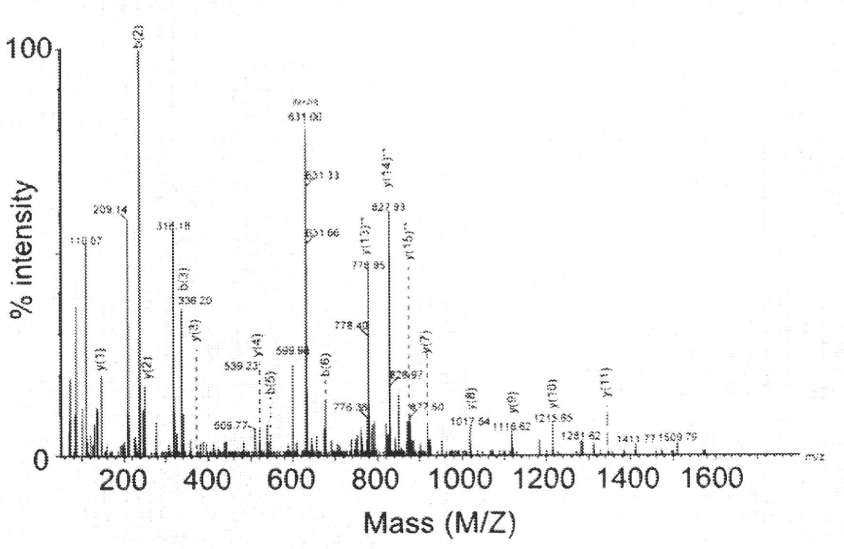


Fig. 2 Identification of β_2GPI-binding protein. (**A**) Identified peptide sequences of gelsolin by MS analysis. Five peptides corresponding to mouse gelsolin were identified. (**B**) Assigned b- or y- ions from amino acid sequence of 127 to 142 of mouse gelsolin are represented. (**C**) MS/MS spectrum of peptide 127 to 142 of mouse gelsolin. Fragment ions corresponding to b- and y- ions from identified sequence (124 to 142) are indicated.

Identification of β_2GPI-associated proteins by LC-MS

Proteins immunopurified with anti-FLAG (M2)-conjugated Sepharose were directly digested with Lys-C endopeptidase and analysed by an online-nanoLC-ESI-quadrupole time of flight hybrid mass spectrometer. Obtained MS/MS data were searched against the non-redundant National Center for Biotechnology Information (nrNCBI) database MASCOT algorithm. Many proteins with a significant MASCOT score were identified, but most of them were intracellular proteins such as molecular chaperones and ribosomal proteins that were abundant and seem to be contaminants (Table S1). These proteins were omitted from the list of identified protein, and proteins expressed on the membrane and/or cell surface were approved as candidates for β_2GPI-binding protein. Eventually, we found five peptides corresponding to gelsolin, and two of the five peptides had reliable MASCOT scores (more than 31, $P < 0.05$,) (Fig. 2A). The MS/MS data of the peptide (127–142 amino acid residue) with the highest MASCOT score are shown in Fig. 2B and C. Gelsolin has two types of localization pattern: one isoform localizes in the cytosol to regulate formation of actin fibre, whereas the other isoform, which has a signal peptide, localizes outside the cell or on the cell surface. We focused on plasma gelsolin as a β_2GPI-binding protein because gelsolin interacts with fibronectin and integrin, which can transduce intracellular signalling in collaboration with several types of kinase such as MAPK and FAK.

