Angiopoietin-Like 2, a Circadian Gene, Improves Type 2 Diabetes Through Potentiation of Insulin Sensitivity in Mice Adipocytes

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Angiopoietin-like (Angptl)2, a member of the Angptl protein family, is predominantly secreted from adipose tissue and the heart. Here, we demonstrate that the expression of Angptl2 in epididymal adipose tissue of C57BL/6J mice shows pulsatility and circadian rhythmicity and that the rhythmicity was disrupted in high-fat-fed and leptin receptor-deficient diabetic db/db mice with insulin resistance. To investigate whether the reduction in Angptl2 expression was related to the progression of diabetes, we treated db/db mice with recombinant Angptl2 for 4 wk during the peak period of Angptl2 expression in C57BL/6J mice. Angptl2-treated mice showed decreases in plasma glucose, insulin, triglyceride, and fatty acid levels and an increase in plasma adiponectin, a therapeutic regulator of insulin resistance, leading to improvements in glucose tolerance. In cultured adipocytes, recombinant Angptl2 increased adiponectin expression and stimulated insulin sensitivity partially by reducing the levels of tribbles homolog 3, a specific Akt kinase inhibitory protein. Conversely, Angptl2 small interfering RNA reduced adiponectin expression, resulting in insulin resistance. In preadipocytes, treatment with Angptl2 small interfering RNA inhibited differentiation to adipocytes and reduced adiponectin expression. Taken together, our results suggest that replenishment of Angptl2 stimulates insulin sensitivity and improves the type 2 diabetic state. (*Endocrinology* 152: 2558–2567, 2011)

A ngiopoietin-like (Angptl) proteins have been identified as several molecules containing a coiled-coil domain and a fibrinogen-like domain, motifs that are structurally conserved in angiopoietins. However, none of these proteins bind to either tyrosine kinase with immunoglobulin-like and EGF-like domains 2 or 1 and are still considered orphan ligands. Several studies have shown that Angptl have the potential to regulate angiogenesis by their action as angiopoietins. In addition, some Angptl are potent regulators of lipid, glucose, and energy metabolism. For example, Angptl3 and Angptl4 regulate triglyceride metabolism by suppressing lipoprotein lipase (1, 2).

There is also evidence that Angptl6/angiopoietin-related growth factor (AGF)-deficient mice have significantly larger both visceral and sc fat depots than wild-type mice (3). Furthermore, AGF suppresses gluconeogenesis in hepatocytes (4). On the basis of these findings, it appears that Angptl plays a role in the regulation of metabolic function.

Energy homeostasis, including glucose and lipid metabolism, is subject to circadian regulation that synchronizes energy intake and expenditure with changes in the external environment throughout the daily 24-h cycle (5). Circadian clocks are present in most tissues of the body,

where they control the expression of 5–10% of tissue-specific mRNA through both transcriptional and post-transcriptional regulation (6, 7). The central clock gene Clock:Bmal1 dimerizes to drive the expression of circadian effector genes implicated in a multitude of physiological functions (8, 9). Mutations in the central clock genes, Clock and Bmal1, result in metabolic changes leading to obesity and the metabolic syndrome (10–12). Kohsaka *et al.* (13) demonstrated recently that a high-fat diet disrupts the expression of circadian clock-controlled genes involved in fuel utilization in the liver and adipose tissue. These reports suggest that circadian genes play a crucial role in obesity and the metabolic syndrome.

In this study, we sought to identify the circadian genes in the Angptl family and found that both Angptl2 and Angptl4 act as circadian genes in adipose tissue (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Several studies have reported that Angptl2 induces sprouting in endothelial cells (14, 15), indicating a role in angiogenesis. Based on amino acid similarity, Angptl2 is notably closer to the antidiabetic factor Angptl6/AGF than it is to Angptl3 or Angptl4, factors which accelerate hyperglycemia (16). From these reports, we speculated that Angptl2 is an antidiabetic factor. To verify this assumption, we investigated whether administration of Angptl2 improved changes associated with diabetes in db/db mice. We also demonstrated that Angptl2 enhanced insulin sensitivity in adipocytes.

Materials and Methods

Animals

All experiments were conducted using 12-wk-old male mice. We obtained 5-wk-old C57BL6J mice and diabetic db/db mice from Charles River Laboratories (Germantown, MD). The mice were maintained on a 12-h light, 12-h dark cycle (LD) in normal cages with food and water *ad libitum*. Zeitgeber time (ZT) was used as the time scale for the LD conditions. ZT0 represents lights on and ZT12 represents lights off. For high-fat feeding, mice were fed D12451 (45% of kilocalories from fat; Research Diets, Inc., New Brunswick, NJ) for 7 wk. To investigate the antidiabetic effect of Angptl2, 8-wk-old db/db mice were injected ip with 0.8 mg/kg body weight Angptl2 at ZT12 for 4 wk. Tissue and serum samples were collected 2 h after the final administration of Angptl2. All experimental procedures were conducted according to the policies of the Animal Ethical Committee of Astellas Pharma, Inc. (Ibaraki, Japan).

Antibodies

The antibodies that recognize Angptl2 were from R&D Systems (Minneapolis, MN); the antiactin antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); the antibodies that recognize phosphorylated Thr308 and Ser473 on Akt, phos-

phorylated Thr202/Tyr204 on Erk1/2, phosphorylated Ser256 on forkhead box protein O1 (FoxO1), phosphorylated Ser636/639 on insulin receptor substrate (IRS)-1, anti-Akt, anti-Erk1/2, anti-FoxO1, and IRS-1 were from Cell Signaling Technology (Beverly, MA); the antibody that recognizes phosphorylated Thr642 on Akt substrate of 160 kDa (AS160) and anti-AS160 were from Upstate (Waltham, MA); and the antibody that recognizes tribbles homolog 3 (Trib3) was from Calbiochem (La Jolla, CA).

Circadian gene experiments

To investigate the circadian expression of genes, we collected epididymal adipose tissue and serum every 4 h over the course of 2 d under LD conditions. We began to sample epididymal adipose tissues at ZT0. Total RNA was prepared using Trizol reagent (Invitrogen/Life Technologies, Inc., Carlsbad, CA). Tissues lysates for Western blotting were prepared using sodium dodecyl sulfate (SDS) sampling buffer [1 mM EDTA, 1% SDS, and 10 mM HEPES-HCl (pH 7.5)] containing Complete (Roche Applied Science, Mannheim, Germany) and Phosphatase inhibitor cocktails (Sigma Chemical Co., St. Louis, MO). Albumin was removed from the serum samples using the ProteoExtract Albumin/IgG Removal kit (Calbiochem).

Quantitative PCR

Quantitative PCR was performed using SYBR Green Reagent on an ABI Prism 7700 machine (Applied Biosystems, Foster City, CA) as described previously (4). The oligonucleotides used for PCR are listed in Table 1.

Preparation of recombinant human Angptl2 protein

Purification of recombinant human Angptl2 protein was carried out as described by Ito and co-workers (15). Briefly, the conditioned medium from transfected HEK293 cells was collected and passed through a 0.22-μm pore size filter (Millipore, Bedford, MA). To purify Angptl2 protein, the filtered conditioned medium was transferred to an anti-FLAG antibody (M2) affinity gel (Sigma Chemical Co.), with only Angptl2 protein being trapped in the gel. After the gel was washed with PBS, the protein was eluted by adding Gly-HCl (pH 3.0) and immediately neutralized with Tris-HCl (pH 8.0). Angptl2 protein was dialyzed in PBS overnight at 4 C. Protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, MA).

Metabolic measurements

Enzymatic assay kits were used to determine the levels of triglycerides (Sigma Chemical Co.) and nonesterified fatty acids (Roche Applied Science). Plasma glucose was measured by an Amplex Red Glucose/Glucose Oxidase Assay kit (Invitrogen, Carlsbad, CA) based on the immobilized glucose oxidase membrane/hydrogen peroxide electrode method. Plasma insulin was determined using a mouse insulin ELISA kit (Morinaga Biochemistry Co., Kanagawa, Japan) and plasma adiponectin by an ELISA kit (Otsuka Pharmaceutical Co., Tokushima, Japan).

Glucose tolerance test

Mice were fasted for 16 h and then injected ip with glucose (2 g/kg body weight) for measurement of plasma glucose at the indicated times.

TABLE 1. Quantitative PCR primers

Gene	Accession no.	Forward primer	Reverse primer
Angptl2	NM_011923	TGCCATTTGTGTCAACTCCAA	CACGATGCCTCCGTCTACCT
Angptl4	NM_020581	GCCAAATTGCTCCAATTTCCT	CGTGGTCTTGGTCCCAGGTA
G6pc	NM_008061	CAGGGCTGTTTGAGGAAAGTG	GGAAGATTCTGCACCGCAAG
Pck1	NM_011044	AGCATGCGCTGAGATCTAGGA	GGTGATTTCCCCTCCCAATC
Cpt1a	NM_013495	AGCGATGCAGAATCTCATTGG	GGGTCTCACTCTCCTTGCCA
Cyp7a1	NM_007824	TGATGTAACGACTGCTGAGCG	AAGTGATGAAACTCAGGCCCC
Acly	NM_134037	GGCTGGGACCATTGTATCCTT	GTCGGCTGCATGAACACAGA
Acacb	NM_133904	TACAGGTGGCTCAGCTGCTG	TGGAGTGGCTCCGTGAGTTT
Fasn	NM_007988	AGTGTTGGTGCAAGCCCTCT	CAAGCAACCTCCACTCCTCTG
Lpl	NM_008509	GAACACCAACCCACATGCAA	AGGCACAACCAGCTTTCTCCT
Il1b	NM_008361	GGCTGGACTGTTTCTAATGCCT	TTTGAACAGAATGTGCCATGGT
Il6	NM_031168	CTGGAGTCACAGAAGGAGTGGC	CCACAGTGAGGAATGTCCACAA
TNF-α	NM_013693	GATTATGGCTCAGGGTCCAACT	CATTCGAGGCTCCAGTGAATTC
MCP—1	NM_011333	CATCTGCCCTAAGGTCTTCAGC	GCATCACAGTCCGAGTCACACT
CD68	NM_009853	CCCAAGGAACAGAGGAAGACTG	GTGTGAACTGTGACATTTCCGTG
aP2	NM_024406	GCCAAGCCCAACATGATCA	ATTCCACGCCCAGTTTGAAG
Adipoq	NM_009605	CTGGCAGGAAAGGAGAGCCT	CCAGTGCTGCCGTCATAATG
Cebpa	NM_007678	CAAAGCCAAGAAGTCGGTGGAC	GCGGTCATTGTCACTGGTCAAC
Cebpb	NM_009883	AATCACCTTAAAGATGTTCCTGCG	TCGAAACGGAAAAGGTTCTCAA
Ppara	NM_011144	AATTAACGGGTAACCTCGAAGTCTG	TAAATGGCTAACCTTGGGCCAC
PParg	NM_011146	GCAGCTACTGCATGTGATCAAGA	GGGTGGGACTTTCCTGCTAATAC
Trib3	NM_175093	GCTGGCAGATACCCATTCCA	CCACAAGTCGCTCTGAAGGTTC
b-actin	NM_007393	TGAGAGGGAAATCGTGCGTGAC	AAGAAGGAAGGCTGGAAAAGAG

Cell culture

Mouse preadipocyte 3T3-L1 cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum. For adipocyte differentiation, the cells were grown to full confluence for 2 d and then induced with differentiation medium containing 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine. After 2 d of induction, the cells were cultured for 2 d in progression medium containing 10 μg/ml insulin. The medium was then changed to DMEM with 10% fetal bovine serum for differentiation at 37 C and an atmosphere containing 5% CO_2.

Transfection

Angptl2 small interfering RNA (siRNA) and negative control siRNA were purchased from Invitrogen. Several siRNA oligonucleotides were screened and tested for their ability to inhibit Angptl2 expression. The most active of these oligonucleotides for mouse Angptl2 (sense, 5′-UGAAAGUGUAGGUGCACUUGUCGGG-3′; antisense, 5′-CCCGACAAGUGCACCUACACUUUCA-3′), mouse Trib3 (sense, 5′-CACAGUUGCUGAAGACAAAGCGACG-3′; antisense, 5′-CGUCGCUUUGUCUUCAGCAACUGUG-3′) effectively blocked the expression of Angptl2 or Trib3. We transfected 3T3-L1 preadipocytes and adipocytes with each of the siRNA at a final concentration of 30 nM using Lipofectamine RNAiMAX (Invitrogen). All the experiments were performed 2 d after transfection.

Glucose uptake assay

Day 7–11 3T3-L1 adipocytes were incubated for 3 h in serum- and glucose-free media. Basal and insulin-induced glucose uptakes were measured by rinsing the cells and then incubating in serum- and glucose-free media for 30 min with or without 1 nM insulin, followed by the addition of unlabeled 2-deoxy-D-glucose (1 mM) and 1 μCi of 2-deoxy-D-[^{14}C] glucose (Amersham Biosciencess/GE Healthcare, Princeton, NJ). After incubation

for 5 min at room temperature, the media were removed, and the cells were washed in cold PBS and lysed in 300 μl of 0.1% SDS. The radioactivity associated with 50 μl was determined by liquid scintillation counting. The results were corrected for nonspecific binding using control cells incubated in the presence of 20 μM cytochalasin B. Glucose uptake was normalized to protein content as measured from the remaining cell lysates with BCA protein assay kit (Pierce).

