ucts during DNA synthesis in the PCR reactions; (3) automated size separation on DNA sequencing (polyacrylamide) gels; (4) laser detection of the two fluorescent single-stranded DNA fragments that represent respective polymorphic alleles; and (5) multiplex processing (up to six SNPs) by gel electrophoresis and detection of fluorescent fragments simultaneously using multiple loading of fragments labeled with different fluorescent colors. These approaches successfully improved the accuracy and success rate of our genotyping assay.

Gonadotropin-releasing hormone is a key molecule in the hypothalamic–pituitary–gonadal axis that controls estrogen status. Dramatic changes in GnRH levels in serum during the perimenopausal period in women underscore the key role of GnRH in governing the entire process of hormonal regulation. Treatment of hypogonadism by GnRH agonists/antagonists sometimes causes estrogen deficiency due to increased negative feedback, with a resulting increase in biochemical markers of bone turnover and bone loss [16–18]. All of these facts indicate that normally regulated GnRH secretion is a requirement for maintenance of normal bone mass.

Estrogen status correlates not only with premenopausal peak bone mass [3] but also with increased bone turnover in postmenopausal women [1,5]. Thus, multiple factors involved in the estrogen regulatory system are assumed to contribute to acquisition of peak bone mass as well as postmenopausal bone loss. A longitudinal study of bone loss in a follow-up study of our cohort and/or an examination of peak bone mass among younger women would help to clarify these issues. In addition, reproducible association or linkage should be tested in a different ethnic group by means of both standard and alternative methods of association studies, including the transmission disequilibrium test (TDT).

Multiple reports have described correlations between variations in signal peptide sequences and altered function of those peptides [19–22]. In general, a signal peptide sequence contains a hydrophobic core region consisting of 6–15 amino acid residues [23]. In GnRH, the 11 hydrophobic amino acids from 7Leu to 18Val (LLAGLILLTWCV) are conserved among mammalian species and among gene families. The 16Trp (wild-type) residue lies at the C-terminal end of the hydrophobic core. Alteration of 16Trp to 16Ser would entail a loss of hydrophobicity from 0.50 to −0.55 units, suggesting that this alteration might cause a shift in translocation efficiency of the peptide from free ribosomes to endoplasmic reticulum or a change in some other aspect of intracellular transport of molecules. Those suggestions require further investigations, which might include, for example, an in vitro translated peptide-translocation assay that would measure the amount of peptide bind to microsomal membrane in vitro, and pulse–chase experiments on cultured cells.

In summary, we have developed the Sd-PCR genotyping method that can transform nucleotide differences (G, A, T, or C) between two alleles at a single site into size differences between the respective alleles, an automated, high-throughput, and inexpensive SNP typing procedure for large-scale, genomewide SNP screening. This procedure was applied to identify a novel osteoporosis susceptibility gene, *GnRH*, whose polymorphic alleles encode an amino acid variation (W16S) that correlated significantly with bone mineral density among postmenopausal women in the general Japanese population as well as among osteoporosis patients. Structural inspection indicated that this amino acid sequence variation could affect processing of the signal peptide. In that case, variation in the *GnRH* gene might explain, at least in part, the pathogenesis of postmenopausal osteoporosis and this information could lead to development of new treatment designs and plans for prevention of the disease.

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ORIGINAL ARTICLE

Association of tumor necrosis factor receptor 1 gene polymorphism with bone mineral density

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Background: Estrogen deficiency in postmenopausal women causes an increased production of proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α. These cytokines are associated with an increase of bone turnover and an acceleration of bone loss. Tumor necrosis factor-α is known to promote osteoclastogenesis via TNFR1, one of the tumor necrosis factor receptors (TNFR). Therefore, the purpose of the present report was to investigate the association of *TNFR1* gene polymorphism with bone mineral density (BMD) in postmenopausal Japanese women.

Methods: The question of whether a polymorphism of the *TNFR1* gene would correlate with osteoporosis in 320 unrelated healthy postmenopausal women in Japan, was investigated. A single nucleotide polymorphism (SNP) located at Pro12 (CCA to CCG) in exon 1 of *TNFR1* was utilized.

Results: The subjects were categorized into three genotypes: AA, AG, and GG. The frequency of each genotype was 72.2%, 23.8%, and 4.0%, respectively. The association of this polymorphism with BMD of the lumbar spine and total body, and several bone metabolic markers was then examined. Concerning the *TNFR1* gene, the AA group had significantly low total body BMD, compared with the AG + GG group (Z score; 0.285 *vs* 0.568; $P = 0.03$), although BMD of the lumbar spine was not statistically different.

Conclusion: These results suggest an association between this SNP of the *TNFR1* gene and BMD, and an involvement of TNFR1 in postmenopausal osteoporosis among Japanese.

Keywords: bone mineral density, genetics, osteoporosis, polymorphism, tumor necrosis factor receptor 1.

Introduction

Osteoporosis is characterized by pathologically low bone mass and an increased risk of fracture.[1,2] Osteoporotic fracture is a serious event in an increasingly aging population. Low bone mass is one of the most significant risk factors for fractures, and it has been suggested that low bone mass may be partly hereditary.[3,4] The report of Morrison *et al.* was the first to demonstrate an association of common allelic variants of the *vitamin D receptor* gene (*VDR*) with bone mineral density (BMD).[5] Following this study, several groups reported on the *VDR* gene polymorphism[6] and BMD, but its genetic implications are still controversial.[7,8]

Estrogen deficiency in postmenopausal women causes an increased production of proinflammatory cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis fac-

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tor (TNF)-α.[9] An increased production of proinflammatory cytokines is associated with an increase of bone turnover and an acceleration of bone loss, which leads to an increased susceptibility to fractures.[10] Thus, we investigated in this report the association with a *tumor necrosis factor receptor* (*TNFR*) gene polymorphism and BMD in postmenopausal Japanese women.

Tumor necrosis factor-α has a tumor necrosing activity.[11] It is secreted from macrophages and monocytes in several pathological conditions, such as systemic inflammation, malignant tumor, and autoimmune diseases.[12] Moreover, TNF-α regulates lipoprotein lipase (LPL) activity, and elevates the serum level of triglyceride.[13] The overproduction of TNF-α, not only from macrophages and monocytes, but also from adipocytes, induces so-called 'insulin resistance'.[14] Tumor necrosis factor receptors are members of transmembrane receptors, and are mediators of various TNF-α actions.[15,16] There are two subtypes of TNFR: TNFR1 and TNFR2.[17,18] Tumor necrosis factor receptor 1 is expressed constitutively in various cell lineages, including osteoblast-progenitor cells and osteoclasts, and is reported to suppress osteoblast formation and promote osteoclast formation.[19–22] In contrast, the roles of TNFR2 in the bone metabolism are still unclear. A *TNFR2* gene polymorphism is reported to be related to glucose metabolism, especially 'insulin resistance'.[23,24] With regards to the involvement of *TNFR2* gene polymorphism in the bone metabolism, it is reported that a polymorphism in the 3′ untranslated region (UTR) is associated with low BMD.[25] However, to our knowledge the association of *TNFR1* gene polymorphism with BMD has not been reported as yet. Therefore, we have studied the association of a single nucleotide polymorphism (SNP) of *TNFR1* gene[26] with BMD.

Methods

Subjects

Genotype analysis was performed using the blood samples obtained from 320 unrelated postmenopausal women (mean age ± SD: 64.9 ± 9.6 years) living in Nagano prefecture, Japan. Subjects who had endocrinological disorders (e.g. hyperthyroidism, hyperparathyroidism), diabetes mellitus, liver disease, renal disease, use of medications that were known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin), or unusual gynecologic history were excluded. Subjects who were affected by collagen diseases were also excluded. All volunteers were unrelated and gave informed consent before the study.