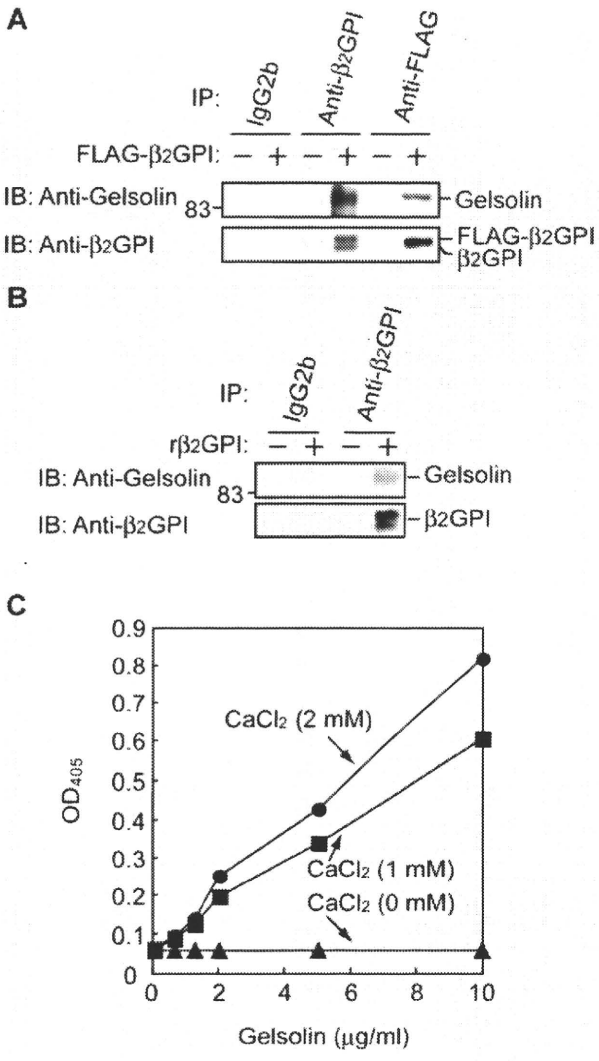


Fig. 3 Biochemical interaction between β_2GPI and plasma gelsolin. (**A**) Interaction between gelsolin and FLAG-β_2GPI. HEK293T cells were transfected with expression plasmids encoding FLAG-tagged β_2GPI and plasma gelsolin. Proteins secreted from transfected cells were subjected to immunoprecipitation with an antibody as indicated, and the resulting precipitates were subjected to immunoblot analysis with anti-gelsolin or anti-FLAG antibody. (**B**) Interaction between endogenous gelsolin and recombinant human β_2GPI. HEK293T cells were transfected with expression plasmids encoding gelsolin. Gelsolin secreted from transfected cells was mixed with human recombinant β_2GPI and subjected to immunoprecipitation with an antibody as indicated, and the resulting precipitates were subjected to immunoblot analysis with anti-gelsolin or anti-β_2GPI antibody. (**C**) The binding between phospholipid-bound β_2GPI and plasma gelsolin was confirmed by ELISA as described in the 'Materials and methods' section.

Interaction of β_2GPI with plasma gelsolin

To confirm the binding between β_2GPI and gelsolin, we examined the interaction between β_2GPI and gelsolin using HEK293T cells. HEK293T cells were transfected with expression plasmids encoding FLAG-tagged β_2GPI and plasma gelsolin. The cell lysates were immunoprecipitated with anti-β_2GPI (WBCAL1; monoclonal aCL/β_2GPI) or FLAG antibodies and then immunoblotted with anti-gelsolin or β_2GPI antibodies (MAB1066). Immunoprecipitation and immunoblot analysis revealed that FLAG-tagged β_2GPI specifically interacted with gelsolin (Fig. 3A). To further verify the interaction between recombinant β_2GPI and endogenous gelsolin, we examined the interaction with endogenous gelsolin. The supernatant including endogenous plasma gelsolin secreted from cultured RAW264.7 cells was mixed with human recombinant β_2GPI and subjected to immunoprecipitation with antibodies as indicated (WBCAL1 or Mock), and the resulting precipitates were subjected to immunoblot analysis with anti-gelsolin or anti-β_2GPI antibody (MAB1066). An *in vitro* pull-down assay showed that plasma gelsolin directly binds to β_2GPI (Fig. 3B). Direct binding of plasma gelsolin to cardiolipin-β_2GPI complex in a calcium-dependent fashion was confirmed by ELISA (Fig. 3C).