Results

Disruption of rhythmic expression of Angptl2 in db/db and DIO mice

To determine whether mRNA expression of Angptl in epididymal adipose tissue showed daily rhythms, fat samples were obtained from 12-wk-old C57BL/6J mice every 4 h throughout a 48-h period. As shown in Fig. 1A, expression of the Angptl2 and Angptl4 genes exhibited 24-h rhythmicity. In addition, peak expression of Angptl2 mRNA at ZT12 corresponded with the peak period of food consumption and was opposite to the phase of Angptl4 mRNA expression, which peaked at ZT0. The rhythmic expression of Angptl2 mRNA (Fig. 1B) and protein (Fig. 1C) was disrupted in the epididymal fat of db/db mice as a result of reduced mRNA expression during the peak period. Angptl2 was expressed not only in fat but also in heart (Supplemental Fig. 2), although the reduction in Angptl2 mRNA was not observed in the heart at ZT12 (Fig. 1D). Serum Angptl2 levels were also found to show circadian expression and were reduced in db/db mice from
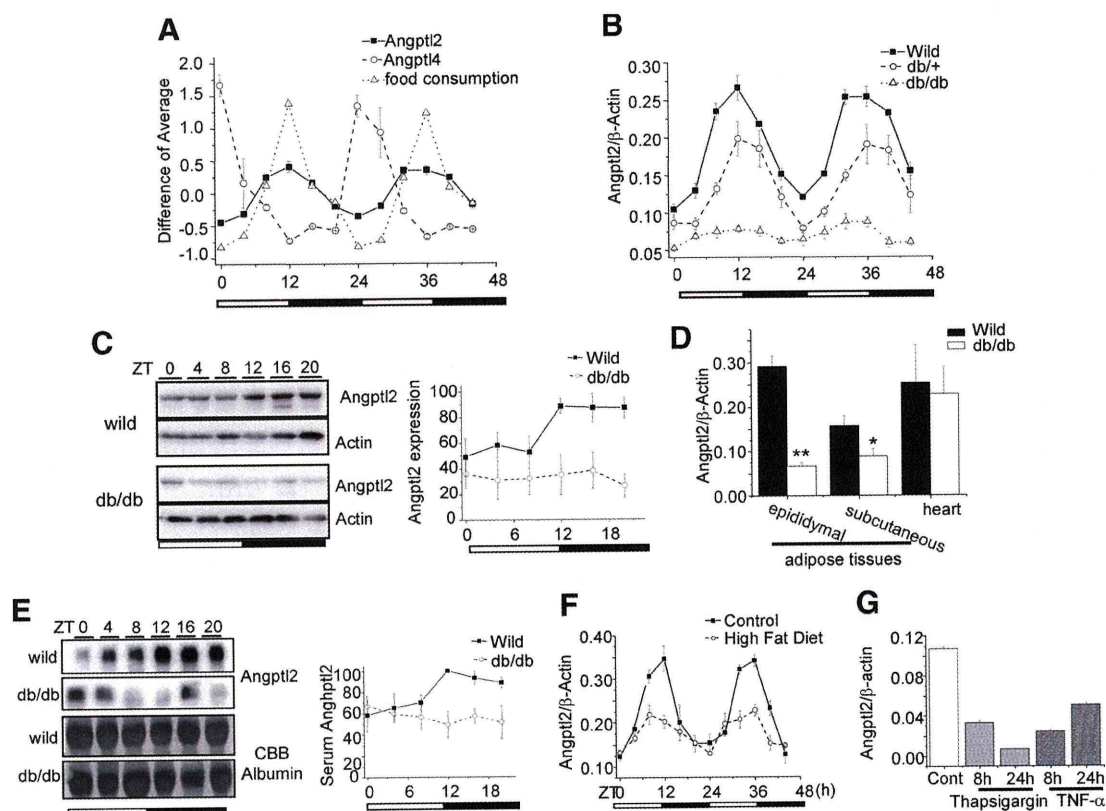
FIG. 1. Disruption of circadian expression of Angptl2 by diabetes and obesity in epididymal adipose tissue. A, Temporal expression profiles of Angptl2 and Angptl4 mRNA in epididymal adipose tissue from C57BL6 mice, determined by quantitative PCR assay (n = 3). Temporal pattern of food intake was calculated at every 4 h. In this and all other figures, the *error bars* represent ±SEM. Male mice aged 12 wk were used in the experiments. Data were normalized so that the average signal intensity of 24 time points was 0. B and C, Temporal expression profiles of Angptl2 mRNA (n = 3) (B) and protein (C) for each genotype. C, Immunoblotting was performed with anti-Angptl2 antibody. Actin was used as an internal control (*left panel*). The graph represents the ratio of band densities, comparing with maximum points (*right panel*) (n = 3). D, Angptl2 mRNA expression in epididymal and sc adipose tissues and heart of wild or db/db mice (n = 3). E, Temporal expression profiles of Angptl2 in serum. Representative Western blottings (*left panel*) and quantitative data (right *panel*) (n = 3) for the Angptl2 are shown. Coomassie Brilliant Blue-stained albumin is as control bands for protein loading. F, Temporal expression profiles of Angptl2 in epididymal adipose tissue of mice fed either a normal (CE-2) or high-fat diet (D12451) for 8 wk (n = 3). G, Angptl2 mRNA expression in differentiated 3T3-L1 adipocytes treated with 2 μM Thapsigargin or 10 ng/ml TNF-α for indicated time (n = 3). **, $P < 0.01$; *, $P < 0.05$ compared with wild mice.

ZT12 to ZT16 (Fig. 1E). Furthermore, the reduced expression of Angptl2 mRNA was also observed at ZT12 in mice with high-fat diet-induced obesity (Fig. 1F). Investigation of the relationship between Angptl2 expression and the major pathoetiology of diabetes showed that Angptl2 mRNA expression in differentiated 3T3-L1 adipocytes was reduced by 8- or 24-h treatment with either 2 μM thapsigargin, an endoplasmic reticulum stress inducer or 10 ng/ml TNF-α, major factor of insulin resistance (Fig. 1G).

Angptl2 improves circulating plasma glucose and lipid levels

To examine the effectiveness of Angptl2 in treating diabetes, db/db mice were administered a daily ip injection of recombinant human Angptl2 at 0.8 mg/kg body weight per day at ZT12 for 4 wk. The control group was treated with vehicle buffer (PBS) for the same interval. The plasma human Angptl2 concentration was approximately 100

ng/ml at 2 h after administration and decreased to less than 50 ng/ml at 12 h (Supplemental Fig. 3). These concentrations are similar to the active concentrations *in vitro* (15). These results also indicated that the administration of Angptl2 caused variations in its plasma concentration over time, as well as the physiological condition. No significant difference in daily food intake (PBS, 5.79 ± 0.188 g/d; Angptl2, 5.80 ± 0.139 g/d) or body weight was observed between the two groups (Fig. 2A). Epididymal adipose tissue weight was slightly decreased (PBS, 2.00 ± 0.117 g; Angptl2, 1.77 ± 0.092 g). A significant decrease in random-fed glucose levels was observed in the 2 wk after administration of Angptl2 compared with controls (Fig. 2A). The Angptl2-treated db/db mice also showed a decrease in serum insulin levels and increases in serum adiponectin levels and mRNA expression in mesenteric adipose tissues to levels similar to those in wild-type mice (Fig. 2B). An ip glucose tolerance test showed that db/db
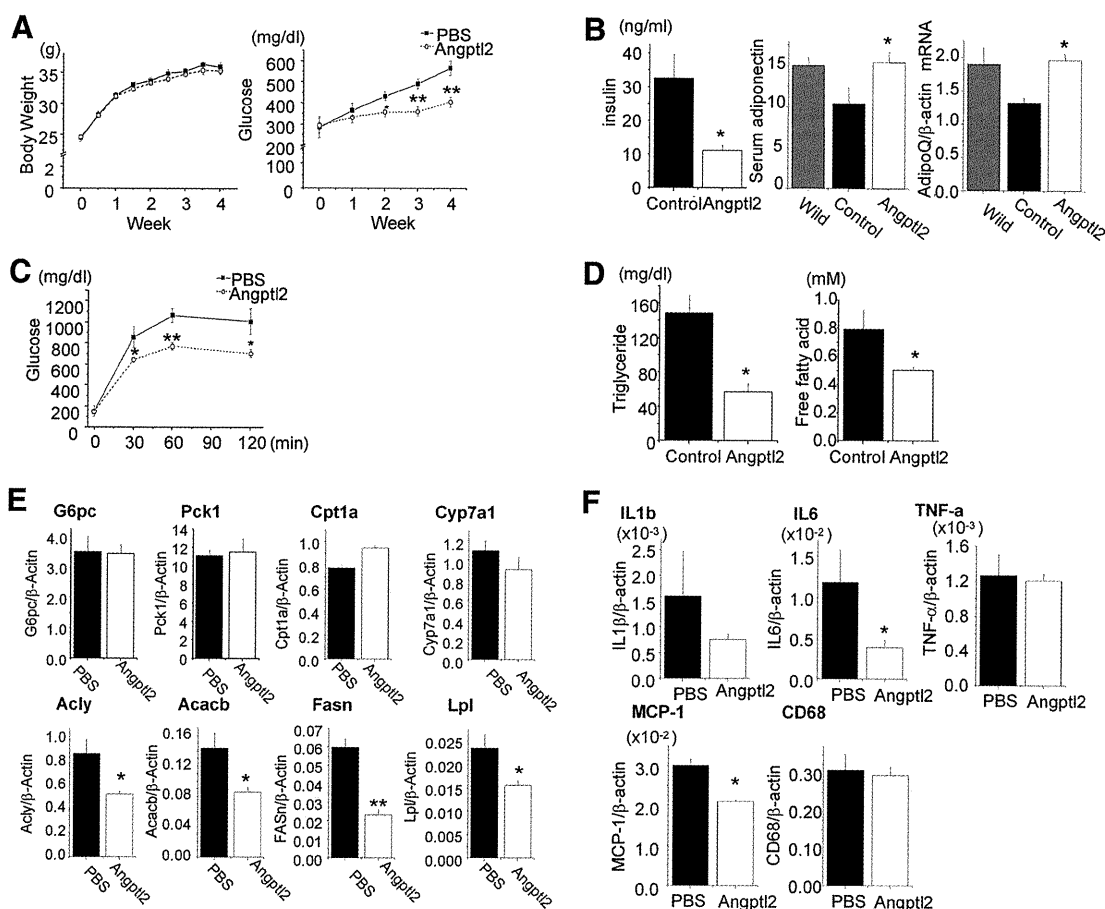
FIG. 2. Effects of Angptl2 on insulin resistance. Angptl2 or PBS control was administered to db/db mice for 4 wk. A, Body weight and fed blood glucose levels of db/db mice administered 0.8 mg/kg body weight per day Angptl2 or PBS at ZT12 for the indicated periods starting at 6 wk of age. B, Plasma insulin (*left*) and adiponectin (*middle*) concentrations and expression of adiponectin in mesenteric adipose tissues (*right*) in wild mice and db/db mice administered Angptl2 or PBS. C, Blood glucose concentrations during ip glucose tolerance tests in db/db mice administered Angptl2 or PBS for 4 wk. The final administration of Angptl2 was conducted at the start of fasting. Mice were fasted for 16 h and then injected ip with glucose (2 g/kg body weight). D, Serum triglyceride and free fatty acid concentrations in db/db mice administered Angptl2 or PBS. E, Quantitative RT-PCR of mRNA encoding fatty acid metabolism-, gluconeogenesis-, and cholesterol metabolism-related genes in liver. F, Quantitative RT-PCR of mRNA encoding inflammatory factors and macrophage markers in mesenteric adipose tissue (n = 5 animals per group). **, $P < 0.01$; *, $P < 0.05$ compared with vehicle control.

mice had improved glucose tolerance after Angptl2 treatment (Fig. 2C). Furthermore, serum triglyceride and free fatty acid levels also decreased with Angptl2 administration (Fig. 2D). We investigated mRNA expression in the liver of genes related to fatty acid synthesis (Acly, Acacb, Fasn, and Lpl) and oxidation (Cpt1a), gluconeogenesis (G6pc and Pck1), and cholesterol metabolism (Cyp7a1) (Fig. 2E). The expression of gluconeogenesis-related and cholesterol metabolism-related genes did not change significantly, whereas the expression of fatty acid synthesis and metabolism-related genes (Acly, Acacb, Fasn, and Lpl) was reduced in the liver of mice administered Angptl2. Inflammations in adipose tissues cause insulin resistance. We next examined the expression of mRNA for inflammatory cytokines (IL-1β, IL-6, and TNF-α), a chemokine (monocyte chemotactic protein-1), and macrophage marker [cluster of differentiation 68 (CD68)] in mesenteric adipose tissue. Administration of Angptl2 in-

duced decreases in IL-6 and monocyte chemotactic protein-1 mRNA expression, whereas the expression of IL-1β, TNF-α, and CD68 remained unchanged (Fig. 2F).