Genotyping

The DNA was extracted from white blood cells of peripheral blood.[27] The DNA fragment containing one base substitution (CCA to CCG) at Pro12 in exon 1 of *TNFR1* was amplified by polymerase chain reaction (PCR) using a Gene Amp PCR kit (Perkin Elmer Cetus, Norwalk, CT, USA). The reaction was carried out in a final volume of 25 μL containing 100 ng of genomic DNA, 10 pmol of each primer, 200 mmol/L dNTP, 10 mmol/L Tris-HCL (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.001% gelatin and 0.1 U Taq DNA polymerase (Takara, Kyoto, Japan). The sense and antisense primers to detect TNFR1 gene polymorphism were 5′-GAGCCCAAATGGGGGAGTGAGAGG-3′ (TNFR1S) and 5′-ACCAGGCCCGGGCAGGAGAG-3′ (TNFR1AS),[26] respectively. Template DNA was amplified by 35 cycles by the following steps: denaturation at 95°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. The PCR products were digested with MspA1I (Takara), which can recognize A to G substitution and electrophoresed in 2% agarose gel.

Clinical data

Bone turnover markers, pyridinoline (Pyr), deoxypyridinoline (Dpyr) in urine[28] and intact-osteocalcin (IOC) in serum,[29] in addition to parathyroid hormone (PTH), calcitonin, 1, 25(OH)2D3, calcium (Ca), phosphate (P) and alkaline phosphatase (AL-P), total cholesterol (TC), triglyceride (TG) were measured. Moreover, the BMD of the lumbar spine (L2–4) and total body was measured by dual-energy X-ray absorptiometry (DXA) using fast scan mode (DPX-L; Lunar, Madison, WI, USA). The Z score (average 0, SD 1) was calculated by the installed software of Lunar DPX-L based on the data from 20 000 Japanese women.[30]

Statistical analysis

Comparison of BMD in Z scores and biochemical markers between the groups of each allelic genotype were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group were normally distributed, we applied the Student's *t*-test, using the STATVIEW-J4.5 software (SAS Institute, Cary, NC, USA).

Results and discussion

After amplification of DNA fragments containing the CCA to CCG substitution at Pro12 in exon 1 of *TNFR1*, the PCR products were digested with MspA1I, which can recognize A to G substitution. The polymorphic MspA1I site was detected as 108-bp and 75-bp fragments. The subjects were categorized into three genotypes: AA, AG, and GG (Fig. 1).

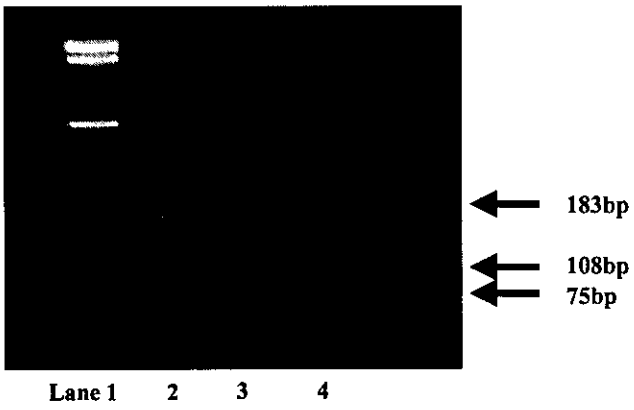

Figure 1 Electrophoresis of polymerase chain reaction (PCR) products revealing polymorphism in tumor necrosis factor receptor (TNFR) 1, after digestion of DNA with MspA1I. In recombinant DNA samples, a single 183-bp product was derived from AA homozygotes (lane 2); 183-, 108-, and 75-bp products were derived from AG heterozygotes (lane 3); and 108- and 75-bp products were derived from GG homozygotes (lane 4). Lane 1, molecular-weight marker.)

Frequencies of alleles in the *TNFR1* gene were 231 AA homozygotes, 76 AG heterozygotes and 13 GG homozygotes. The risk ratio of the Hardy–Weinberg formula was 0.0421 and slightly significant, possibly due to recruitment of subjects from a relatively small isolated area in Japan. We analyzed the correlation of the genotypes with BMD of the lumbar spine and total body between two groups of 231 AA homozygotes and 89 AG heterozygotes + GG homozygotes. As a result, the AA group had significantly lower total body BMD, compared with the AG + GG group. (Z score; 0.285 *vs* 0.568; $P = 0.03$) (Table 1). There was a high level of deoxypyridinoline (Dpyr) in the urine in the AA group (Dpyr; 7.60 *vs* 6.93; $P = 0.059$).

The cytokines, such as IL-1, IL-6, and TNF-α, are thought to be important promoters for bone loss in postmenopausal women. Fontova *et al.* reported the association of femoral BMD with *IL-1 receptor antagonist* (*IL-1ra*) and *TNF-α* gene polymorphism.[31] Genotype combination of A2 allele (A2+) of the *IL-1ra* gene and

Table 1 Comparison of Z scores of lumbar spine (L2–L4) and total body bone mineral density, background and biochemical data of the subjects between the groups of genotypes

Variable	Genotype		*P*
	AG + GG ($n = 89$)	AA ($n = 231$)	
Age (years)	64.7 ± 8.5	65.0 ± 10.0	NS
Body height (cm)	149.4 ± 6.1	150.8 ± 6.4	NS
Bodyweight (kg)	50.8 ± 8.1	51.1 ± 8.2	NS
Years after menopause	15.4 ± 8.4	15.6 ± 10.4	NS
BMI (kg/m^2)	22.5 ± 3.3	22.7 ± 3.1	NS
% fat	31.8 ± 8.4	32.4 ± 7.6	NS
Lumbar spine BMD (Z score)	0.203 ± 1.2	0.041 ± 1.45	NS
Total body BMD (Z score)	0.568 ± 0.98	0.285 ± 0.95	0.03
Serum			
Ca (mg/dL)	9.13 ± 0.41	9.15 ± 0.38	NS
P (mg/dL)	3.55 ± 0.44	3.48 ± 0.45	NS
AL-P (IU/l)	163.6 ± 49.4	175.7 ± 54.7	NS
I-OC (mg/dL)	7.35 ± 3.3	7.77 ± 4.0	NS
Intact PTH (pg/mL)	38.6 ± 14.2	37.3 ± 14.2	NS
Calcitonin (pg/mL)	22.6 ± 9.5	23.1 ± 9.9	NS
1, 25 (OH)2D3 (pg/mL)	34.9 ± 11.3	33.4 ± 12.7	NS
TC (mg/dL)	200.7 ± 33.1	196.7 ± 36.1	NS
TG (mg/dL)	160.4 ± 92.9	144.4 ± 78.6	NS
Urinary			
Pyr (pmol/μmol Cr)	32.2 ± 8.87	34.7 ± 11.4	0.09
Dpyr (pmol/μmol Cr)	6.93 ± 1.98	7.60 ± 2.82	0.06

NS, not significant; BMI, body mass index; BMD, bone mineral density; AL-P, alkaline phosphatase; I-OC, intact osteocalcine; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; Pyr, pyridinoline; Dpyr, deoxypyridinoline.
Values are given as mean ± SD.