Plasma gelsolin enhances the localization of β_2GPI on the cell surface

It has been reported that plasma gelsolin binds to fibronectin, which belongs to the family of extracellular matrix (ECM) proteins and plays important roles in cellular adhesion, proliferation, differentiation and migration [32]. First, we confirmed the expression of β_2GPI (recombinant human β_2GPIs or FLAG-tagged β_2GPIs) on the cell surface by flowcytometric analysis. When RAW264.7 cells were incubated with β_2GPI at 37°C for 1 hr, interaction of β_2GPI on the cell surface was observed (Fig. 4A and B). To determine whether gelsolin affects the expression of β_2GPI on the cell surface, we examined the expression level of β_2GPI on the cell surface. The expression level of β_2GPI on the cell surface was enhanced in the presence of gelsolin compared to that in the absence of gelsolin (Fig. 4C). It has been shown that fibronectin, which binds to gelsolin, associates with the extracellular domain of the integrin family. To determine whether the cell surface expression of β_2GPI depends on integrin, we examined the expression level of β_2GPI with anti-integrin $\alpha_5\beta_1$ antibody as an inhibitory antibody. Anti-integrin $\alpha_5\beta_1$ antibody inhibited the expression of β_2GPI on the surface of RAW264.7 cells (Fig. 4D and E). Furthermore, to determine whether anti-integrin $\alpha_5\beta_1$ antibody affects the expression of β_2GPI without addition of recombinant β_2GPI, RAW264.7 cells cultured with serum-free medium were incubated with anti-integrin $\alpha_5\beta_1$ antibody, and then the cells were stained by aCL/β_2GPI. Flowcytometric analysis showed that without addition of recombinant β_2GPI, anti-integrin $\alpha_5\beta_1$ antibody does not affect the staining by anti-β_2GPI antibody (WBCAL1) (Fig. 4F). These findings indicate that gelsolin