Angptl2 is a regulator of adipogenesis in 3T3-L1 cells

We next investigated the effects of Angptl2 on premature and mature adipocytes using 3T3-L1 cells. Consistent with previous work that has shown mice treated with Angptl2 have increased serum adiponectin levels, expression of the adipocyte-specific protein, Angptl2 was highest in preadipocytes and in fully mature adipocytes, on d 0 and 7 of adipocyte differentiation (Fig. 3A). Based on this finding, we hypothesized that Angptl2 regulates adipocyte differentiation in an autocrine or paracrine manner. To test this hypothesis, we transfected confluent preadipocytes with Angptl2 siRNA or a negative control siRNA. Angptl2 siRNA reduced the expression levels of mRNA and
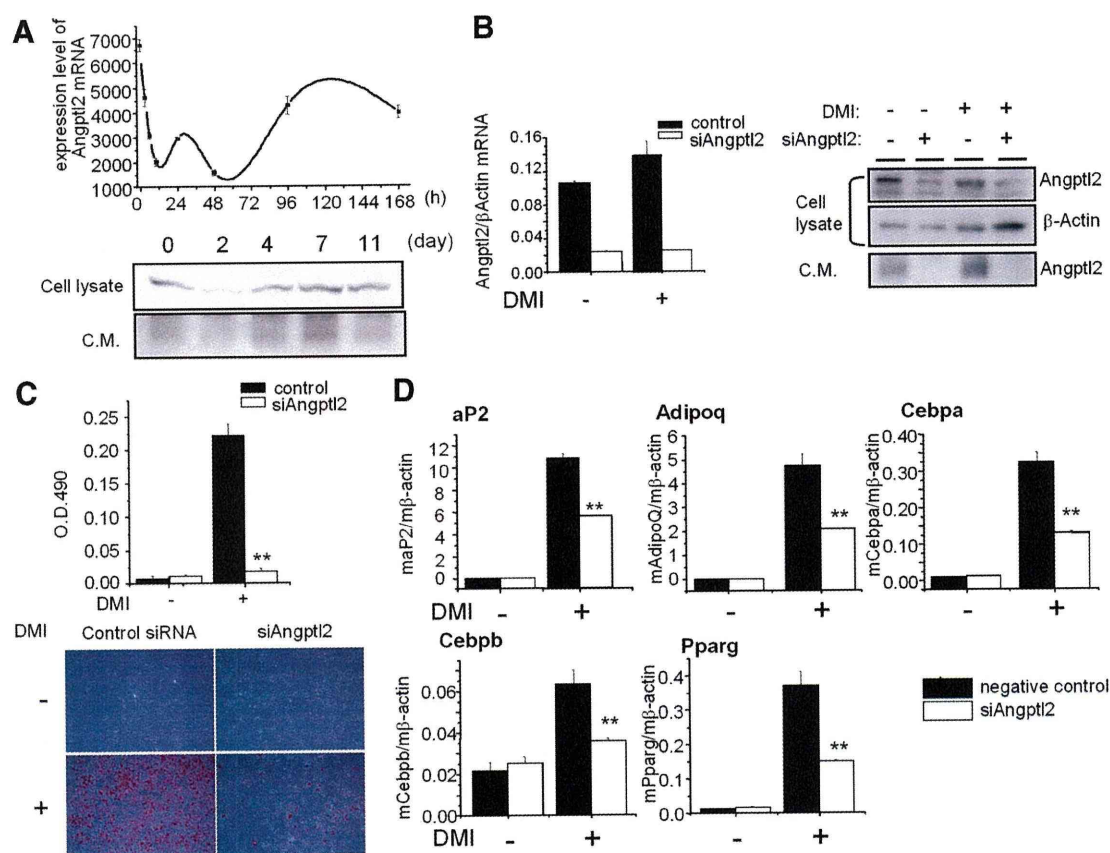
FIG. 3. Effects of Angptl2 on 3T3-L1 preadipocyte differentiation. A, Levels of Angptl2 mRNA (*upper panel*) and protein in the cell lysates or culture medium (*lower panel*) during differentiation of 3T3-L1 cells by dexamethasone, isobutylmethylxanthine and insulin (DMI). B, Effects of Angptl2 siRNA on mRNA (*left panel*) and protein (*right panel*) expression or secretion. Two days after siRNA transfection, cells were induced adipocyte differentiation. Cell lysates were corrected at d 7. C, Staining of neutral lipids by Oil Red O. *Upper panel* is the amount of neutral lipid as determined by measurement at OD 490. D, Expression of genes associated with adipose differentiation (n = 3). **, $P < 0.01$; *, $P < 0.05$ compared with negative control siRNA.

protein (Fig. 3B) of several target genes 48 h after transfection. After this time, differentiation media were added to the cells, and differentiation allowed to proceed normally. Seven days after induction of differentiation, neutral lipid content stained with Oil Red O was reduced markedly in cells receiving Angptl2 siRNA treatment compared with negative controls (Fig. 3C). Expression of adipocyte differentiation marker genes, including aP2, Adipoq, peroxisome proliferator-activated receptor (PPAR)α, PPARγ, and CCAAT-enhancer-binding proteins (CEBPα and CEBPβ) (Fig. 3D), were also reduced in cells receiving Angptl2 siRNA treatment compared with negative controls. However, treatment with 1 mg/ml Angptl2 during differentiation did not affect the degree of differentiation (Supplemental Fig. 4). Angptl2 is highly expressed in 3T3-L1 adipocytes, and the differentiation medium was not changed for 2–3 d to induce adipocyte differentiation. Therefore, we believe that the amount of secreted Angptl2 in the culture medium of PBS-treated cells was sufficient to induce differentiation in these cells. These results also suggest that Angptl2 is not an aggressive inducer but rather an auxiliary factor in adipocyte differentiation.

Angptl2 increases insulin sensitivity by stimulating insulin-dependent phosphorylation of Akt and its substrates but not IRS or ERK1/2

To investigate the effects of Angptl2 on adiponectin expression, we transfected 3T3-L1 adipocytes with Angptl2 siRNA. This was effective in reducing the expression of Angptl2 mRNA and protein (Fig. 4A). Treatment with Angptl2 siRNA also decreased the expression of adiponectin mRNA, and correspondingly, we also observed that the concentration of adiponectin protein in the culture medium was reduced (Fig. 4B). Moreover, treatment with 1 μg/ml Angptl2 for 72 h increased the expression and release of adiponectin in 3T3-L1 adipocytes (Fig. 4C). Akt is a key molecule in insulin-dependent glucose uptake (17). A crucial event in this process is the translocation of GLUT4 (SLC2A4) to the plasma membrane, a process which acts as a regulator of glucose uptake in cells. This trafficking process is mediated, in part, as a result of
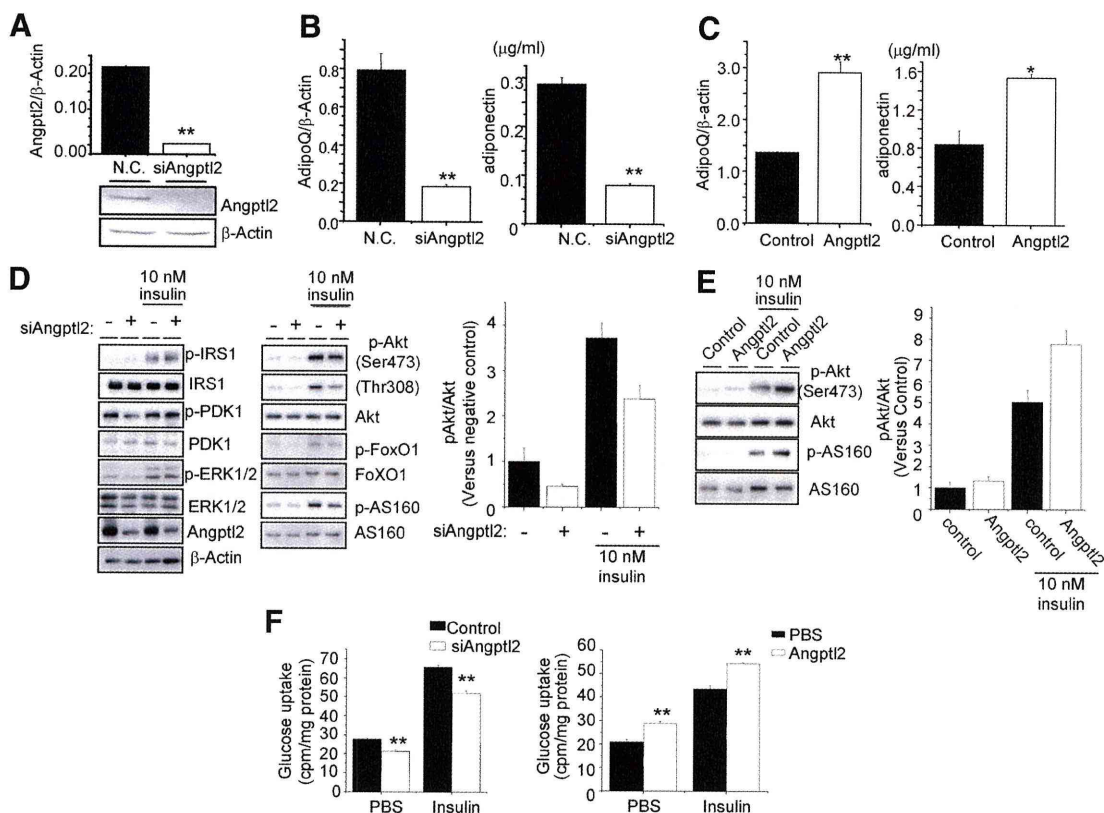
FIG. 4. Effects of Angptl2 on insulin sensitivity in 3T3-L1 adipocytes. A, Effects of Angptl2 siRNA on expression of mRNA (*left panel*) and protein (*right panel*) (n = 3). B and C, Levels of adiponectin mRNA expression and protein concentration in culture medium of 3T3-L1 adipocytes treated with or without Angptl2 siRNA (B) or recombinant Angptl2 (C) (n = 4). For treatment with recombinant Angptl2, the culture medium with or without 1 μg/ml Angptl2 was changed every day for 3 d. D and E, Effects of Angptl2 siRNA (D) and 1 μg/ml recombinant Angptl2 (E) on phosphorylation levels of insulin signaling molecules, determined by immunoblotting. The graph represents the ratio of band densities, comparing phosphorylated Akt to total Akt protein (n = 3). F, Effect of Angptl2 siRNA (*left*) and 1 μg/ml recombinant Angptl2 (*right*) on insulin-induced glucose uptake (n = 3). **, $P < 0.01$; *, $P < 0.05$ compared with negative control siRNA or PBS control. N.C., Negative control.

AS160 phosphorylation by Akt (18). To examine the effects of Angptl2 on insulin-induced activation of the Akt signaling pathway, we measured the phosphorylation levels of insulin signaling proteins. Treatment with Angptl2 siRNA inhibited insulin-induced phosphorylation of Akt and several Akt substrates, including FoxO1 (19) and AS160 (Fig. 4D). However, phosphorylation of IRS-1 and pyruvate dehydrogenase kinase 1, which are upstream of Akt, and of ERK1/2, another insulin signaling pathway, was not influenced by treatment with Angptl2 siRNA (Fig. 4D). Similar results were obtained using two other Angptl2 siRNA (Supplemental Fig. 5). In addition, treatment with 1 μg/ml Angptl2 enhanced the insulin-induced phosphorylation of Akt, FoxO1, and AS160 (Fig. 4E). Similarly, insulin-induced glucose uptake was decreased by treatment with Angptl2 siRNA, whereas glucose uptake was increased by treatment with 1 μg/ml Angptl2 (Fig. 4F).