GG allele of the *TNF-α* gene was associated with greater hip BMD at femoral neck and Ward triangle levels ($P = 0.02$). We focused on *TNFR* gene polymorphisms in the TNF-α signal in the present study. Here, we have noticed the influence of a polymorphism of the *TNFR1* gene on bone metabolism. The AA group of *TNFR1* polymorphism had significantly lower total body BMD, compared with the AG + GG group, although there was no significant difference in BMD of the lumbar spine between these two groups. Lower BMD in postmenopausal women can be considered as a result of abnormally rapid bone loss and/or lower peak bone mass. At present, two hypotheses could be proposed: (i) this silent polymorphism may be linked with other mutations in exons, which contributes to the change of the TNFR1 protein function or may be linked with a mutation in regulatory elements affecting the levels of expression through variable transcriptional regulation; or (ii) the polymorphism in the *TNFR1* gene may be linked with a mutation of another undefined gene adjacent to the *TNFR1* gene, which cause low BMD directly or indirectly. Our results suggest that TNFR1 may be involved in the bone metabolism among postmenopausal Japanese women, and that the SNP in the *TNFR1* gene is useful as a genetic marker for osteoporosis. In addition, the contribution of other genetic factors should be analyzed further by utilizing other candidate genes that are known to play important roles in the bone metabolism.

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Association of a single-nucleotide polymorphism in low-density lipoprotein receptor-related protein 5 gene with bone mineral density

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Abstract Low-density lipoprotein receptor-related protein 5 (LRP5) is an important regulator of osteoblast growth and differentiation, affecting peak bone mass in vertebrates. Here, we analyzed whether the *LRP5* gene was involved in the etiology of postmenopausal osteoporosis, using association analysis between bone mineral density (BMD) and an *LRP5* gene single-nucleotide polymorphism (SNP). Association of an SNP in the *LRP5* gene at IVS17-1677C > A (intron 17) with BMD was examined in 308 postmenopausal Japanese women (65.2 ± 9.6 years; mean ± SD). The subjects bearing at least one variant A allele (CA + AA; n = 142) had significantly lower Z scores for total body and lumbar BMD than the subjects with no A allele (CC; n = 166) (total body, 0.08 ± 1.09 versus 0.50 ± 1.03; P = 0.0022; lumbar spine, −0.42 ± 1.43 versus −0.02 ± 1.42; P = 0.013). These findings suggest that the *LRP5* gene is a candidate for the genetic determinants of BMD in postmenopausal women, and this SNP could be useful as a genetic marker for predicting the risk of osteoporosis.

Key words wnt · LRP5 · osteoporosis · bone mineral density · polymorphism

Introduction

Osteoporotic fracture is a serious event in an increasingly aging population. Low bone mass is one of the most significant risk factors. Twin and sibling studies have revealed that the proportion of variance of bone mineral density (BMD) accounted for by genetic factors is around 50%–90% [1–6]. These studies have suggested that the variation in BMD among individuals is largely caused by genetic factors. Therefore, genetic markers that are correlated with BMD would be useful for predicting future bone loss and for clarifying the mechanism of bone loss in osteoporosis. After an association of BMD with vitamin D receptor (VDR) genotypes was reported [7], polymorphisms in several other genes were investigated [8]. These genes included those implicated in bone formation by the regulation of osteoblast growth and differentiation, such as transforming growth factor beta 1 (TGFβ1) [9], collagen type Ia1 (COLIA1) [10], parathyroid hormone (PTH) [11], and p57Kip2 (CDKN1C) [12]. Considering the polygenetic nature of BMD distribution and the multiplicity of endocrine factors known to regulate bone mass and bone turnover, it is important that the panel of candidate genes could be expanded to elucidate the whole genetic background of osteoporosis.

The Wnt signaling pathway plays a pivotal role in embryonic development and oncogenesis [13,14]. Studies using *Drosophila*, *Xenopus*, and mammalian cells have established a canonical signaling pathway [15–17]. Both genetic and biochemical results have provided solid evidence indicating that FZ proteins function as Wnt receptors. Wnt proteins bind Frizzled (FZ) and prevent glycogen synthase kinase 3 (GSK3)-dependent phosphonylation of β-catenin, leading to the stabilization of β-catenin. Meanwhile, the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) were found to be also required for the Wnt signaling pathway as Wnt co-receptors [18,19]. Recent reports have demonstrated that the Wnt-β-catenin signaling pathway regulates bone density through LRP5 [20–23]. Inactivating mutations in LRP5 decrease bone mass and cause the autosomal-recessive disorder osteoporosis-pseudoglioma syndrome in humans [20] and mice [21]. Conversely, activating mutations in LRP5 are linked to autosomal-dominant high-bone mass traits [22,23]. These data suggest that LRP5, which modulates

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Wnt signaling, controls bone metabolism in vivo in mammals. To examine the possible contribution of the *LRP5* gene to the etiology of involutional osteoporosis, we investigated an association between polymorphism in this gene and BMD in Japanese women.

Subjects and methods

Subjects

Genotypes were analyzed in DNA samples obtained from 308 healthy postmenopausal Japanese women (mean age ± SD; 65.2 ± 9.6 years) living in Nagano prefecture, Japan. Exclusion criteria included endocrine disorders such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease, use of medications known to affect bone metabolism (e.g., corticosteroids, anticonvulsants, heparin), or unusual gynecologic history. All women were non-related volunteers and provided informed consent before this study.

Measurement of BMD and biochemical markers

The lumbar spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry, using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentrations of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), intact osteocalcin (I-OC; enzyme-linked immunosorbent assay [ELISA]; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin, 1, 25$(OH)_2D_3$, total cholesterol (TC), and triglyceride (TG). We also measured urinary pyridinoline (PD; HPLC method) and deoxypyridinoline (DPD; HPLC method). The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. The Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20000 Japanese women.

SNP Selection

A polymorphic variation of the *LRP5* gene was extracted from the JSNP-database (http://snp.ims.u-tokyo.ac.jp/index.html), and was denoted as IVS17-1677C > A according to its localization on the gene.

Genotyping procedure

Genotypes of IVS17-1677C > A were determined using the SNP-dependent (Sd)-polymerase chain reaction (PCR) method, a modified allele-specific PCR of polymorphic sequence as previously described [24,25]. Two allele-specific primers (AS-primers) and one reverse primer were prepared per single-nucleotide polymorphism (SNP). The AS-primers (long and short) have a five-base difference between them; each has a polymorphic nucleotide of the SNP sequence at the 3′ ends, and an additional artificial mismatch introduced near the 3′ end. Primer sequences used were as follows: IVS17-1677C > A FL-primer: 5′-TTTTTGGGCGGTAAAT ACACGTCTCTCGAG-3′; IVS17-1677C > A FS-primer: 5′-CCGCGGTAAATACACGTCTCTCGAT-3′; and IVS17-1677C > A reverse-primer: 5′-GTTTCC GTCAGAAC GCTGCACTA-3′.

This primer set allowed distinct discrimination of alleles. For the assay, a genomic DNA sample (10ng) was amplified with 250nM of each primer (two polymorphic forward, and a reverse) in a 10-μl reaction mixture containing 10mM dNTPs, 10mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 1U Taq DNA polymerase, and 0.5mM fluorescence-labeled dCTP (ROX-dCTP; Perkin-Elmer, Norwalk, CT, USA). The Sd-PCR reaction was carried out in a thermal cycler (Gene-amp system 9600; Perkin-Elmer) with initial denaturalization at 94°C for 4min, followed by 5 cycles of stringent amplification (94°C for 20s, 64°C for 20s, 72°C for 20s) and then 25 cycles at 94°C for 20s, 62°C for 20s, 72°C for 20s), terminating with a 2-min extension at 72°C. Allele discrimination was carried out by electrophoresis and laser scanning of the DNA fragments on an ABI Prism 377 DNA system, using GeneScan Analysis Software ver2.1 (Applied Biosystems, Foster City, CA, USA). To confirm the accuracy of the Sd-PCR method, direct resequencing was carried out using the ABI Prism BigDye Terminator system (Applied Biosystems).