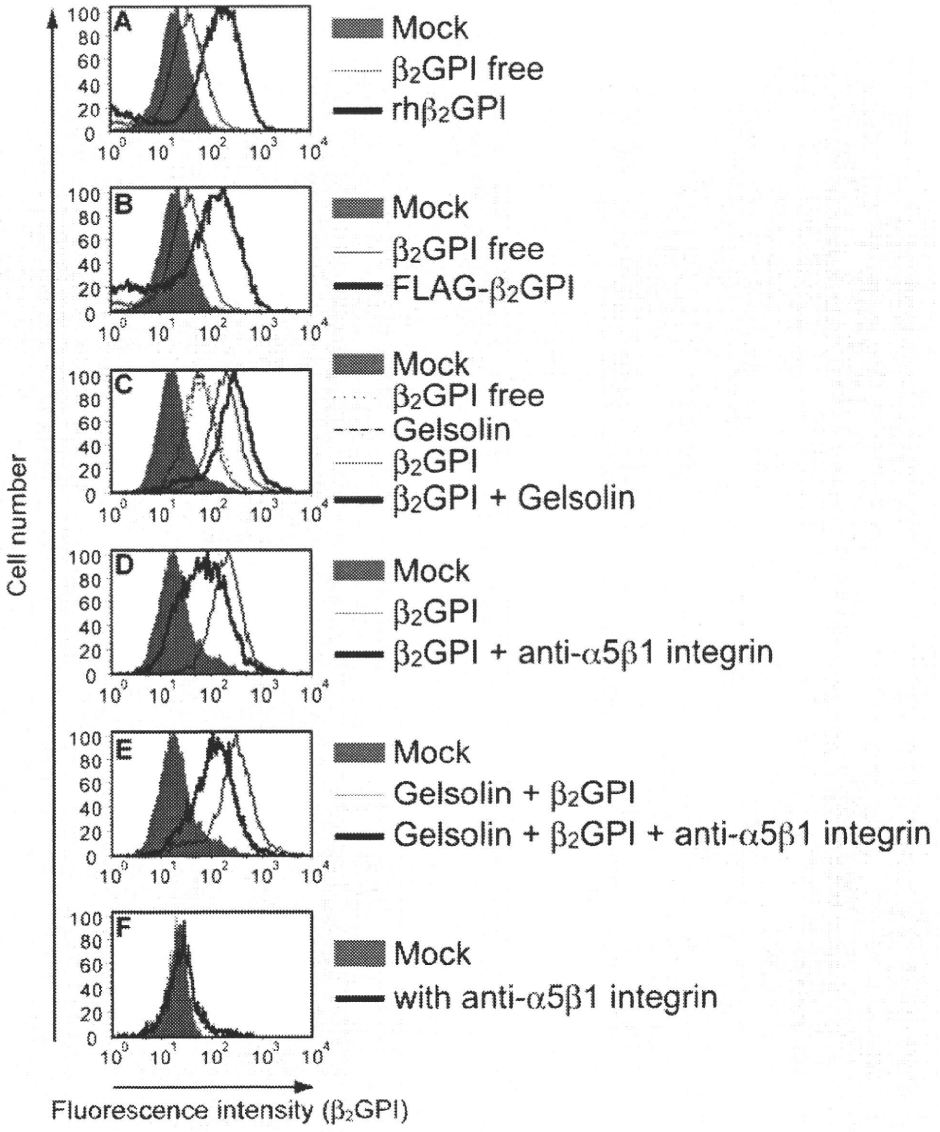


Fig. 4 Interaction of β_2GPI and plasma gelsolin on cell surface. (**A** and **B**) β_2GPI binding to the cell surface. RAW264.7 cells cultured with serum-free medium were incubated with or without recombinant human β_2GPI or FLAG-β_2GPI, and then binding of β_2GPI to the cell surface was detected by aCL/β_2GPI (WBCAL1). Mock has no primary antibody. Binding to the cell surface by recombinant human β_2GPI (**A**) showed almost the same intensity as that by secreted FLAG-β_2GPI (**B**). (**C**) Gelsolin affects the binding of β_2GPI to the cell surface. RAW264.7 cells were incubated with or without recombinant FLAG-β_2GPI and gelsolin, and then binding of β_2GPI to the cell surface was detected by aCL/β_2GPI. (**D** and **E**) Binding of β_2GPI to the cell surface was inhibited by anti-integrin $\alpha_5\beta_1$ antibody. RAW264.7 cells were incubated with or without recombinant FLAG-β_2GPI, gelsolin and anti-integrin $\alpha_5\beta_1$ antibody, and then binding of β_2GPI to the cell surface was detected by aCL/β_2GPI. **(F)** RAW264.7 cells cultured with serum-free medium were incubated with anti-integrin $\alpha_5\beta_1$ antibody, and then the cells were stained by aCL/β_2GPI.

enhanced the cell surface expression of β_2GPI and that the interaction is mediated by integrin on the cell surface.

Intracellular signalling *via* aCL/β_2GPI antibody is dependent on integrin $\alpha_5\beta_1$

We previously reported that p38-MAPK was phosphorylated in RAW264.7 cells stimulated by human monoclonal aCL/β2GPI [22]. To determine whether a cell surface complex including gelsolin activates RAW264.7 cells, we investigated the phosphorylation of p38-MAPK. Stimulation to RAW264.7 cells by aCL (WBCAL1) showed that p38-MAPK phosphorylation was not induced by plasma gelsolin alone but was induced by aCL/β_2GPI stimulation and was further enhanced by plasma gelsolin plus aCL/β_2GPI stimulation (Fig. 5A). However, anti-integrin $\alpha_5\beta_1$ antibody attenuated phosphorylation of p38-MAPK by plasma gelsolin plus aCL/β_2GPI stimulation (Fig. 5A). These findings indicate that aCL/β_2GPI caused phosphorylation of p38-MAPK in collaboration with gelsolin and integrin on the cell surface. Furthermore, to determine the effect on downstream molecules such as focal adhesion kinase FAK, the phosphorylation of FAK by aCL/β_2GPI was investigated. Stimulation of aCL/β_2GPI and plasma gelsolin resulted in an increased level of phosphorylation of FAK, whereas anti-integrin $\alpha_5\beta_1$ antibody attenuated the phosphorylation of FAK (Fig. 5B). Taken together, the results suggest that anti-β_2GPI