Angptl2 regulates Akt activity as a result of reduced Trib3 expression

Phosphorylation of IRS-1 and pyruvate dehydrogenase kinase 1, which are upstream of Akt, and of ERK1/2, an-

other insulin signaling pathway, was not influenced by treatment with Angptl2 siRNA. This result suggests that Angptl2 influences an Akt-specific regulatory protein. Therefore, we investigated whether Angptl2 affected the expression of Trib3, a specific Akt inhibitory protein (20). Trib3 mRNA expression was increased by Angptl2 siRNA treatment and decreased by treatment for 48 h with 1 μg/ml Angptl2 (Fig. 5A). The expression of Trib3 protein was also increased by Angptl2 siRNA treatment (Fig. 5B). To investigate the relationship between Trib3 and Angptl2-regulated insulin resistance, we cotransfected Trib3 siRNA with Angptl2 siRNA. The suppression of Akt phosphorylation caused by treatment with Angptl2 siRNA was partially recovered by cotransfection with Trib3 siRNA (Fig. 5C). Meanwhile, the enhanced insulin-induced phosphorylation of Akt by Angptl2 treatment was not observed in cells treated with Trib3 siRNA (Fig. 5D). Furthermore, expression of adiponectin mRNA was also increased by Trib3 siRNA (Fig. 5E). These data indicated that Angptl2 partially increased insulin sensitivity via reduced Trib3 expression in 3T3-L1 adipocytes. We then examined expression of Trib3 in adipose tissues ad-
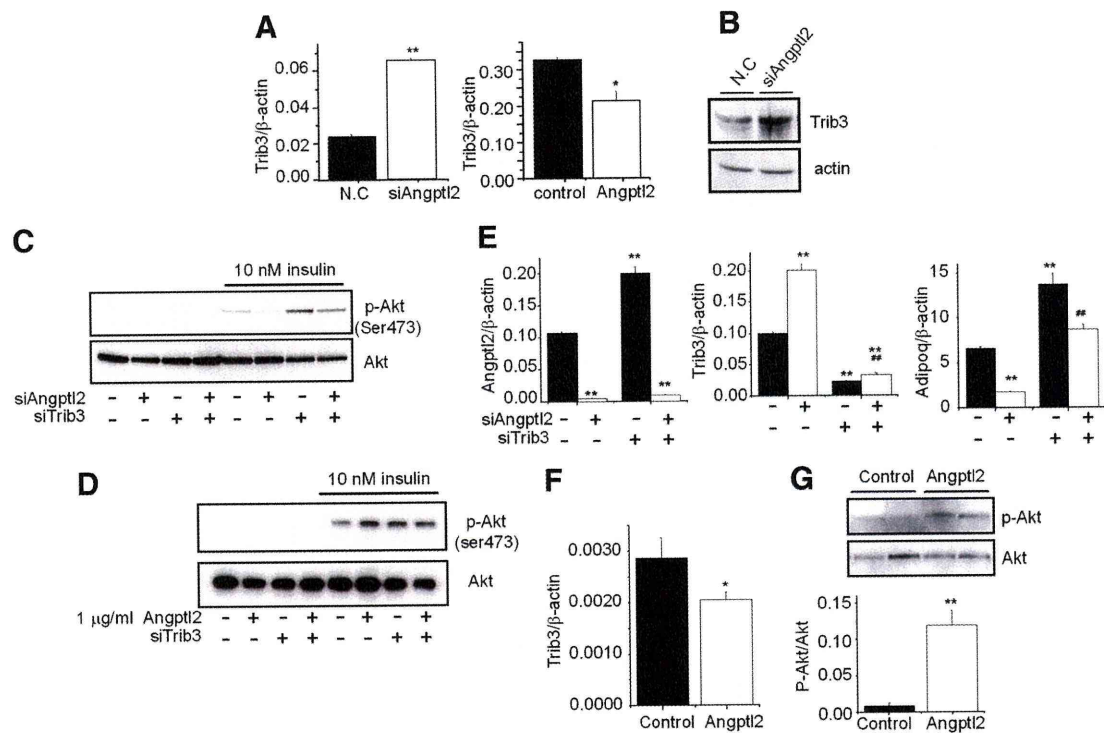
FIG. 5. Effects of Trib3 on Angptl2-induced stimulation of insulin sensitivity. A and B, Levels of Trib3 mRNA (A) and protein (B); n = 3 per group. C, Effects of cotransfection of Angptl2 and Trib3 siRNA on insulin-induced Akt phosphorylation. D, Effect of Trib3 siRNA on recombinant Angptl2-induced stimulation of insulin-dependent Akt phosphorylation. E, Effect of Trib3 siRNA on Angptl2 siRNA-induced reduction in adiponectin mRNA. F, Levels of Trib3 mRNA in mesenteric adipose tissues of mice administered with 0.8 mg/kg body weight per day Angptl2 or PBS at ZT12 for 4 wk (n = 5). G, Levels of phosphorylated Akt in mesenteric adipose tissues 2 h after final administration of Angptl2 or PBS, determined by Western blotting (*upper panel*). The graph represents the ratio of band densities, comparing phosphorylated Akt to total Akt protein (*lower panel*) (n = 3). **, $P < 0.01$; *, $P < 0.05$ compared with negative control siRNA or PBS control. N.C., Negative control.

ministrated with Angptl2. In adipose tissues of mice administrated Angptl2, the expression of Trib3 was reduced (Fig. 5F), and phosphorylated Akt was increased 2 h after the final administration (Fig. 5G).

Discussion

In this study, we observed circadian expression of Angptl2 in epididymal adipose tissue, with the peak phase occurring at ZT12. This peak phase was consistent with the active period of feeding behavior, and the rhythmic expression was disrupted after the weight gain and insulin resistance by reduced gene expression during the peak phase. It was notable that the administration of Angptl2 to *db/db* mice during the peak period of expression reduced plasma glucose, insulin, and lipid levels. The reductions of plasma insulin and lipid levels were observed at least for 12 h after administration (Supplemental Fig. 6). Furthermore, the difference in random-fed glucose level between PBS and Angptl2 group was gradually increased during 4-wk administration. These results suggested that treatment with Angptl2 improved glucose metabolism in organs/tissues and reduced plasma insulin level. Because of

the improvement of hyperinsulinemia, excessive fat synthesis and lipid storage might be suppressed in liver and adipose tissues of Angptl2-treated mice. To investigate whether this reduction was possibly, due to improvements in insulin sensitivity, we examined the effects of Angptl2 on insulin sensitivity in 3T3-L1 adipocytes. We found that Angptl2 increased the expression of adiponectin and insulin-dependent glucose uptake by regulating insulin sensitivity in adipocytes. These results indicate that the effects of Angptl2 on glucose tolerance in *db/db* mice might be due to improvements in insulin resistance, although further *in vivo* studies are needed to confirm this finding.

An important component of nutrient homeostasis is the coordination of daily rhythms of rest and activity, feeding behavior, energy utilization, and energy storage across the 24-h light/dark cycle. Recent molecular studies observed 24-h variation in the expression of mRNA encoding several genes involved in energy homeostasis. In the liver, coordinated circadian expression of glucose transporters, the glucagon receptor, and enzymes involved in rate-limiting steps in the metabolism of hexose sugars have been reported, with the peak phase of expression occurring during the feeding period (21). Similarly, in adipose tissue, the

expression of diabetes-related genes, such as leptin (22), the adiponectin receptor (23), PPAR (24), and plasminogen activator inhibitor-1 (25), was observed to follow circadian patterns. Likewise, we have demonstrated for the first time that Angptl2 is a novel circadian gene involved in the pathology of type 2 diabetes mellitus. On the basis of these reports, we hypothesized that nutrient homeostasis may be maintained by a daily cycle of increases and decreases in the expression of metabolic regulating factors. We therefore examined the effects of Angptl2 on diabetes *in vivo* by administration of the protein, rather than using transgenic or knockout mouse models.

In 3T3-L1 adipocytes, Angptl2 induced insulin sensitivity of Akt. Although the mechanism of this action is not entirely clear, Trib3 expression is known to be increased by treatment with Angptl2 siRNA. There is also evidence that Trib3 siRNA partially recovers Angptl2 siRNA-induced insulin resistance in 3T3-L1 adipocytes. In addition, Trib3 has also been shown to interfere with insulin signaling by binding to and inhibiting Akt (22, 26). Trib3 expression can be increased by numerous stimuli, including starvation (27), PPAR-mediated activation (26), and chronic alcohol ingestion (28), all of which are linked to insulin insensitivity. Overexpression of Trib3 in a cell line moderately decreased insulin-induced phosphorylation and activation of Akt (20). Furthermore, it has been reported that Trib3 suppresses adipocyte differentiation by negatively regulating PPARγ transcriptional activity (29). Taken together, these findings indicate that the regulation of Trib3 expression may be one of the mechanisms by which Angptl2 improves insulin resistance. However, long-term treatment with Angptl2 did not stimulate phosphor-Akt, whereas chronic treatment with Angptl2 did acutely stimulate phosphor-Akt (Supplemental Fig. 7). Furthermore, acute treatment with Angptl2-induced Akt phosphorylation was suppressed by pretreatment with LY294002, a phosphatidylinositol 3 kinase inhibitor (Supplemental Fig. 7). These results suggest that different mechanisms exist between acute and continuous treatment-induced Akt phosphorylation. Thus, further studies are needed to investigate the mechanisms involved in Angptl2-stimulated insulin signaling.

In contrast to our findings, a recent study showed that Angptl2 promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance (30). In this article, Angptl2-transgenic mice showed inflammation in adipose tissue and insulin resistance. Conversely, Angptl2 knockout mice showed reduced inflammation after high-fat feeding. Angptl2 activates chemotaxis of monocytes/macrophages result in inflammation (30). Although the results of their and our studies seem to be discrepant, we suggest that the transgenic and knockout mice used in their study represent a distinct disease state from that in db/db mice. Angptl2 transgenic and knockout mice develop diabetes or a diabetic-resistant state as they grow, whereas db/db mice show severe diabetes and inflammatory state by 8 wk of age, before the administration of Angptl2 in our study. Therefore, Angptl2 transgenic and knockout mice are useful to investigate the potential role of Angptl2 in the pathogenesis of diabetes, whereas the administration of Angptl2 in db/db mice helps us to examine the role of Angptl2 in the progression of diabetes. Here, we demonstrated that the administration of Angptl2 improved blood glucose levels, lipid metabolism, and insulin resistance in db/db mice. In cultured adipocytes, as in db/db mice, treatment with Angptl2 improved insulin sensitivity. The *db/db* mice show severe inflammation, including the activation of chemotaxis. Thus, the administration of Angptl2 may not further accelerate monocyte/macrophage chemotaxis. In fact, the infiltration of macrophages into adipose tissues and CD68 expression were not affected by Angptl2 administration. On the other hand, Angptl2 did decrease plasma lipid and free fatty acid levels and lowered the expression of cytokines and chemokines. Taken together, these results suggest that Angptl2 improves the inflammatory state and insulin sensitivity in *db/db* mice. Taken together, our results suggested that Angptl2 plays differing roles in different stages in the pathogenesis of type 2 diabetes.

In summary, we demonstrated that Angptl2 increases both adiponectin expression and insulin sensitivity, thereby reducing blood glucose levels and lipid content. These results suggest that Angptl2 plays a crucial role in regulating insulin sensitivity and is, therefore, involved in the progression of type 2 diabetes mellitus. Characterization of the Angptl2 receptor and the cellular mechanisms underlying Angptl2-induced modulation of metabolic gene expression may enable us to develop new therapies for metabolic diseases.

Acknowledgments

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Histone chaperone Spt6 is required for class switch recombination but not somatic hypermutation

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Activation-induced cytidine deaminase (AID) is shown to be essential and sufficient to induce two genetic alterations in the Ig loci: class switch recombination (CSR) and somatic hypermutation (SHM). However, it is still unknown how a single-molecule AID differentially regulates CSR and SHM. Here we identified Spt6 as an AID-interacting protein by yeast two-hybrid screening and immunoprecipitation followed by mass spectrometry. Knockdown of Spt6 resulted in severe reduction of CSR in both the endogenous Ig locus in B cells and an artificial substrate in fibroblast cells. Conversely, knockdown of Spt6 did not reduce but slightly enhanced SHM in an artificial substrate in B cells, indicating that Spt6 is required for AID to induce CSR but not SHM. These results suggest that Spt6 is involved in differential regulation of CSR and SHM by AID.

The Ig genes in antigen-stimulated B lymphocytes are diversified by two distinct genetic alteration mechanisms, namely somatic hypermutation (SHM) and class switch recombination (CSR) (1, 2). SHM causes the accumulation of point mutations in the rearranged variable (V) region genes, leading to generation of antibodies with higher affinity after cellular selection by a limited amount of antigen (1). CSR replaces the heavy chain constant region (C_H) gene proximal to the V_H gene, namely $C\mu$ with one of the downstream C_H genes by recombination between the switch (S) regions located 5′ to each C_H gene, thereby producing antibodies with diverse effector functions without changing their antigen specificity (2).

Both SHM and CSR require activation-induced cytidine deaminase (AID), which is specifically expressed in activated B cells (3). It is well accepted that AID initiates single-strand DNA breaks essential for SHM and CSR through its cytidine deaminase activity. AID can also introduce mutations in such non-Ig loci as c-myc, Pim1, Pax5, bcl-6, and RhoH (4, 5). The number of target loci of AID seems to be larger than expected but still limited (5). Although extensive analyses have been done to uncover the exact molecular mechanism how AID induces DNA strand breaks at restricted loci, it is unknown how a single-molecule AID differentially regulates CSR and SHM or how the Ig genes and other target loci are preferentially targeted in the whole genome. To answer these questions, extensive studies were carried out to identify cofactor(s) that may account for the target specificity of the AID function. Several AID-interacting proteins have been reported, including RNA polymerase II (6), replication protein A (7), protein kinase A (8, 9), DNA-PKcs (10), MDM2 (11), CTNNBL1 (12), Spt5 (13), and PTBP2 (14). Unfortunately, however, none of these proteins could show any functional correlation to support the target specificity of AID. There is no clear mechanism to limit the number of target loci. Most of the proteins like RNA polymerase II, protein kinase A, Spt5, and PTBP2 are rather ubiquitous and interact with many proteins other than AID. PTBP2 is a splicing factor, and Spt5 is one of the transcription elongation factors that associate with RNA polymerase II. Replication protein A, DNA-PKcs, and MDM2 are proteins involved in general DNA repair. CTNNBL1 was later shown to be dispensable for CSR (15).