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the minor A-allele and the group with only the major C-allele encoded at that locus were subjected to analysis. Coefficients of skewness and kurtosis were calculated to test deviation from a normal distribution. Because the clinical and biochemical traits in each genotypic group were normally distributed, we applied Student's *t*-test, using StatView-J4.5 software (SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

Association of LRP5 *gene polymorphism in intron 17 with BMD*

We analyzed the genotypes for the *LRP5* IVS17-1677C > A polymorphism (rs3781586 in the National Center for Biotechnology Information [NCBI] dbSNP data-

Table 1. Comparison of background and biochemical data between subjects bearing at least one A allele (AA + CA) and subjects with no A allele (CC) at IVS17-1677 (intron 17)

Items	Genotype (mean ± SD)		*P* value
	CC	CA + AA	
No. of subjects	166	142	
Age (years)	65.1 ± 9.6	65.4 ± 9.9	NS
Height (kg)	151.0 ± 6.2	150.3 ± 6.4	NS
Body weight (kg)	50.7 ± 8.4	50.3 ± 8.1	NS
Lumbar spine BMD (g/cm^2)	0.92 ± 0.20	0.87 ± 0.19	0.025
Lumbar spine BMD (Z score)	−0.02 ± 1.42	−0.42 ± 1.43	0.013
Total body BMD (g/cm^2)	1.00 ± 0.11	0.96 ± 0.12	0.015
Total body BMD (Z score)	0.50 ± 1.03	0.08 ± 1.09	0.0022
Ca (mg/dl)	9.2 ± 0.43	9.2 ± 0.45	NS
P (mg/dl)	3.4 ± 0.46	3.4 ± 0.48	NS
ALP (IU/l)	183.7 ± 62.6	195.4 ± 71.0	NS
I-OC (ng/ml)	7.6 ± 4.2	8.3 ± 3.7	NS
PD (pmol/μmol of Cr)	36.1 ± 24.7	34.8 ± 12.0	NS
DPD (pmol/μmol of Cr)	7.6 ± 5.2	7.4 ± 2.4	NS
Intact PTH (pg/ml)	35.1 ± 16.4	35.8 ± 16.6	NS
Calcitonin (pg/ml)	22.8 ± 11.1	23.4 ± 11.7	NS
1,25 $(OH)_2D_3$ (pg/ml)	37.5 ± 12.6	34.3 ± 10.4	NS
TC (mg/dl)	198.7 ± 37.5	195.7 ± 39.2	NS
TG (mg/dl)	141.5 ± 81.4	136.8 ± 71.4	NS
Percent fat	32.1 ± 7.9	31.6 ± 7.4	NS
BMI	22.2 ± 3.2	22.2 ± 2.9	NS

Statistical analysis was performed according to the method described in the text
BMD, bone mineral density; Ca, calcium; P, phosphate; ALP, alkaline phosphatase; I-OC, intact-osteocalcin; PD, pyridinoline; DPD, deoxypyridinoline; PTH, parathyroid hormone; TC, total cholesterol; TG, triglyceride; BMI, body mass index; NS, not significant

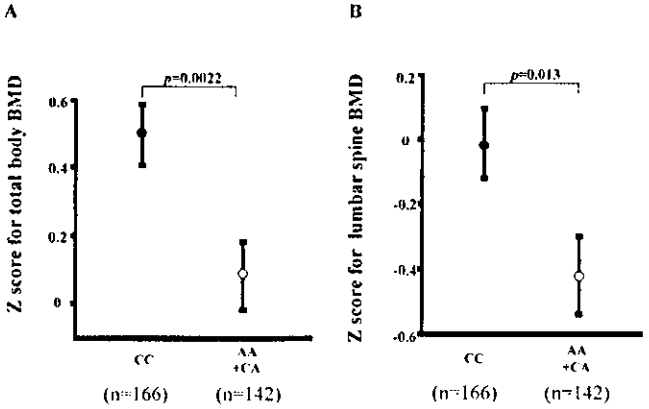

Fig. 1. Z Score values for total body and lumbar bone mineral density (*BMD*) in the groups with each genotype of the *LRP5* gene in intron 17 (IVS17-1677C > A). **A** Z Score values for total body BMD are shown as the *solid circle* for genotype CC at IVS17-1677 and as the *open circle* for genotype AA + CA at IVS17-1677. Values are expressed as means ± SE. Numbers of subjects are shown *in parentheses*. **B** Z Score values for lumbar spine BMD are shown in the same manner as in **A**

base) in 308 subjects, using Sd-PCR methods [25]. Among the 308 postmenopausal volunteers, 24 were AA homozygotes, 118 were CA heterozygotes, and 166 were CC homozygotes. Allelic frequencies were 0.731 for the C allele and 0.269 for the A allele in this population.

We compared Z scores for BMD of total body and lumbar spine between subjects bearing at least one chromosome with the A allele (genotype AA + CA; n = 142) and subjects with no A allele (CC; n = 166). The former subjects had significantly lower Z scores for total body BMD (0.08 ± 1.09 versus 0.50 ± 1.03; P = 0.0022, Fig. 1A) and lumbar BMD (−0.42 ± 1.43 versus −0.02 ± 1.42; P = 0.013; Fig. 1B). As shown in Table 1, the background data were not significantly different between these groups.

Discussion

We investigated the influence of a genetic variation of the *LRP5* gene on bone mineral properties. The allelic frequencies of an SNP in intron 17 (0.731 for IVS17-1677C and 0.269 for IVS17-1677A) in Japanese postmenopausal women were in Hardy-Weinberg equilibrium. The allelic frequencies of this SNP in the general Japanese population were reported in the JSNP database (IMS-JST137897). The database reported that the allelic frequencies were 0.726 for IVS17-1677C and 0.274 for IVS17-1677A, indicating that the allelic frequencies in the present study were in line with the JSNP database.

Recently, patients with homozygous *LRP5* gene disruption were reported [20]. There are many types of mutations affecting bone mass accrual during growth, causing the autosomal recessive disorder osteoporosis-pseudoglioma syndrome. Regarding the effect on the bone, these patients showed a marked decrease in their BMD. In addition, Kato et al. [21] created and characterized *LRP5* gene knockout mice. Interestingly, *LRP5* gene knockout mice showed lower bone mass density than wild-type mice because of decreasing osteoblast proliferation. In their report, Kato et al. [21] observed the presence of LRP5 protein in osteoblasts lining the endosteal and trabecular bone surfaces, but not in osteoclasts, by immunohistochemistry in wild-type mice. Recently, a gain-of-function mutation (G171V) in the *LRP5* gene was described in two kindreds with an enhanced bone density [22,23]. In vitro studies showed that the normal inhibition of Wnt signaling by another protein, Dickkopf-1 (Dkk1), was defective in the presence of this mutation, resulting in increased signaling due to unopposed Wnt activity. Thus, LRP5 may be one of the cellular mediators involved in bone formation, by regulating the proliferation and differentiation of osteoblasts.

In the present study, significant correlation was observed between BMD and a polymorphism in intron 17 (IVS17-1677C > A). To our knowledge, this is the first report that a common SNP in the *LRP5* gene affected BMD. However, it is still unclear how BMD is affected by this intronic polymorphism of the *LRP5* gene. For explaining this, three hypotheses could be proposed. (i) This intronic polymorphism may be linked with exon mutations and may contribute to changing LRP5 protein function. (ii) This polymorphism may be linked with mutations of regulatory elements and may affect the levels of expression through transcriptional regulation. (iii) The polymorphism in the *LRP5* gene may be linked with mutation of another unidentified gene adjacent to the *LRP5* gene which causes low BMD directly or indirectly.

In conclusion, our finding suggests that the *LRP5* gene may be a candidate for the genetic determinants of BMD in postmenopausal women. Examining *LRP5* gene variation will, it is hoped, enable us to understand one of the mechanisms of involutional osteoporosis. Wnt and LRP5 signaling have been implicated in other diseases, including cholesterol and glucose metabolism-related diseases [26]. The variant presented here may be involved in the risk of such diseases, as well as osteoporosis.