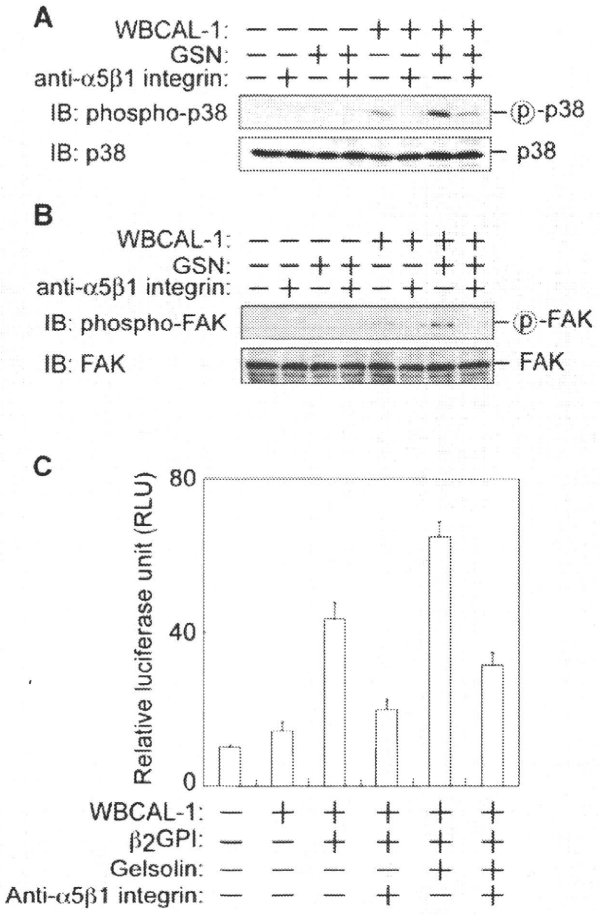


Fig. 5 Change in intracellular signalling by gelsolin and aCL/β_2GPI. (**A**) Gelsolin and integrin affect phosphorylation of p38 MAPK by anti-β_2GPI antibody. RAW264.7 cells were incubated for 2 hrs as indicated after serum-free culture for 16 hrs and then stimulated with aCL/β_2GPI (WBCAL1) for 30 min., and then phosphorylation of p38 MAPK was determined by immunoblot analysis using specific antibodies against total-p38 and phospho-p38. (**B**) Anti-integrin $\alpha_5\beta_1$ antibody inhibits phosphorylation of FAK by aCL/β_2GPI. RAW264.7 cells were stimulated with aCL/β_2GPI for 10 min. and then phosphorylaion of FAK was determined by immunoblot analysis. (**C**) aCL/β_2GPI increases NF-κB activity. RAW264.7 cells stably expressing κB luciferase reporter were inoculated into a 24-well dish and stimulated as indicated. After stimulation at 37°C for 4 hrs, κB luciferase activity was measured.

antibody affects the integrin signalling including its downstream signal molecule FAK, followed by activation of p38-MAPK.

It has been reported that the p38-MAPK pathway is linked to the NF-κB pathway [33]. To determine whether aCL/β_2GPI antibody functions with gelsolin, we examined its effect on relative luciferase activity by NF-κB. The transcriptional activity of NF-κB was further increased by stimulation with the combination of gelsolin and one of the aCL/β_2GPI antibodies, WBCAL1, whereas it was inhibited by anti-integrin $\alpha_5\beta_1$ antibody (Fig. 5C). These findings indicate that aCL/β_2GPI causes the engagement of integrin with gelsolin, resulting in activation of the p38-MAPK pathway and NF-κB pathway.

Discussion

We identified plasma gelsolin as a novel β_2GPI-binding protein on the cell surface of monocytes. Affinity purification using an anti-FLAG-β_2GPI-conjugated column clarified that β_2GPI interacts with gelsolin on monocytes, and then the binding of β_2GPI with gelsolin was confirmed by immunoprecipitation. Moreover, flowcytometric analysis demonstrated that gelsolin enhances the affinity of β_2GPI on the cell surface. Gelsolin is expressed as two isoforms, cytoplasmic gelsolin and plasma gelsolin, which are encoded by a single gene and produced by alternative translation [34]. Both gene products of gelsolin have six homologous repeats (S1–S6), each of which contains 120–130 amino acid residues, and plasma gelsolin has an extra 23 amino acid residues at the amino-terminus [35, 36]. Cytoplasmic gelsolin is known as an actin-depolymerizing factor and plays a crucial in removal of actin released by tissue injury. Plasma gelsolin has another function as a carrier protein for bioactive mediators such as lysophosphatidic acid (LPA), lipopolysaccharide (LPS), amyloid β protein (Aβ) and platelet-activating factor (PAF) to protect cells from exposure to excess stimulation [37–41]. Plasma gelsolin also interacts with fibronectin and especially colocalizes at a region where inflammation arises [32]. Fibronectin forms a dimeric glycoprotein in plasma and a dimeric or multimeric form that interacts with integrin $\alpha_5\beta_1$ on the cell surface. Fibronectin is involved in cell adhesion, morphological change and migration processes, including wound healing, blood coagulation, host defense and metastasis.