AID has been shown to have the nuclear localization signal and nuclear export signal in its N terminus and C terminus, respectively (16, 17). The deletion of the nuclear localization signal region of AID results in loss of the AID functions for both SHM and CSR (16). A series of mutations at the N terminus of AID also causes defects in CSR as well as SHM (18). Although no mutations at the N terminus of AID have been shown to cause CSR-specific loss of the AID function, some AID mutants with point mutations in the N-terminal region retain substantial CSR activity but severely damage SHM activity, which is most likely due to a combination of partial loss of DNA cleavage activity and less efficient cleavage of the V region compared with S regions (18–20). Conversely, a S3A mutaiton augments both CSR and SHM (21). On the other hand, the deletions and/or mutations in the nuclear export signal region (residues 183–198) result in loss of the AID function for CSR but not SHM, probably because AID with the C-terminal deletion has normal DNA cleavage activity (19, 22). The results suggest that AID has at least two functions: DNA cleavage of V and S region associated with the N-terminal region, and CSR-specific activity associated with the C-terminal region. In addition, the C-terminal region was shown to be responsible for interaction with poly $(A)^+$ RNA (23). We proposed that the C-terminal region of AID might be responsible for generation of recombination synapsis factor (19). Therefore, we assumed that cofactors interacting with the C-terminal region of AID might be responsible for CSR-specific activity rather than DNA cleavage, whereas cofactors interacting with the N-terminal region of AID might be responsible for DNA cleavage of both V and S regions.

In the present study, we screened AID association molecules by yeast two-hybrid screening and coimmunoprecipitation. We then assessed their functional involvement in CSR and SHM.

Because AID associates with a numerous molecules, we used AID mutants as negative controls and chose the association molecules specific to wild-type AID. Among these molecules we identified Spt6, whose interaction was blocked by AID mutations at the N terminus. Knockdown of Spt6 resulted in great reduction of CSR in both the endogenous Ig locus in B cells and an artificial substrate in fibroblasts. Surprisingly, however, knockdown of Spt6 did not reduce but slightly enhanced SHM in an artificial substrate in B cells. These results indicate that Spt6 is involved in differential regulation of CSR and SHM by AID.

Results and Discussion

Proteins Physically Interacting with AID. To identify AID-interacting proteins that may account for the target specificity of AID function in CSR and SHM, we overexpressed mouse (m) and human (h) AID in a mouse B-cell line, CH12F3-2A, which can switch from IgM to IgA upon stimulation. We used AID tagged with GFP-FLAG (GF) at its C terminus to avoid masking the FLAG epitope by putative large AID association proteins. The addition of GF to the C terminus of AID had little effect on the function of AID to induce both CSR and SHM. Cytoplasmic extracts of CH12F3-2A with AID-GF were fractionated by centrifugation through a glycerol density gradient (10–50%, vol/vol). Each fraction collected was analyzed for the presence of AID-GF by GFP fluorescence (Fig. S1A). The distribution of AID-GF was broad. Similar broad distribution of endogenous AID was observed in CH12F3-2A extracts using an anti-AID antibody (Fig. S1B). However, the increase of the NaCl concentration from 150 mM to 500 mM reduced the overall size distribution and sharpened the distribution profile (Fig. S1C). The results indicate that AID interacts with a large number of cytoplasmic proteins, some of which can be removed at 500 mM NaCl. The complex formation was not due to GFP because GF alone formed a small and sharp peak. RNase A treatment reduced the size of the peak only slightly at 150 mM NaCl but hardly at 500 mM NaCl, indicating that there are some AID protein complexes containing RNA but the majority of the AID complex is formed through the protein–protein interaction.

We then immunoprecipitated AID-GF interacting molecules with the anti-FLAG antibody from the cytoplasmic extracts and fractionated by SDS/PAGE, followed by MS. As shown in Fig. 1A, a huge variety of proteins were coimmunoprecipitated with AID-GF compared with GF. Therefore, we decided to compare coimmunoprecipitates between AID and its loss-of-function mutant. We used the human AID mutant P13 (M139V) defective in both SHM and CSR activities (22). Similarly diverse proteins with similar intensity were coimmunoprecipitated with the P13 mutant when we used an equal number of cells for wild-type and mutant AID (Fig.1A).

Among coimmunoprecipitates, Spt6, Trim28, Nucleolin, Skiv2l2, Zfp84, CRM1, and eEF1α were clearly more abundant in wild-type AID-GF (Fig. 1A). We also obtained the following two groups of coimmunoprecipitates from CH12F3-2A cells: (i) proteins involved in the nuclear–cytoplasmic transport, including importin 4 and importin β3 (Ranbp 5); and (ii) proteins involved in the translation or degradation of proteins, and chaperones, including eEF1α, Hsp70, Stip1, TCP1, CCTq, and KIAA1967. Identification of proteins in the group (i) and CRM1 indicates that the coimmunoprecipitations in the current condition is suitable to detect expected functional partners of AID because AID has been reported to be actively exported from nucleus to cytoplasm in a CRM1-dependent manner (16, 17). The degradation-related molecules and chaperons in group (ii) were coimmunoprecipitated probably because overexpressed wild-type and mutant AID-GF were targets of the degradation or inactivation machinery. Although eEF1α also showed a striking difference between wild-type AID and P13 mutant, we suspected that eEF1α was directly associated with mRNA to which AID interacts as reported previously (23). In fact the treatment of cell extracts with RNase A before immunoprecipitation significantly reduced eEF1α from the coimmunoprecipitates with AID-GF (Fig. 1B).

In a separate series of experiments, we expressed hAID-FLAG (F) and hAID mutants including L172A-F, ΔN10-hAID-F
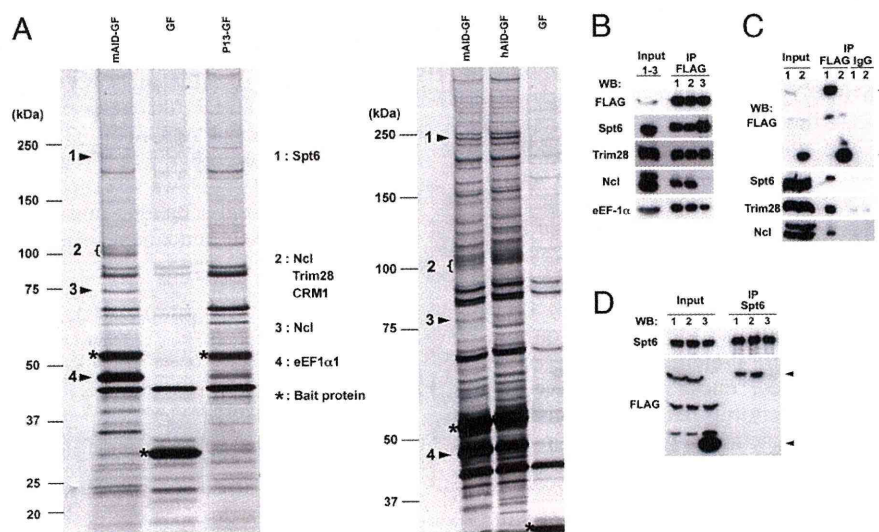
Fig. 1. Identification of AID-interacting proteins. (A) Silver staining of proteins coimmunoprecipitated with mouse AID-GFP-FLAG (mAID-GF), human AID-GF (hAID-GF), GF, and P13-GF from cytosolic extracts prepared from CH12F3-2A cells expressing either of the AID-GFs. Bands were excised and analyzed by MS. Proteins that appeared to be obviously more abundant in mAID-GF and hAID-GF are indicated. Nucleolin appears at two positions owing to possible modification. (B) Western blot analyses of immunoprecipitates with anti-FLAG M2 antibody from mAID-GF–expressing CH12F3-2A extracts treated with DNase I (lane 2) or RNase A (lane 3). Immunoprecipitates from untreated extracts are shown in lane 1. (C) Western blot analyses of immunoprecipitates either with anti-FLAG M2 antibody or with mouse IgG from cytosolic extracts prepared from CH12F3-2A cells expressing mAID-GF (lane 1) or GF (lane 2). Arrows indicate mAID-GF or GF. (D) Western blot analyses of immunoprecipitates with anti-Spt6 mAb from cytosolic extracts prepared from CH12F3-2A cells expressing mAID-GF (lane 1), hAID-GF (lane 2), or GF (lane 3). Two nonspecific bands appeared in input by FLAG Western blot.

(N-terminal 10 residue truncation), and JP8Bdel-F (Table S1) in 293T cells and compared proteins immunoprecipitated with the anti-FLAG antibody by MS. We picked up seven proteins that were specifically coimmunoprecipitated with hAID-F in all four repeated experiments. The list of such proteins is shown in Table S2. Surprisingly, none of them overlapped with the above experiments in CH12F3-2A cells. The discrepancy could be at least in part due to the difference in tags to AID and cells used.

Spt6 Is Required for CSR in B Cells and Fibroblasts. We first focused on Spt6, Trim28, and Nucleolin because they are involved in nucleic acid metabolism and most distinctly associated with wild-type AID. We confirmed that Spt6, Trim28, and Nucleolin were coimmunoprecipitated with AID-GF but not GF by Western blotting (Fig. 1B). The control IgG did not precipitate any of them (Fig. 1C). The association of Spt6 and Trim28 with AID-GF was not reduced by either RNase A or DNase I treatment of the cell extracts before immunoprecipitation, whereas the association of Nucleolin with AID-GF was almost completely abolished by RNase A treatment, suggesting that RNA bridges AID with Nucleolin (Fig. 1B). In addition, the association between Spt6 and AID was further confirmed by detection of AID-GF in immunoprecipitates with an anti-Spt6 antibody (Fig. 1D).

Interaction of AID with Spt6 was also supported by yeast two-hybrid screening. We screened a human lymph node cDNA library fused to the GAL4 activation domain (AD), with hAID fused to the GAL4 DNA-binding domain (BD) as bait, and a mouse pre B-cell cDNA library fused to the GAL4 AD, with mAID fused to the GAL4 DNA-BD as bait. The C-terminal fragments of hSPT6 (3817–5178 nt) and mSpt6 (4050–5178 nt), which contain Src homology 2 domain, were isolated from the human and mouse libraries, respectively (Fig. S2). This interaction was further confirmed by coimmunoprecipitation of hAID with hSPT6 fragments fused with GST.

We therefore examined the functional involvement of Spt6 in CSR by knocking down its expression in CH12F3-2A cells. Knockdown of Spt6 or AID significantly reduced CSR efficiency (Fig. 2 A and B). However, 1.5 μg of Spt6 siRNA decreased AID mRNA and germline transcript (GLT) of Cα significantly, although GLT of Cμ was intact (Fig. 2C). Therefore, we further examined the effects of Spt6 knockdown using the AID-ER (fusion protein of AID and the hormone-binding domain of the estrogen receptor) system, in which CSR can be induced rapidly upon 4-hydroxy tamoxifen (OHT) addition without de novo transcription and translation of AID. Knockdown of Spt6 with 0.6 μg of siRNA reduced the efficiency of CSR almost in parallel with the degree of Spt6 protein reduction (Fig. 3 A–C). However, Stp6 knockdown did not affect the amounts of AID-ER protein and GLTs (Fig. 3 C and D). The results indicate that Spt6 is required for CSR.

To further confirm the involvement of Spt6 in CSR, we used the artificial switch substrate in a mouse fibroblast cell line, NIH 3T3 (24). We knocked down Spt6 using a mixture of siRNAs in NIH 3T3 cells expressing the artificial switch substrate of CSR and AID-ER. siRNAs against Spt6 significantly reduced CSR efficiency in the artificial switch substrate compared with control siRNAs against LacZ (Fig. 4A). Consistently, postswitch transcripts were decreased by Spt6 knockdown, although it did not reduce the amounts of AID-ER mRNA and preswitch transcripts Pre-Tr1 and Pre-Tr2 (equivalent to GLTs) (Fig. 4B). These results further confirmed that Spt6 is required for CSR.

Other Candidates Are Not Required for CSR. We then examined involvement of other AID-binding proteins in CSR by knockdown assay. Knockdown of Trim28 also reduced CSR but simultaneously decreased AID mRNA in CH12F3-2A cells (Fig. S3 A and B). We thus examined the effect of Trim28 knockdown in the AID-ER system. Trim28 knockdown in the AID-
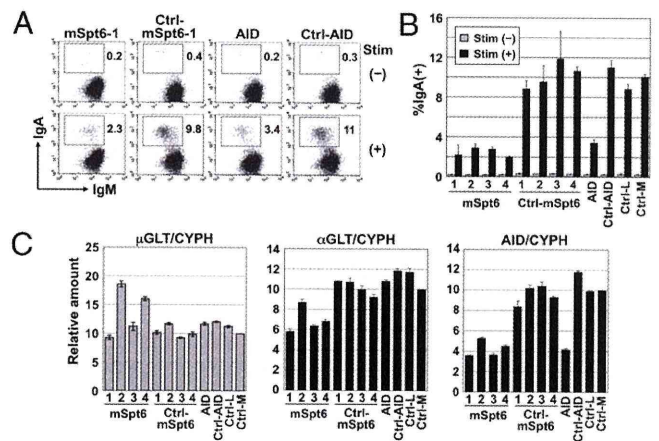
Fig. 2. CSR is inhibited by Spt6 knockdown in CH12F3-2A cells. (A and B) Spt6 knockdown severely reduced CSR efficiency. CH12F3-2A cells (1.5 × 10⁶) were introduced with 1.5 μg of siRNAs against mSpt6, scrambled siRNA for them, an siRNA against mAID, a scrambled siRNA for it, or negative control siRNAs with low (36%) and medium (48%) GC contents (Ctrl-L and Ctrl-M, respectively). The GC contents of oligos mSpt6-1, -2, and -4 are medium (45–55%), and those of oligos mSpt6-3 and AID are low (35–45%). Twenty-four hours after siRNA introduction, cells were stimulated with CD40L, IL-4, and TGF-β for 24 h. The percentages of IgA⁺ cells in the live population are indicated. Representative FACS profiles are shown (A). The mean ± SD values were obtained from triplicate experiments (B). (C) siRNAs against Spt6 reduced the amount of αGLT and AID transcripts in Spt6-knockdown cells. Quantitative PCR analyses for μGLT, αGLT, and AID transcripts in Spt6-knockdown cells. Values were normalized by cyclophilin (CYPH). Unstimulated and stimulated cells were analyzed for μGLT and for αGLT and AID transcripts, respectively.