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Protein Phosphatase 5 Is a Negative Regulator of Estrogen Receptor-Mediated Transcription

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Estrogen receptors (ERs) are transcription factors that can be modulated by both estrogen-dependent and growth factor-dependent phosphorylation. A yeast two-hybrid screening identified a serine/threonine protein phosphatase (PP5) as an interactant of ERβ (1–481), a dominant negative ERβ mutant. Glutathione *S*-transferase pull-down assays, mammalian two-hybrid assays, and immunoprecipitation studies showed that PP5 directly binds to both ERα and ERβ via its tetratricopeptide repeat domain. E domains of ERα and ERβ, without containing activation domain core regions in transcription activation function 2, were required for the binding to PP5. In ERα-positive breast cancer MCF7 cells, estrogen- and epidermal growth factor-dependent phosphorylation of ERα on serine residue 118, a major phosphorylation site of the receptor, was reduced by expressing PP5 but enhanced by PP5 antisense oligonucleotide. Estrogen-induced transcriptional activities of both ERα and ERβ and mRNA expression of estrogen-responsive genes, including pS2, c-*myc*, and cyclin D1, were suppressed by PP5 but enhanced by PP5 antisense oligonucleotide. A truncated PP5 mutant consisting only of its tetratricopeptide repeat domain acted as a dominant negative PP5 that enhanced serine residue 118 phosphorylation of ERα and transactivations by ERα and ERβ. We present the first evidence that PP5 functions as an inhibitory regulator of ER phosphorylation and transcriptional activation *in vivo*. (*Molecular Endocrinology* 18: 1131–1143, 2004)

THE PHYSIOLOGICAL FUNCTIONS of estrogen are mediated by the two estrogen receptors, ERα and ERβ (1). ERs are transcription factors that regulate expressions of estrogen-targeted genes in response to hormone binding. Like other transcription factors, ERs are phosphoproteins (2). It has been observed that direct phosphorylation of ERα is induced upon addition of ligands (3) as well as mediated by protein kinases in a ligand-independent manner (4–7). Serine 118 (S118) is a major phosphorylation site within A/B domain, or N-terminal transcription activation function (AF)-1 of ERα, the mutation of which reduces transactivation by ERα (3, 8). Previous literature shows that S118 is phosphorylated by MAPK (4) or by Cdk-activating kinase, a cyclin-dependent kinase that phosphorylates the *Pol*II C-terminal tail domain (7). S118 phosphorylation is also induced by ligand binding to the receptor in a MAPK- or Cdk-activating kinase-independent manner (9).

The molecular mechanism(s) by which the phosphorylation of ERs is regulated remain(s) to be clarified. The reversible phosphorylation of proteins is catalyzed by protein kinases and phosphatases. Among the enzymes, serine/threonine protein phosphatases belong to the PPP family that specially targets phosphorylation of serine/threonine residues (10). The PPP family of phosphatases is comprised of several members including protein phosphatase 1 (PP1), PP2A, PP2B, PP2C, and PP4–7 (11). PP5 (serine/threonine protein phosphatase), another member of the PPP family, has a unique character in that it consists of a single polypeptide chain containing a phosphatase catalytic domain near its C terminus and four tetratricopeptide repeat (TPR) domains as a regulatory region in its N terminus (12). The TPR domain consists of a highly degenerate 34-amino acid repeat initially identified in several cell-cycle gene products and in proteins involved in the regulation of RNA synthesis (13, 14). TPR domains mediate protein-protein interactions

Abbreviations: AD core, activation domain core; AF, activation function; AS, antisense; E_2, 17β-estradiol; dccFCS, dextran-coated charcoal-treated fetal calf serum; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GR, glucocorticoid receptor; GST, glutathione *S*-transferase; PP5, serine/threonine protein phosphatase type 5; S118, serine 118; S118A, mutation of S118 to alanine; S118E, substitution of S118 to glutamic acid; Scr, scrambled; SDS, sodium dodecyl sulfate; SSC, sodium saline citrate; TPR, tetratricopeptide repeat; VP16, herpes simplex viral protein 16; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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(15), and there is evidence that the TPR domain of PP5 targets the phosphatase to other proteins, including heat shock protein 90-glucocorticoid receptor complex (16), apoptosis signal-regulating kinase 1 (17), the atrial natriuretic peptide receptor (18), the anaphase-promoting complex (19), and PP2A (20).

Here we show that PP5 directly binds to ERα and ERβ and inhibits transcriptional activities of the receptors. ERα and ERβ interact with the TPR domain of PP5 through their E domains without including activation domain (AD) core regions of helix 12 within C-terminal transcription AF-2, which was previously shown to be important for binding to coactivators (21–23). 17β-Estradiol (E_2)-dependent or epidermal growth factor (EGF)-dependent phosphorylation of ERα on S118 is inhibited by PP5 but enhanced by a truncated mutant of PP5 consisting of only the TPR domain. PP5 suppresses the transactivations by ERα and ERβ but not those by ERα mutants with one amino acid substitution, S118A (mutation of S118 to alanine) or S118E (substitution of S118 to glutamic acid (3). PP5 inhibits E_2-induced mRNA expression of estrogen-targeted genes including pS2, c-*myc*, and cyclin D1. Furthermore, an antisense (AS) oligonucleotide against PP5 that could reduce endogenous PP5 expression enhanced both E_2-dependent and EGF-dependent phosphorylation of ERα, transactivation by ERα, and E_2-induced mRNA expression of estrogen-targeted genes. Our results may present a novel molecular mechanism that PP5 is a key regulator of the signaling pathways of ERα and ERβ in a negative manner.

RESULTS

Identification of PP5 that Interacts with a Dominant Negative ERβ

To identify novel binding partners of ERs, we used the yeast two-hybrid system based on a Lex A-ERβ (1–481) fusion protein to screen a cDNA library derived from estrogen-depleted MCF7 breast cancer cells. ERβ (1–481) mutant is a dominant negative form of ER that has potential to repress both ERα- and ERβ-mediated transactivation (24). Among positive clones, three independent clones encoding PP5 were obtained. The interactions of the PP5 clones between ERβ (1–481) were confirmed by the galactose-dependent growth of yeast strain EGY48, which was cotransformed with pSH18–34 LacZ reporter plasmid, pEG202NLS-ERβ (1–481), and the PP5 clones in galactose-inducible pJG4–5 (Fig. 1).

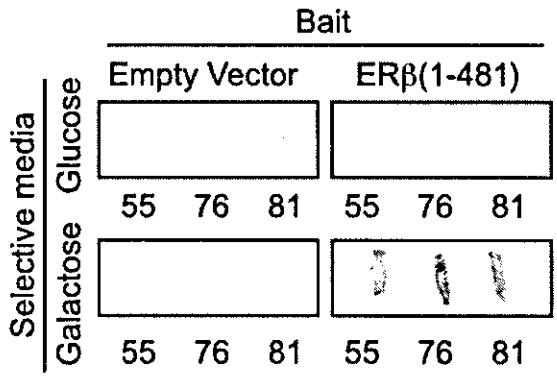

Fig. 1. Specific Interaction between PP5 and ERβ (1–481) in Yeast

Galactose-inducible plasmids pJG4–5 including PP5 clones (nos. 55, 76, and 81) obtained from yeast two-hybrid screening were transformed into yeast EGY48 along with LacZ reporter plasmid and pEG202NLS containing ERβ (1–481) or its empty vector. The specific interaction between PP5 clones and ERβ (1–481) construct was observed in galactose-containing medium through β-galactosidase staining.