Integrins are heterodimeric membrane proteins composed of an α chain and a β chain. Each specific integrin induces a variety of responses in different cell types. Integrin α5 chain undergoes post-translational cleavage to yield disulfide-linked light and heavy chains that join with the β_1 chain to form a fibronectin receptor [42]. Integrins provide dynamic, physical links between the ECM such as fibronectin and cytoskeletons. In addition to adhesion, integrins are known to participate in cell surface-mediated signalling in concert with other cell surface receptors, including growth factor receptors such as epidermal growth factor, lysophosphatidic acid or thrombin, and are involved in proliferation, survival, morphological change, migration and gene expression. Ligation of ECM to integrins triggers assembly of cytoskeleton proteins (such as tallin, actin and paxillin) and intracellular tyrosine kinase FAK and results in a large variety of signal transduction events. Integrin-mediated signals is likely to be necessary in normal cells, such as human umbilical vein endothelial cells or mammary epithelial cells, to block apoptosis *via* the Akt pathway and activate cells *via* the MAPK pathway [43–45]. In

Ntera2 neuronal cells, $\alpha_5\beta_1$-mediated adhesion to fibronectin decreased apoptosis. Previous studies have shown that expression of $\alpha_5\beta_1$ promotes apoptosis of human hematopoietic cell lines, monocyte-differentiated HL-60 cell lines and mouse macrophage RAW264.7 cell lines [46–48]. However, it has been reported that fibronectin could not mediate the binding of β_2GPI to the cell surface in endothelial cells [27, 49]. We confirmed direct interaction of phospholipid-bound β_2GPI and gelsolin by ELISA, and the binding was found only in the presence of calcium. The interaction of β_2GPI with gelsolin in our study suggests that engagement of β_2GPI by anti-β_2GPI antibody caused the complex formation including gelsolin, fibronectin and integrin $\alpha_5\beta_1$, followed by activation of the p38-MAPK pathway and NF-κB pathway.

Zeisel *et al.* reported that FAK and myeloid differentiation protein 88 (MyD88) pathways were inter-linked and initiate a pro-inflammatory response through NF-κB activation [50]. In a previous study, we demonstrated that the p38 MAPK-dependent signalling pathway participates in aPL-mediated TF expression. A specific inhibitor of p38 MAPK decreased TF mRNA expression induced by aCL/β_2GPI stimulation, indicating a crucial role of the p38 MAPK pathway in APS. Raschi *et al.* reported that a dominant-negative form of TNF-receptor-associated factor 6 (TRAF6) and MyD88 abrogated NF-κB activation induced by monoclonal aCL/β_2GPI, suggesting that aCL/β_2GPI reacts to β_2GPI associated with a member of the TLR or interleukin-1 receptor family. The present study demonstrated that gelsolin is a scaffolding protein that links β_2GPI and integrin/fibronectin and that integrin is also important for activation of the p38 MAPK and NF-κB pathways by aCL/β_2GPI.

Several inhibitors for integrins have been developed and investigated in animal models of inflammatory diseases, and some of these inhibitors (*e.g.* anti-integrin αIIbβ_3, anti-integrin $\alpha_4\beta_7$) are used clinically as anti-platelet agents or anti-inflammatory bowel disease agents [51]. Recently, arginine-glycine-aspartic acid (RGD) peptides that bind to integrin $\alpha v\beta_3$, $\alpha v\beta_5$ or $\alpha_5\beta_1$ have become available for the treatment of inflammatory arthritis [52]. This is a first report to prove how the β_2GPI-aCL/β_2GPI interaction on monocyte surface occurs with its partner molecule, gelsolin. Despite the fact that we could not show direct procoagulant alteration of cells by aCL/β_2GPI with integrin $\alpha_5\beta_1$ blockade in this study, either on monocytes or on endothelial cells, our findings provide a clue for establishing specific treatments by down-regulating the p38 MAPK pathway *via* integrin $\alpha_5\beta_1$ and therapeutic benefits for patients with autoimmmune diseases, including APS.

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Supporting Information

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

Table S1. Peptide sequences of β_2 GPI-associated proteins identified by MS analysis.

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