ER system did not affect CSR, suggesting that Trim28 blocked CSR by inhibiting AID transcription (Fig. S3 C and D). Involvement of Trim28 in the transcriptional regulation of AID was further confirmed by the fact that both AID transcription and CSR are drastically reduced in Trim28-deficient B cells (Fig. S3 E and F). We concluded that the association of Trim28 with AID is not related to AID function. Knockdown of Trim28 did not show any significant effects on CSR in NIH 3T3 cells either (Fig. 4 A and B).

Knockdown of Nucleolin did not significantly affect CSR efficiency, either in CH12F3-2A cells or NIH 3T3 cells, although Nucleolin protein was dramatically reduced by knockdown. Knockdown of Skiv2l2 only slightly reduced CSR efficiency, both in CH12F3-2A cells and NIH 3T3 cells, whereas knockdown of Zfp84 did not significantly affect CSR in CH12F3-2A cells. Therefore, we concluded that Nucleolin, Skiv2l2, and Zfp84 do not play major roles in CSR, although we could not exclude the possibility that residual amounts of target proteins were still sufficient to support CSR. We then examined whether candidates identified from 293T cells by specific coimmunoprecipitation with hAID-F are involved in CSR (Table S2). Knockdown of these candidates was carried out in CH12F3-2A cells, but none of them affected CSR significantly except for hnRNPA1, which we could not knockdown. We could therefore identify only Spt6 that has functional relevance for the CSR activity of AID among all candidates detected by physical association with AID.

Spt6 Is Dispensable for SHM in B Cells. We next examined the effect of Spt6 knockdown on SHM in a human B-cell line BL2. To assess SHM efficiency sensitively and quickly, we took advantages of a modified GFP substrate of SHM (Fig. 5A). In addition, we used a C-terminal truncation mutant of AID, JP8Bdel, which has stronger SHM activity but marginal CSR activity (19, 22). In this system, OHT-activated JP8Bdel-ER protein caused loss of GFP

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Fig. 3. CSR is inhibited by Spt6 knockdown in mAID-ER–expressing CH12F3-2A cells without affecting the amount of AID. (*A* and *B*) Spt6 knockdown reduced CSR efficiency. CH12F3-2A cells expressing mAID-ER (1.5×10^6) were introduced with 0.6 μg of siRNAs against mSpt6, scrambled siRNA for them, an siRNA against mAID, a scrambled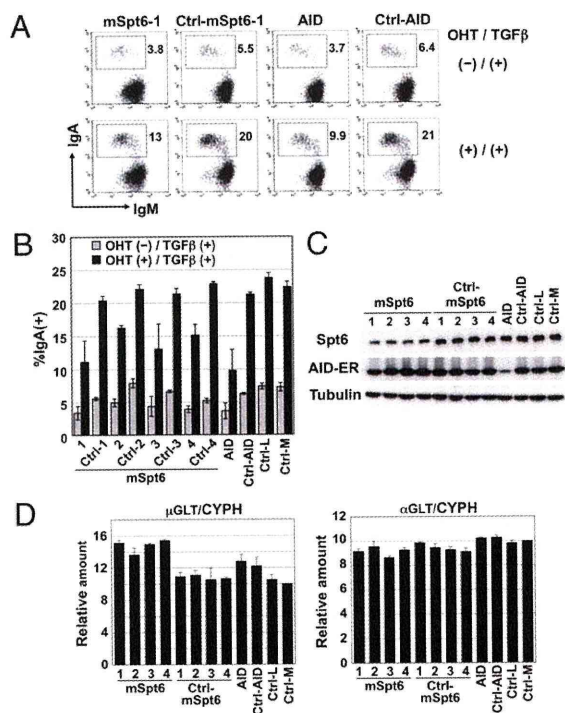 siRNA for it, or negative control siRNAs with low and medium GC contents (Ctrl-L and Ctrl-M, respectively). Twenty-four hours after siRNA introduction, cells were stimulated with OHT and TGF-β for 24 h. The percentages of IgA⁺ cells in the live population are indicated. Representative FACS profiles are shown (*A*). Mean ± SD values were obtained from triplicate experiments (*B*). (*C*) siRNAs against Spt6 efficiently reduced the amount of Spt6 protein but did not affect the amount of AID-ER protein. (*D*) Quantitative PCR analyses for μGLT and αGLT in Spt6-knockdown cells. Stimulated cells were analyzed. CYPH, cyclophilin.

Fig. 4. Spt6 knockdown inhibited CSR in the artificial switch substrate SCI (μ, α) in NIH 3T3 cells. (*A*) NIH 3T3 cells expressing mAID-ER and SCI (μ, α) were introduced with d-siRNAs against mSpt6, mTrim28, mAID, and LacZ together with a DsRed-expressing plasmid as a transfection indicator. Twenty-four hours after transfection, cells were stimulated with OHT for 36 h. The percentages of switched CD8α⁺ cells in the live and DsRed⁺ population are indicated. Mean ± SD values were obtained from triplicate experiments. (*B*) Quantitative PCR analyses for pre- (Pre-Tr1 and Pre-Tr2) and postswitch transcripts (Post-Tr), AID-ER, Spt6, and Trim28 transcripts. CYPH, cyclophilin.

fluorescence due to the accumulation of deleterious mutations in the GFP gene. Excessive mutations induced by JP8Bdel caused cell death. AID knockdown by three different siRNA oligos inhibited loss of GFP fluorescence, as well as the accumulation of point mutations in the GFP gene and cell death, confirming that these events were dependent on AID function and thus useful indicators for SHM (Fig. 5 *B* and *C* and Table S3).

Surprisingly, SPT6 knockdown did not inhibit but rather slightly augmented the frequency of GFP-negative cells, as well as actual mutation frequency in the GFP gene and cell death, without affecting the amount of JP8Bdel-ER protein (Fig. 5 *C* and *D* and Table S3). Although the difference was modest, the relative increase of mutation frequencies correlated well with the knockdown efficiency of each oligo against SPT6. It should be noted that both fluorescence and point mutations in the GFP gene were not reduced by SPT6 knockdown in the absence of OHT, indicating that SPT6 knockdown did not affect transcription of the SHM target. These results clearly showed that Spt6 is not required for SHM but rather inhibitory to SHM.

Spt6 Interacts with AID Through Its N Terminus. Because the C-terminal region of Spt6 interacts with AID, we then examined whether a specific region of AID is responsible for the association with Spt6. Deletion of the N-terminal residues 2–26 of AID abolished the association with Spt6, suggesting that this region contains residues involved in the interaction with Spt6

(Fig. 6 *A* and *B* and Table S1). We further tested AID mutants that carry deletion or mutation(s) in the N-terminal region and found that the deletion of residues 2–10 but not residues 2–5 of AID abolished the association with Spt6, suggesting that residues 6–10 may be responsible for the association with Spt6. Amino acid substitution experiments showed that only M6 was critical within residues 6–10 to the association with Spt6. Other mutations in the N-terminal region [G23S, V18S-R19V, W20K, and P7 (R24W)], which have been shown to reduce SHM more drastically than CSR or to abolish both, did not affect the association with Spt6 (Fig. 6*B* and Table S1). In agreement with Fig. 1, P13-GF actually lost the binding capacity with Spt6.

Next, we tried to examine the involvement of the C-terminal region (residues 183–198) of AID in the association with Spt6. To avoid insolubility of the C-terminally deleted (ΔC) AID by the spontaneous accumulation in the nucleus, we combined the deletion or mutation(s) in the N-terminal region with the deletion of the C-terminal region because most N-terminal mutants (P7, V18S-R19V, and W20K) did not accumulate in the nucleus even when nuclear export was blocked by leptomycin B (16, 18). Such N-terminal mutants that additionally lacked the C-terminal region (P7-ΔC, V18S-R19V-ΔC, and W20K-ΔC) still associated with Spt6, indicating that the C-terminal region of AID is not involved in the association with Spt6. A human AID mutant P20 carrying a 34-aa insertion after residue 182 did not show any reduction in the association with Spt6, although P20 has severe defect in CSR but little effect on SHM (22).

Dissociation of AID Mutant Activities from Their Spt6 Binding. Studies on series of AID mutants clearly demonstrated that the C-terminal region of AID is required for CSR-specific function other than DNA cleavage. This function is assumed to be related to synapsis formation of cleaved ends (19). On the other hand, the N-terminal region of AID is required for DNA cleavage of both V and S regions. Because Spt6 is required only for CSR, the interaction of Spt6 with N-terminal residues 6–10 of AID was puzzling. To

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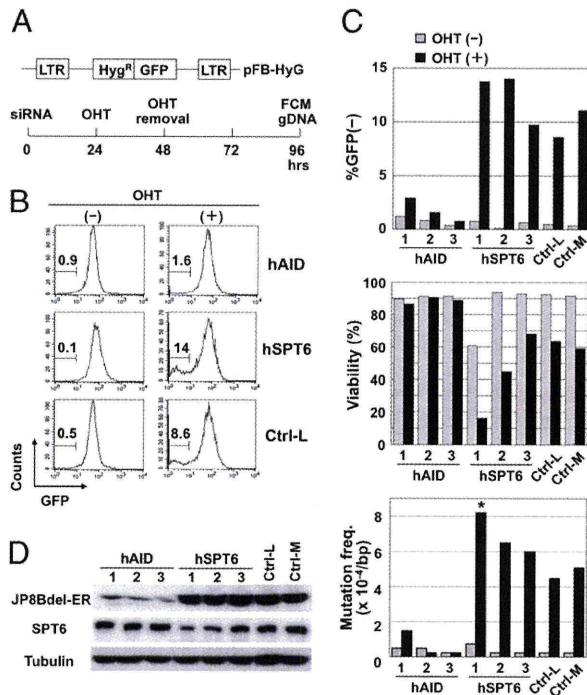
Fig. 5. Spt6 knockdown augmented SHM in BL2 cells. (A) Schematic representation of the artificial SHM substrate and the SHM assay procedure. BL2 cells expressing JP8Bdel-ER and the artificial SHM assay substrate were introduced with siRNAs, stimulated for 24 h with OHT, and incubated for an additional 48 h in the absence of OHT. Then cells were harvested for flow cytometry (FCM) and genomic DNA extraction. (B and C) SPT6 knockdown augmented the SHM efficiency in the artificial substrate. BL2 cells expressing JP8Bdel-ER and the artificial SHM assay substrate (1.5 × 10⁶) were introduced with 3.0 µg of siRNAs against hSPT6, siRNAs against hAID, or negative control siRNAs with low (36%) and medium (48%) GC contents (Ctrl-L and Ctrl-M, respectively). The GC contents of oligos hAID-1, -2, -3, and hSPT6-3 are medium (45–55%) and those of oligos hSPT6-1 and -2 are low (35–45%). The percentages of GFP⁻ cells are indicated (B). Graphical summary of the percentages of GFP⁻ cells, viability, and mutation frequencies in the GFP sequence (C). Statistical significance was evaluated against the corresponding control oligo by χ² test. *P < 0.05. Data are representative of three independent experiments. (D) Two siRNA oligos against SPT6 (1 and 2) reduced the amount of SPT6 protein but did not affect the amount of JP8Bdel-ER protein. Note that the other siRNA oligos against SPT6 (3) did not substantially reduce the amount of SPT6 protein.

Fig. 6. AID interacts with Spt6 through its N terminus. (A) Schematic representation of wild-type and mutant AID-GF constructs. (B) Western blot analyses of immunoprecipitates with anti-FLAG from cytosolic extracts of CH12F3-2A cells expressing wild-type AID-GF or mutant AID-GFs. All AID constructs are of human origin except for mWT and mG23S. An equal amount of wild-type and mutant AID-GF proteins was analyzed by adjusting the loading amounts of immunoprecipitates. (C) AID-deficient splenocytes were stimulated with LPS for 48 h and infected with retroviruses expressing mutant AID-GFs. Cells were stimulated for additional 48 h in the presence of LPS and IL-4. The percentages of IgG1⁺ cells in the GFP⁺ population are indicated. Mean ± SD values were obtained from triplicate experiments. (D) NIH 3T3 cells harboring an SHM substrate pI were infected with retroviruses expressing wild-type and mutant AID-GFs and cultured for 7 d. Genomic DNA was extracted for sequencing analysis. GF was used as mock. Statistical significance was evaluated against WT by χ² test. *P < 0.001, **P < 0.05.

is not clear whether this interaction is essential for the DNA cleavage function of AID.