ERs Directly Bind to and Are Substrates for PP5

To characterize whether PP5 directly interacts with ERs *in vitro*, we performed glutathione *S*-transferase (GST) pull-down assays using bacterially expressed GST or GST-fused PP5 mutants and *in vitro* translated ERα and ERβ (Fig. 2). Both ERα and ERβ were pulled down by full-length PP5, but not by GST alone. GST-PP5 (28–165), GST-PP5 (2–181), and GST-PP5 (2–312) can bind both ERs, whereas neither GST-PP5 (2–71) nor GST-PP5 (181–499) can bind the receptors (Fig. 2, B and C). The data suggest that both ERα and ERβ directly bind to PP5, and PP5 interacts with ERs through its TPR domains.

We next examined whether the interaction between PP5 and ERs has ligand dependency (Fig. 2, D and E). The amounts of pulled-down ERα or ERβ did not change for GST-PP5 or GST-PP5 (28–165) in the presence or absence of E_2, indicating that the association of PP5 with ERs does not require ligand activation.

We further investigated which domain(s) of ERs is/are responsible for the association with PP5 *in vivo* by performing mammalian two-hybrid assays. Cotransfection of expression constructs for the herpes simplex viral protein 16 (VP16) transactivation domain fused to PP5 and the GAL4 DNA binding domain fused to full-length or fragments of ERα/ERβ was performed into 293T cells, and the binding ability of PP5 with ER fragments was assessed by measuring luciferase activity that is derived from a GAL4-driven luciferase reporter (Fig. 3). The most significant luciferase activity was observed when PP5 interacted with E domains of ERs without containing the activation domain (AD) core regions within AF-2 [*i.e.* ERα (302–530) and ERβ (248–481)] (Fig. 3B). ER fragments including ABCD domains, ABC domains, and the AD core regions within E/F domains showed no binding activity to PP5.

The finding that TPR domains in PP5 are requisite sites for interaction with ERs was further confirmed by coimmunoprecipitation experiments, using 293T cells cotransfected with green fluorescent protein (GFP)-tagged ERα along with Flag-tagged full-length PP5 or

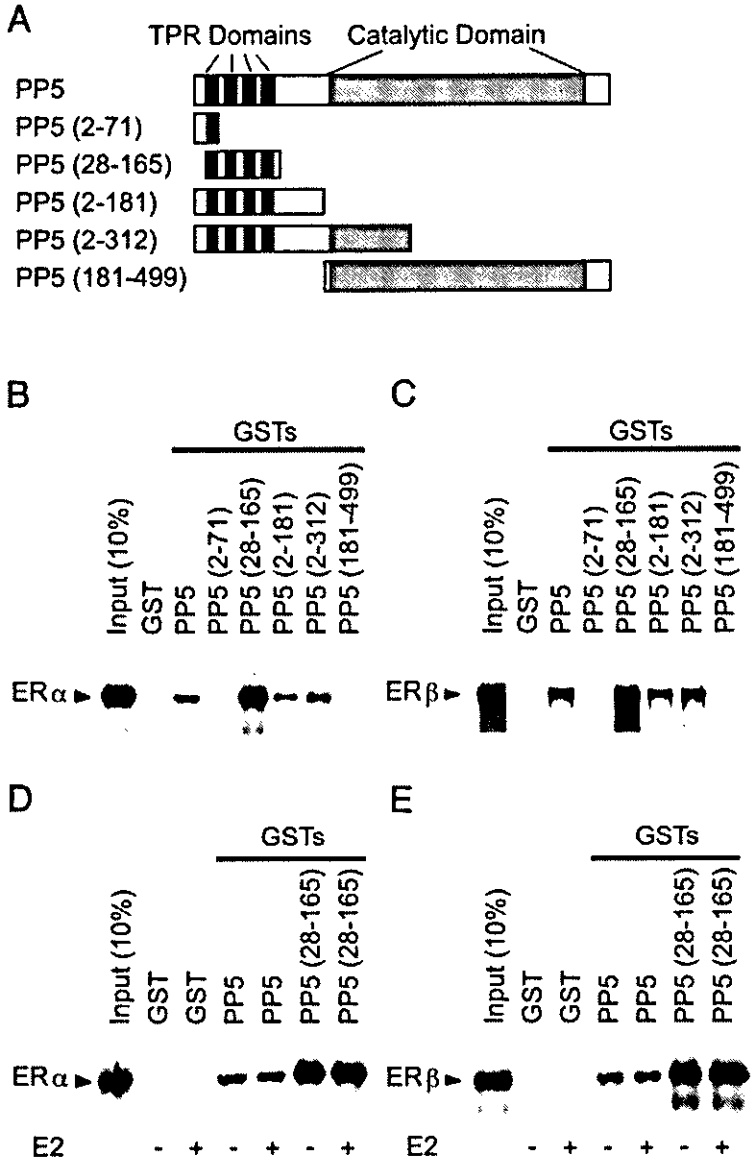

Fig. 2. ERα and ERβ Proteins Are Pulled Down by GST Fusion Proteins Containing PP5 or Its TPR Domains

A, Schematic diagrams of human PP5 constructs. PP5 cDNAs were inserted into pGEX4T-1 downstream of and in frame with the GST tag and GST or GST fusion proteins were expressed in *Escherichia coli*. *Solid* and *shaded boxes* indicate TPR domains and catalytic domain, respectively. B and C, *In vitro* translated ERα (B) and ERβ (C) proteins labeled with ^{35}S-methionine were incubated with GST or GST fusion proteins containing full-length PP5 or PP5 mutants. Labeled proteins corresponding to 10% of input and materials bound to glutathione-Sepharose were separated by 10% SDS-PAGE and detected by radioautography. D and E, Estrogen stimulation does not affect the *in vitro* interaction of full-length PP5 or its TPR domains [PP5 (28–165)] with ERα (D) and ERβ (E). GST pull-down assays were performed as described above except for the addition of 10 nM E_2 in the binding solution.

a PP5 mutant with only its TPR domains (Fig. 4B). The *in vivo* interaction between endogenous PP5 and ERα in human breast cancer MCF7 cells was also shown by coimmunoprecipitation study (Fig. 4C).

PP5 Inhibits Phosphorylation of ERα on S118

To assess the phosphatase activity of PP5 on ERs, we next examined whether PP5 modulates the phosphorylation of ERα on S118, which is a major phosphorylation site for ERα. In MCF7 cells, maximal phosphorylation levels of ERα were observed 60 min and 5 min after E_2 stimulation and EGF stimulation, respectively, analyzed by immunoblotting using a specific antibody against ERα phosphorylated at S118 (ERα-P-S118). In cells transfected with PP5, the phosphorylation level of ERα 60 min after E_2 stimulation was 29% reduced compared with control cells transfected with a control

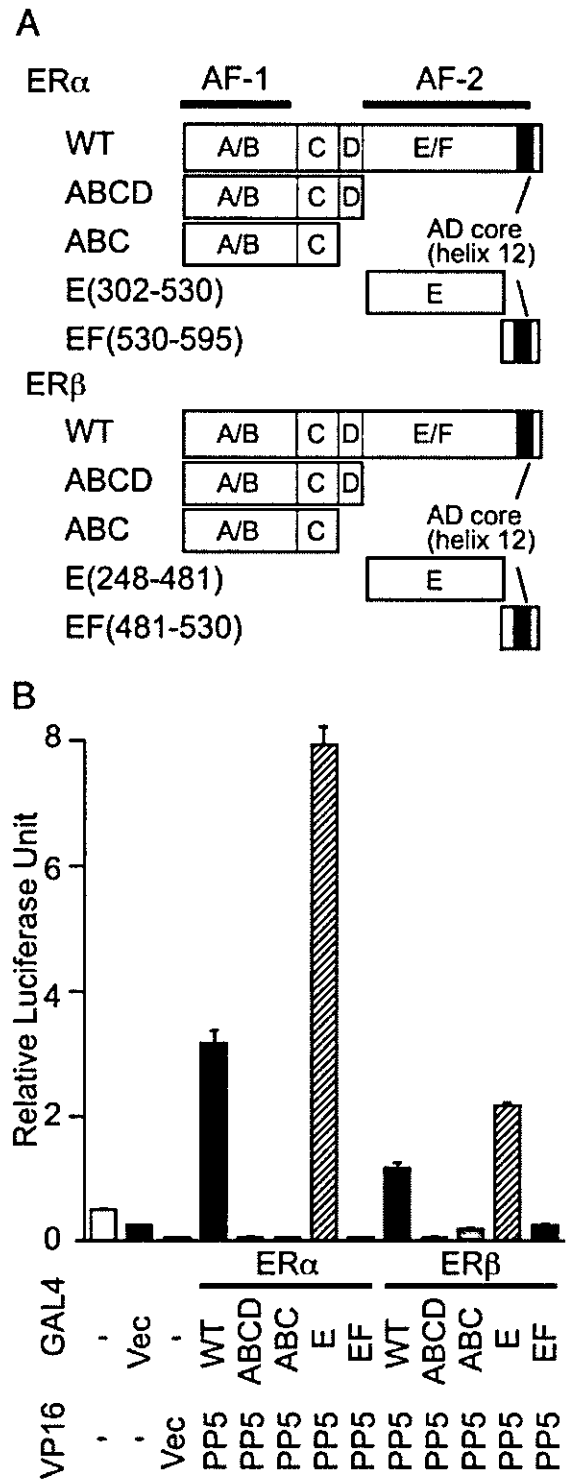

Fig. 3. Interaction of PP5 with ERα and ERβ in Mammalian Two-Hybrid Assays

A, Structure of full-length and truncated mutants of human ERα and ERβ. The cDNAs encoding wild-type (WT) ERα and ERβ or cDNA fragments encoding structural ABCD domains (amino acids 1–302), ABC domains (amino acids 1–263), E domain (amino acids 302–530), and a part of EF domains (amino acids 530–595) of ERα, and ABCD domains (amino acids 1–248), ABC domains (amino acids 1–213), E domain (amino acids 248–481), and a part of EF domains (amino acids 481–530) of ERβ were subcloned into an expression vector pCMX-GAL4 downstream of and in frame with GAL4 DNA binding domain. B, Mammalian two-hybrid assays were carried out using expression vectors of the pCMX-GAL4 containing full-length or mutants of ERα and ERβ (GAL4-ER), and pCMX-VP16 containing PP5 (VP16-PP5), in which PP5 was inserted into pCMX-VP16 downstream of and in frame to the VP16 transactivation domain in pCMX-VP16. 293T cells were cotransfected with 0.7 μg pRL-CMV vector for internal control, 0.8 μg TK-MH100 × 4Luc, 0.2 μg VP16-PP5, and 0.1 μg each pCMX-GAL4 constructs of ERα or ERβ in a well of 24-well plates. The cells were cultured for 24 h and the luciferase assay was performed. Data are the mean ± SD of three independent experiments performed in triplicate.

vector (Fig. 5A), and the phosphorylation level 5 min after EGF stimulation was 29% reduced relative to control cells (Fig. 5B). The maximal S118 phosphorylation levels induced by either E_2 (Fig. 5A) or EGF (Fig. 5B) were approximately 65% and 80% enhanced, respectively, in MCF7 cells transfected with the TPR domains of PP5 compared with control cells. To confirm the inhibitory activity of PP5 on ERα phosphorylation at S118, we investigated the effect of PP5 AS oligonucleotide on the phosphorylation levels of ERα in MCF7 cells. Either E_2-dependent (Fig. 5C) or EGF-dependent (Fig. 5D) phosphorylation of ERα was enhanced by treatment with PP5 AS compared with control mismatch scrambled (Scr) or PP5 sense (Sense) oligonucleotides (~150% increase by 60-min E_2 incubation in AS *vs.* Scr, ~80% increase by 5-min EGF stimulation in AS *vs.* Scr). The results indicate that PP5 has an inhibitory activity on ERα phosphorylation at S118, and the disruption of PP5 activity either by TPR alone or PP5 AS oligonucleotide causes an enhancement of S118 phosphorylation levels.

Suppression of ER Transcription Activity by PP5

To determine whether PP5 affects estrogen-regulated transcription, we next examined transcriptional activity of ERs and ER mutants using an estrogen response element (ERE)-driven luciferase reporter cotransfected with full-length PP5 or truncated PP5 mutants (Fig. 6). PP5 attenuated the ER transcriptional activity in a concentration-dependent manner, as E_2-dependent transactivations by both ERα and ERβ in 293T cells transfected with 50 ng of PP5 were approximately 50% decreased compared with control cells without PP5 transfection (Fig. 6A). Transfection with equal amounts of TPR domain cDNA (5 ng) relative to ER expression vector did not affect E_2-dependent transactivation, whereas 10-fold excess dose of TPR cDNA (50 ng) relative to ER cDNAs elicited a 30–40% increase in E_2-dependent transactivations by both ERα and ERβ compared with control cells. Another PP5 mutant with its catalytic domain alone did not affect ERα and ERβ transactivations. Because levels of S118 phosphorylation of ERα were modulated by PP5 ex-