How Does Spt6 Differentially Regulate CSR and SHM? The target specificity of known specific recombination is determined by combination of *cis* elements (the DNA sequence/structure) and *trans* elements (DNA binding proteins and the chromatin modification mark of the target locus). In VDJ recombination in the Ig genes, the recombination signal sequence is widely distributed in the genome, but the chromatin modification [i.e., histone3 lysine4 trimethylation (H3K4me3)] recognized by RAG2 is essential to cleave the accurate target (26, 27). In meiotic recombination, Spo11 (topoisomerase II) cleaves at loosely conserved DNA target sequences that are also recognized by zinc finger-histone methyltransferase (PRDM9) to generate H3K4me3 at the target chromatin (28–31). Without PRDM9, meiotic recombination is abortive. We have also shown that H3K4me3 at the target S region is essential for CSR (32). The FACT complex composed of SSRP1 and Spt16 is a histone chaperone and modulates the histone transmodification cascade. We have shown that the FACT complex is essential for CSR (32). In the absence of FACT, H3K4 trimethyl modifications are reduced at the Sµ and Sα regions, which is associated with S region cleavage defect.

From these studies, it is likely that Spt6 can determine the target specificity of CSR at least by two strategies: (*i*) recognition of DNA sequence or (*ii*) modification of chromatin. Because Spt6 does not bind DNA directly, it is unlikely that Spt6 directly recruits a DNA cleaving enzyme to any DNA region. In addition, Spt6 associates with RNA polymerase II, which binds both V and S regions. Spt6 is thus unlikely to guide AID specifically to S regions. Because Spt6 is another histone chaperone protein, it is important to examine whether Spt6 also affects the histone modification cascade and thus causes defect in CSR. It is also interesting to analyze why Spt6 is slightly inhibitory to SHM. The

monitor the function of the mutants at residues 6–10, each was fused with GFP in the retroviral expression vector and introduced to AID-deficient spleen cells (Fig. 6C). We also tested the SHM activities of these mutants in a GFP substrate expressed in NIH 3T3 cells (Fig. 6D and Table S4). The point mutation at the residue 6 (M6A) was totally defective for both CSR and SHM. The mutations at residues 7, 9, and 10 reduced SHM as well as CSR, albeit to a less extent. By contrast, the R8A mutant rather augmented CSR and SHM activities.

Although all of R8A, R9A, and K10R mutants had significant modification of their activities, none of them changed the interaction with Spt6 (Fig. 6B). Conversely, N7A augmented interaction with Spt6, although N7A reduced both CSR and SHM activities. M6A that abolished Spt6 interaction lost both SHM and CSR, although Spt6 is involved in only CSR. Human AID mutation M6T also lost both CSR and SHM (25). It is possible that M6A mutation altered the gross structure of AID to abolish the DNA cleavage function, resulting in the loss of both CSR and SHM. Although the N-terminal region of AID seems to be responsible for its interaction with the C-terminal region of Spt6, it
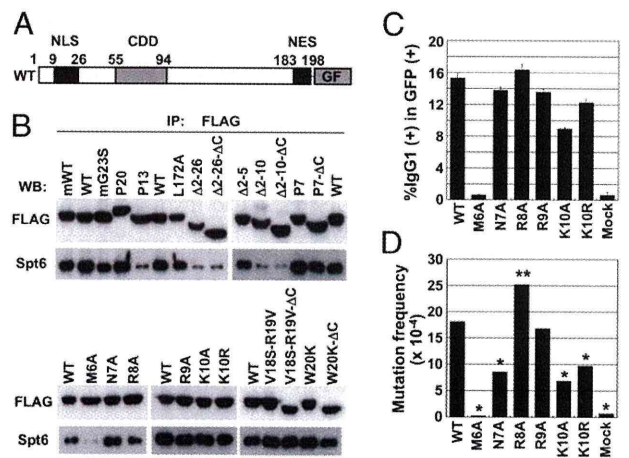
histone modification cascade in the S region and V region may be different, which triggers interesting possibilities for differential regulation of SHM vs. CSR.

There are several other possible mechanisms whereby Spt6 differentially regulates CSR and SHM. Because Spt6 has also been reported to direct Iws1-dependent mRNA splicing and export (33, 34), CSR-related function of Spt6 could involve mRNA splicing and export. Stp6 is also involved in transcriptional regulation of a large number of genes, some of which may be responsible only for CSR. Further analyses are required to uncover the precise role of Spt6 in regulation of CSR but not SHM.

Materials and Methods

RNA Interference. A diced siRNA (d-siRNA) pool was prepared using BLOCK-iT Complete Dicer RNAi Kit according to the manufacture's instructions (Invitrogen). Primers used to amplify template cDNAs for AID, Trim28, and Spt6 of mouse origin were as follows: mAID-F: 5'-CAA GGG ACG GCA TGA GAC CTA CCT-3'; mAID-R: 5'-TCT CGC AAG TCA TCG ACT TCG TAC-3'; mTrim28-F: 5'-CCA AGG AGG TTC GAA GCT CGA TCC-3'; mTrim28-R: 5'-GGA CCT TCA GTC AGA GGC ATC AAC-3'; mSpt6-F: 5'-CAG CAG TTC CTC TAC GTG CAA ATG-3'; and mSpt6-R: 5'-ACT GGA TCA AGG CCT GGC TGT AAG-3'. Stealth siRNAs were introduced into CH12F3-2A or BL2 cells using Amaxa Nucleofector (Amaxa Biosystems). Stealth siRNAs were purchased from Invitrogen: mSpt6-1, -2, -3, and -4 (MSS209819, 209820, 209821, NM_009297_stealth_3806), AID (MSS235859), hSPT6-1, -2, and -3 (HSS110374, 110375, 110376), hAID-1, -2, and -3 (HSS126211, 126212, 126213), mTrim28-1, -2, -3, and -4 (MSS211796, 211797 211798, NM_011588_stealth_1165), and hTRIM28-1, -2, -3, and -4 (HSS115468, 115470, NM_005762_stealth_883, NM_005762_stealth_2386). The efficiency of nucleofection was confirmed to be more than 90% by introducing fluorescein-labeled siRAN oligo.

CSR Assay. CH12F3-2A cells were stimulated for 24 h with CD40L, TGF-β, and IL-4 24 h after introducing siRNA. The surface expression of IgM and IgA was analyzed by staining cells with FITC-conjugated anti-mouse IgM (Southern Biotechnology Associates) and PE-conjugated anti-mouse IgA (Southern Biotechnology Associates). Flow cytometric analyses were performed with a FACSCalibur, and data were analyzed by CellQuest software (BD Biosciences). Live cells were selected for the analyses by forward- and side-scatter intensity and propidium iodide (PI) gatings. CH12F3-2A cells expressing AID-ER were stimulated with OHT and TGF-β for 24 h after introducing siRNA, and the surface expression of IgM and IgA was analyzed by flow cytometry as described above. NIH 3T3 cells expressing the artificial switch substrate SCI(μ, α) and mAID-ER were introduced with d-siRNA and a DsRed-expressing plasmid as a transfection indicator using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were stimulated with OHT 24 for 36 h and stained with allophyco-cyanin–conjugated anti-mouse CD8α (eBioscience). The amounts of μGLT and αGLT were evaluated by quantitative PCR as described previously (32).

SHM Assay. The hygromycin phosphotransferase and EGFP cDNA were fused in-frame in pFB to generate an artificial SHM substrate, pFB-HyGFP. BL2 cells were introduced with AID JP8Bdel-ER and pFB-HyGFP by retroviral infection. A clone expressing AID JP8Bdel and pFB-HyGFP was chosen after selection with puromycin and hygromycin. The clone was stimulated for 24 h with OHT 24 h after introducing siRNA and incubated for an additional 48 h in the absence of OHT. Expression of GFP and survival were evaluated by flow cytometry. Live cells were selected for the analyses by forward- and side-scatter intensity and PI gatings. Genomic DNA was extracted, and GFP sequence was amplified and analyzed. NIH 3T3 cells harboring a SHM substrate pI were infected with retroviruses expressing wild-type and mutant AID-GFs and cultured for 7 d. Genomic DNA was extracted and GFP sequence was amplified and analyzed.

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Statistical analysis of features associated with protein expression/solubility in an *in vivo Escherichia coli* expression system and a wheat germ cell-free expression system

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Recombinant protein technology is an important tool in many industrial and pharmacological applications. Although the success rate of obtaining soluble proteins is relatively low, knowledge of protein expression/ solubility under 'standard' conditions may increase the efficiency and reduce the cost of proteomics studies. In this study, we conducted a genome-scale experiment to assess the overexpression and the solubility of human full-length cDNA in an *in vivo Escherichia coli* expression system and a wheat germ cell-free expression system. We evaluated the influences of sequence and structural features on protein expression/solubility in each system and estimated a minimal set of features associated with them. A comparison of the feature sets related to protein expression/solubility in the *in vivo Escherichia coli* expression system revealed that the structural information was strongly associated with protein expression, rather than protein solubility. Moreover, a significant difference was found in the number of features associated with protein solubility in the two expression systems.

Obtaining highly concentrated, soluble proteins' preparations is necessary for conducting various structural and biophysical studies or using proteins as materials for pharmaceutical or industrial products. *Escherichia coli*, which is easy to handle and manipulate genetically, is the preferred host for overexpressing recombinant proteins, since it can be cultivated rapidly and inexpensively. Moreover, it generally yields high levels of recombinant proteins (*1*). Since the proteins are expressed by the host, one reason for non-expression is a deleterious interaction with the host's metabolism. In addition, a common reason for insolubility is the formation of inclusion bodies. Therefore, the success rate for obtaining soluble proteins is relatively low. For that reason, the construction of protein overexpression systems is an important experimental challenge.

To overcome these unfavourable circumstances, several solutions have been proposed, based on the results of experimental studies: using a different strain of *E. coli*; modifying the N-terminal (*2*) and C-terminal sequences (*3*); fusion with solubility enhancing tags (*4*) and coexpression with molecular chaperones (*5*). Similarly, various alternative cell-based expression systems have been developed. Such systems utilize yeast, insect cells or murine myeloma cells as hosts (*6*). In recent years, cell-free methods for protein synthesis with extracts from prokaryotic (*7*) or eukaryotic (*8*) cells have become an alternative to cell-based methods. The distinctive feature is an *in vitro* translation system. Cell-free expression systems are popular in proteomics and biotechnology, because of their high levels of protein expression and ease of handling (*9, 10*).

In theoretical computational science, clear sequence differences between proteins that remain soluble and those that form inclusion bodies have been reported, thereby yielding some successes in predicting protein solubility solely from amino acid sequences (*11–17*). The first attempt to determine the interconnection between amino acid sequences and protein solubilities was performed by Wilkinson and Harrison (*11*). They observed that protein solubility is strongly associated with the charge average and the turn-forming residue fraction. Subsequent studies revealed several factors associated with protein expression and solubility. Such knowledge under 'standard' conditions may provide a clue for determining priority targets in a large-scale proteomics analysis. However, the difference between the factors related to protein expression

and solubility has remained unclear for genome-scale analyses. In addition, no study has revealed the differences in these factors among protein expression systems.

In this study, we estimated and compared the minimal sets of sequence and structural features associated with protein expression/solubility in an *in vivo E. coli* expression system and a wheat germ cell-free expression system, from an analysis of genome-wide experimental data. The results provide useful information for proteomics analyses.

Materials and Methods

Protein expression experiments

We conducted genome-scale experiments that assessed the overexpression and the solubility of human full-length complementary DNA (cDNA) in an *in vivo E. coli* expression system and a wheat germ cell-free expression system.

In the *E. coli* expression system, the 17,739 human open reading frames [ORFs as Gateway entry clones (9)] were first subcloned into the pDEST17 vector (T7 promoter, amino-terminal His-tag fusion), using LR Clonase (Invitrogen). *E. coli* BL21 star (DE3) pLysS was transformed with the reaction products. The SOC expression mixture was then plated on LB agar plates containing ampicillin. For each ORF, LB medium was inoculated with a single colony and grown overnight at 37°C. The overnight culture was diluted 1:100 into SB medium, grown at 37°C for 3 h and cooled to 20°C. Protein expression was then induced by adding isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM. After 16 h at 20°C, the cells were harvested and suspended in BugBuster (Novagen Inc.). Some of the lysate was reserved as the whole cell sample. The lysate was centrifuged at 15,000*g* for 5 min. The supernatants were prepared as the soluble fraction samples. These protein samples were denatured with SDS-sample buffer and fractionated by 12.5% sodium dodecyl sulfate–poly-acrylamide gel electrophoresis (SDS–PAGE). Proteins were visualized with CBB R-250. Protein expression and solubility were judged by visual inspection, according to whether a clear band was detectable at the expected position calculated from the molecular weight in the whole cell and soluble fraction samples. When a clear band in the whole cell sample lane was obviously observed in the presence of bands derived from

E. coli, it was regarded as overexpression. Conversely, when a specific band was not detected at the expected position, it was regarded as no expression. In the case of a smeared band, it was regarded as low expression. On the same basis, the protein solubility was evaluated using the band in the soluble fraction sample lane.

In the wheat germ cell-free expression system, we expressed 8850 human proteins, for which the entry clones were chosen randomly from our human Gateway entry clone resources (9, 18). The expressed proteins were fused with a carboxy-terminal His-tag (destination vectors: pEW-3H) and were labelled with radioactive amino acids (^{14}C-Leu or ^{35}S-Met). The wheat germ extract was purchased from Toyobo and Cell Free Sciences Co., Ltd. The expressed proteins were separated into soluble and insoluble fractions by centrifugation at 19,000*g* for 20 min. The samples were separated by SDS–PAGE, and the protein expression and solubility were measured by detecting the specific activities of the ^{14}C-Leu and ^{35}S-Met radioisotopes (RI), using a BAS 2000 scanner (Fuji). The rate of dissolution was estimated as described below

$$\text{Rate of dissolution (\%)} = (\text{signal intensity of soluble fraction}/ \\ \text{signal intensity of whole sample}) \times 100 \quad (1)$$

When the rate of dissolution was >60%, the protein was regarded as being highly soluble. In contrast, a protein with the rate of dissolution of <40% was regarded as an insoluble protein.

Data sets

We prepared two data sets, based on the number of experiments per sequence, for the statistical analysis. One group, designated as 'data set_S' ('S' means single), comprised sequences for which the protein expression and solubility were experimentally assessed one time. The other group, designated as 'data set_M' ('M' means multiple), comprised sequences for which the experiments were conducted two or more times.

The two data sets were constructed as follows (Fig. 1). First, the clones bearing the same sequence among the experimental expression data were selected (Fig. 1, Step1). The redundancy checks were executed on the nucleotide level and the amino acid level for protein expression and solubility, respectively. If the same sequences existed, they were regarded as one sequence. In the case of protein expression in the *in vivo E. coli*, 474 out of 17,739 clones were redundant, and 227 sequences remained after checking the sequence redundancy. Next, the sequences for which the experimental results were not the same were removed, to use only the reproducible data (Fig. 1, Step2). In the case of protein expression in the *in vivo E. coli*, 44 sequences were removed. The representative sequences were

Clones that assessed protein expression/solubility

Fig. 1 **Construction process of data set.** Each table represents the number of data set size in each step.

Table I. Data set sizes for statistical analyses.

Expression system	Data set	Expression		Solubility	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)
E. coli	Data set_ME	113 (61.7)	70 (38.3)	71 (37.6)	118 (62.4)
	Data set_SE	7631 (58.7)	5366 (41.3)	2725 (35.7)	4909 (64.3)
Wheat Germ	Data set_MW	208 (99.5)	1 (0.5)	86 (63.2)	50 (36.8)
	Data set_SW	7062 (97.2)	201 (2.8)	2653 (69.5)	1166 (30.5)

Numbers in parentheses signify ratios of positive data and negative data for respective data sets.

then selected in each data set, to avoid the bias of similar sequences (Fig. 1, Step3). The sequences with pair-wise sequence identity of >80%, using CD-hit (*19*), having similar length >80% were assumed to be in a cluster. The longest sequence in each cluster was selected as the representative sequence of each cluster. This collection of sequences was defined as data set_M. On the other hand, data set_S was constructed from the data from which the redundant clones from the expression data had been removed. In the case of protein expression in the *in vivo E. coli*, 17,265 (=17,739—474) sequences were used. The data that showed a smeared band were removed by visual inspection (Fig. 1, Step2) (see 'Results' section). In the case of protein expression in the *in vivo E. coli*, 2703 sequences were removed. Next, in the same manner as for data set_M, the representative sequences were selected from each cluster consisting of similar sequences (Fig. 1, Step3). This collection of sequences was defined as data set_S. The data set size is shown in Table I.

In this study, data set_M was used for estimating the features associated with the protein expression and solubility; data set_S was used for assessing whether a set of selected features corresponds to the general characteristics on a genomic scale. The initial letter of the expression system was added to the end of the data set name, to distinguish them. For example, 'data set_SE' consists of the sequences for which experimental evaluations were performed one time in the *in vivo E. coli* expression system.

Estimation of the features associated with protein expression/solubility

We defined 437 features to investigate the factors associated with protein expression/solubility in the two kinds of expression systems. The features were divided into two groups, based on the information used for producing them, except for the size of the polypeptide.

The first group was derived from sequence information, from both the nucleotides and amino acids. The nucleotide information included the occurrence frequencies of four single nucleotides, 64 codons and the GC contents. Similarly, the amino acid information contained the occurrence frequencies of 20 single amino acids and the property groups, defined by their chemical properties (eight groups: [GALVI][FYW][ST][DE][NQ][RHK][CM][P]) and physical properties (five groups: [GAVLIP][FWY][STCMNQ][DE][RKH]) (Supplementary Table SI). Additionally, the repeat was defined as the maximum number of consecutive same amino acids or property groups. The values of these features were computed for the entire chains and both terminal regions, defined as 60 bases (meaning 20 amino acid residues), because modification of the terminal regions influences protein expression and solubility (*2–4*). The use of a His-tag fusion raises the possibility that the features in the N-terminal region of the *in vivo E. coli* expression system and the C-terminal region in the wheat germ cell-free expression system may not be evaluated properly. We considered the His-tag to have the same influence on any sequences, since we conducted the protein expression experiments under the same conditions. Therefore, we evaluated them under this hypothesis. In total, the first group was composed of 396 features.

The second group was derived from structural information, obtained with several prediction using amino acid information. The structural information included the secondary structures—α-helix, β-sheet and others predicted by PHD (*20*)—along with the transmembrane regions [predicted using TMHMM (*21*)] and the disordered regions [predicted using POODLE-L (*22*)]. For the secondary structures, the ratio of each element to the entire chain was computed. For the disordered regions, their number of occurrences, lengths and proportions in relation to the entire chain were

computed. For the transmembrane regions, the number of occurrences in the entire chain was computed. The structure information also included the occurrence frequencies of single amino acids and the same property groups on the protein surface. The accessible surface area was predicted using RVPnet (*23*). In total, the second group included 40 features.

We estimated which features are associated with protein expression/solubility by analyzing data set_ME and data set_MW. For all features, the statistical difference between positive and negative data was determined using the Student's *t*-test. The positive data of protein expression and solubility mean that a clear band was found in the whole cell sample and the soluble fraction sample. The negative data signify the opposite. A difference of $P < 0.05$ was considered significant.

Assessing the generality of the features

To evaluate whether the set of features selected in the previous section corresponds to the general characteristics of protein expression/solubility on a genome-scale in the two expression systems, we built a statistical model that distinguishes between overexpression and low expression, using sequence information only. Similarly, a statistical model to discriminate between soluble and insoluble proteins was built as well. In this study, we applied the random forest (RF) algorithm (*24*) to produce the statistical models.

First, the sequence in the training and evaluation data set was expressed as a multi-dimensional vector that defined the selected features in the previous section as descriptors. The numbers of elements in a vector were 64 and 45 for protein expression and solubility in the *in vivo E. coli* expression system, respectively (see 'Results' section). In contrast, the sequence was expressed by 32 elements in the wheat germ cell-free expression system (see 'Results' section). The statistical models were then built by training data sets. The default values were used as the RF parameters.

The classification abilities of the statistical models for both expression systems were estimated, using two kinds of evaluation methods. One method was a 5-fold cross validation test using data set_M only. The other method was an expanded test. The statistical models were constructed using data set_M. The classification abilities of these models were then estimated, using data set_S. Finally, the classification abilities obtained from the two evaluation methods were compared. Moreover, in order to validate the features, the classification abilities of these models were compared with that of the Wilkinson and Harrison model (*11*). The model was used to predict the *in vivo* solubility of recombinant proteins in *E. coli*:

$$CV = 15.43\frac{N+G+P+S}{n} - 29.56\left|\frac{(R+K)-(D+E)}{n} - 0.03\right| + 1.71,$$

where N, G, P, S, R, K, D and E are the numbers of asparagines, glycines, prolines, serines, arginines, lysines, aspartic acids and glutamic acids, respectively and *n* is the total number of residues in the sequence. If $CV < 0$, then the protein is predicted to be soluble. If $CV > 0$, then the protein is predicted to be insoluble.

Results

Comprehensive assessment of protein expression/solubility of human full-length cDNA in two expression systems

The human full-length cDNA was expressed in the two expression systems. The results were analysed
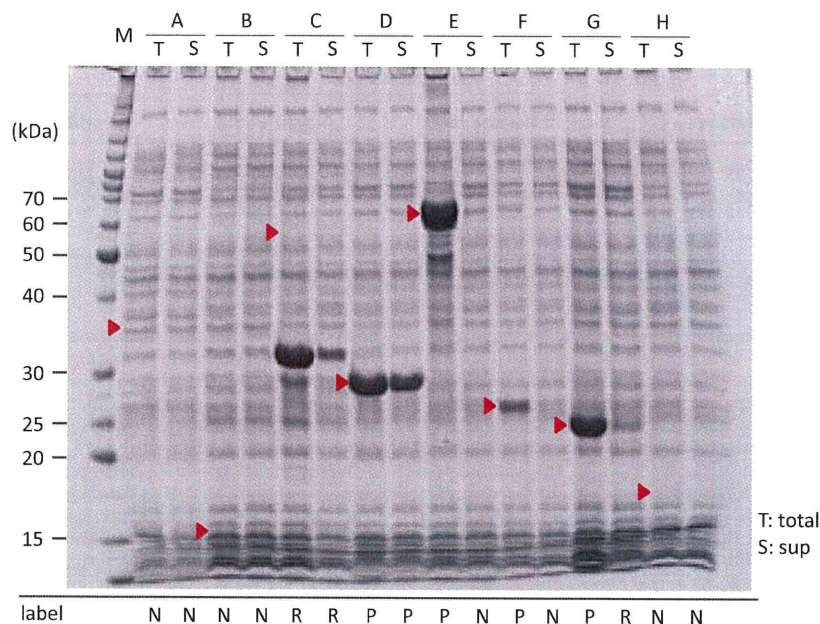
Fig. 2 Example of an SDS–PAGE analysis for eight proteins expressed in the *in vivo E. coli* expression system. M and A¯H, respectively show molecular weight markers and samples. The T and S lanes, respectively show samples obtained from whole cell samples and soluble fraction samples. The red triangles represent the expected positions calculated from the molecular weights. P and N in the label signify positive and negative data, respectively. R in the label shows data removed from statistical analyses.

using SDS–PAGE (Fig. 2). The gels containing the fractionated proteins expressed in the wheat germ cell-free expression system can be seen at the site HGPD (http://riodb.ibase.aist.go.jp/hgpd/cgi-bin/index.cgi) (*18*). When a clear band is present at the expected position calculated from the molecular weight, such as in lane T of sample D in Fig. 2, the data are considered to be positive. However, when an expected band in SDS–PAGE cannot be detected, such as that in lane T of sample A in Fig. 2, the data are considered to be negative. Data were removed from the following analysis if a smeared band (lane S of sample G in Fig. 2) was observed or a clear band existed at an unexpected position (lane T of sample C in Fig. 2), in order to avoid ambiguity in the experimental data. When the SDS–PAGE results were visually inspected to check the protein expression in the *in vivo E. coli* expression system, 44 of 227 raw data sets were removed between the multiple measurements. Similarly, 16.7 and 23.0% of the raw data were excluded, respectively, from the protein solubility in the *in vivo E. coli* and the wheat germ cell-free expression systems.

The sizes of data set_ME and data set_MW are smaller than those of data set_SE and data set_SW (Table I), but data set_ME and data set_MW are more reliable experimental data. In the *in vivo E. coli* expression system, ~60 and 35% of the proteins, respectively, were expressed and soluble. In contrast, almost all of the proteins were expressed in the wheat germ cell-free expression system: ~65% of the proteins were soluble (Table I). The wheat germ cell-free expression system exhibited higher performance in obtaining soluble proteins. For the wheat germ cell-free

expression system, only the protein solubility data were used in the following statistical analyses.

Estimation of the features associated with protein expression/solubility in the two expression systems

The sizes of the polypeptides used to assess the protein expression/solubility experimentally in the *in vivo E. coli* expression system were investigated (Fig. 3A). The average size of the overexpressed polypeptides was significantly longer ($P < 0.05$) than that of the polypeptides with low expression, but no statistically significant difference was found between the sizes of the soluble and insoluble polypeptides. Conversely, in the wheat germ cell-free expression system, the average size of the insoluble polypeptides was significantly longer than that of the soluble polypeptides (data not shown).

Similarly, some sequence and structural features associated with protein expression/solubility were identified from statistical analyses of data set_ME and data set_MW (Fig. 4). In this study, data set_M is not suitable for analyzing the nucleotide information associated with the protein solubility, because data set_M of solubility included sequences that are not identical on the nucleotide level. Consequently, the analysis of the nucleotide information was performed only for the protein expression.

From the perspective of nucleotide information, no GC content or single nucleotide was selected in the *in vivo E. coli* expression system, but 18 out of 61 codons were chosen to have significant contribution to protein expression. Only three rare-frequency codons in the *E. coli* genetic code, among eight tested, passed the Student's *t*-test having significant