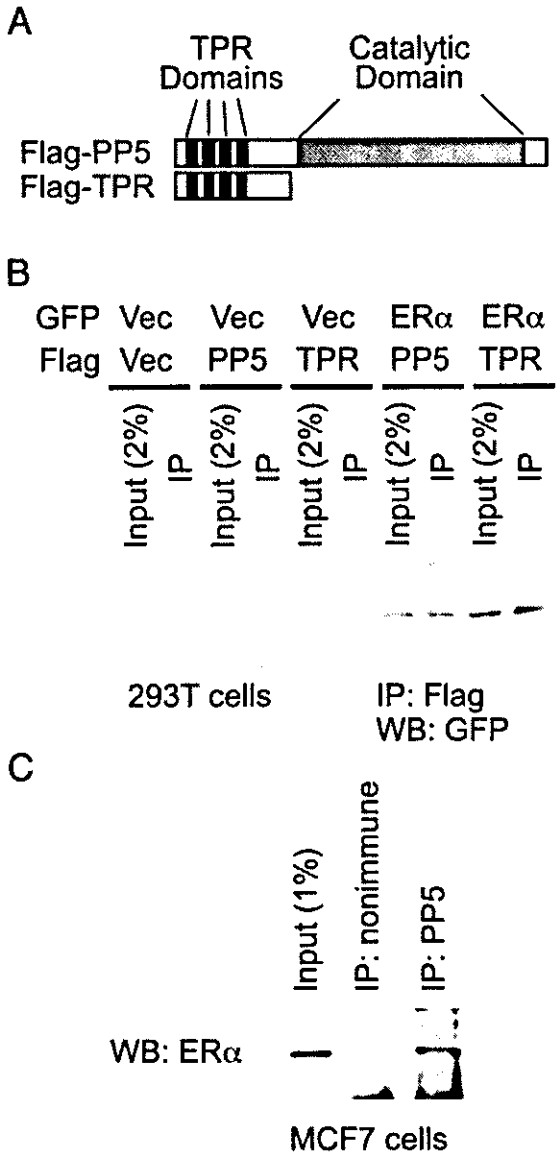

Fig. 4. Interaction and Phosphorylation Inhibition of ERα with PP5

A, Schematic representation of the full-length and TPR domain of PP5. B, Coimmunoprecipitation experiments of PP5 with ERα in 293T cells. Flag-tagged full-length and TPR domain of PP5 or control pcDNA3 vector containing Flag tag (Vec) and GFP-tagged ERα or control pEGFP-C2 vector (Vec) were transfected for 24 h in 293T cells. Cell extracts were obtained after a chemical cross-linking with 200 μg/ml of dithiobis[succinimidyl propionate] (DSP). Extract (1 mg) was incubated with an anti-Flag antibody M2 for 3 h at 4 C and then incubated with protein G-Sepharose beads for 1.5 h at 4 C. Proteins in the immune complexes or 20 μg of the cell extracts (corresponding to 2% of input) were resolved by SDS-PAGE, and the Western blot (WB) was probed with an antibody against GFP. Signals were visualized by an enhanced chemiluminescence system. C, Coimmunoprecipitaion of PP5 with ERα in MCF7 cells. Cell extract (1 mg) from MCF7 cells that were chemically cross-linked with DSP was incubated with either anti-PP5 antibody (PP5/PPT) or nonimmune serum for 3 h at 4 C and then incubated with protein G-Sepharose beads for 1.5 h at 4 C. Proteins in the immune complexes or 10 μg of the cell extracts (corresponding to 1% of input) were resolved by SDS-PAGE, and the Western blot was probed with an antibody against ERα (H-184). IP, Immunoprecipitation.

pression in Fig. 5, we next investigated whether PP5 affects transactivations by ERα mutants on S118. Mutation of S118 to alanine (S118A) causes a reduction in E_2-dependent transcriptional activation in a number of cell types, whereas substitution of S118 to glutamic acid (S118E) resulting in a constitutive negative charge leads to transactivation at levels higher than wild-type ERα (3). E_2-dependent transactivation by S118A was 30% reduced compared with wild-type ERα, whereas that by S118E was approximately 30% enhanced compared with wild-type ERα (Fig. 6B). PP5 expression did not further alter E_2-dependent transactivation by either S118A or S118E, suggesting that PP5 exerts its phosphatase activity on S118. To confirm the inhibitory activity of PP5 on ER-dependent transactivation, we examined the effect of PP5 AS oligonucleotide on the transcriptional activity by ERα (Fig. 6C). The E_2-stimulated ERα transactivation was 2-fold increased by PP5 AS transfection compared with Scr or PP5 sense oligonucleotides transfection, indicating that PP5 negatively regulate ligand-dependent transactivation by ERα.

PP5 Attenuates Induction of Estrogen-Responsive Genes

The ligand-induced ER activation leads to transcriptional induction of estrogen-responsive genes. We next investigated whether PP5 affects E_2-induced expression of the estrogen-responsive genes including pS2 (25), c-*myc* (26), and cyclin D1 (27, 28) by Northern blot analysis (Fig. 7A). Adenoviral expression of PP5 in MCF7 cells markedly suppressed the E_2 (10 nM)-induced mRNA expression of pS2, c-*myc*, and cyclin D1 under the serum-depleted condition, compared with the results of a control adenovirus (Ad-GFP) or vehicle alone. Each mRNA level was normalized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the same sample (Fig. 7, B–D). Approximately 40% reduction of pS2 expression was observed in adenoviral PP5-transduced MCF7 cells 24 h after E_2 stimulation, compared with the gene expression at the same period in control cells including adenoviral GFP-transduced cells and vehicle-transfected cells (Fig. 7B). Regarding c-*myc*, the maximal mRNA expression in Ad-PP5 cells was observed at 2 h after E_2 treatment, and it was only 30% of the maximal expression levels in the control cells (Fig. 7C). The maximal expression of cyclin D1 mRNA in Ad-PP5 cells was delayed compared with that in control cells: the former was at 12 h whereas the latter was at 2 h after E_2 treatment (Fig. 7D). The maximal level of cyclin D1 in Ad-PP5 cells was approximately half of that in vehicle-transfected cells.

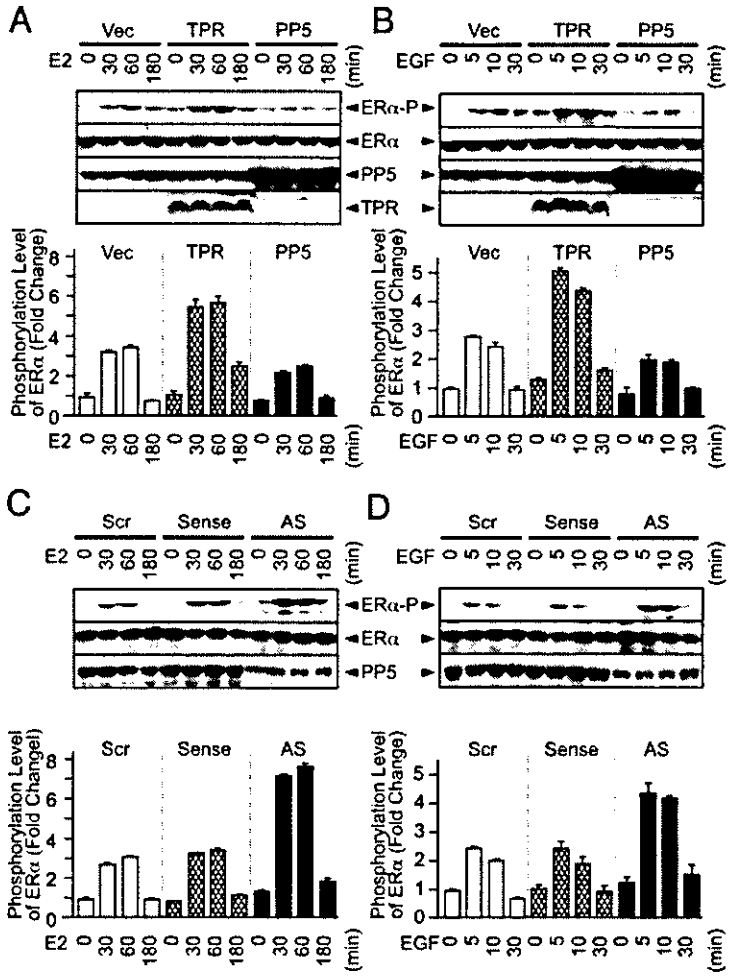

Fig. 5. PP5 Inhibits Phosphorylation of ERα on S118 in MCF7 Cells

MCF7 cells were plated at a density of 1.4×10^5 cells per well on six-well plates overnight, transfected with 1 μg expression plasmids (A and B) or with 1 μg oligonucleotides (C and D) for 12 h, serum starved for 24 h, and subsequently treated with E_2 (10 nM)(A and C) or EGF (100 ng/ml)(B and D) for indicated times. Cell extracts were collected using 80 μl of sample buffer for SDS-PAGE. Extracts (25 μl) were separated by SDS-PAGE and detected by antibody against phosphorylated human ERα on S118 (ERα-P), antibody against whole ERα (H-184), and anti-PP5 antibody (PP5/PPT). A–D, *Upper panels* show representative data from three independent experiments of Western blots after chemiluminescent detection. A–D, *Lower panels* show phosphorylation levels at ERα S118 normalized by total protein amounts of ERα. Quantification of signal intensities was performed using LAS 1000 image analyzer, and data are expressed as the mean ± SD of three independent experiments.

Because S118 phosphorylation levels and ERα transactivation were augmented by treatment with PP5 AS oligonucleotide, we further investigated whether PP5 AS enhances mRNA expression of estrogen-responsive genes (Fig. 8). Transfection with PP5 AS increased basal levels of pS2 (Fig. 8B), c-*myc* (Fig. 8C), and cyclin D1 (Fig. 8D) mRNAs in serum-starved condition (~170%, ~100%, and ~80% increase for pS2, c-Myc, and CycD1, respectively; AS *vs.* Scr). E_2 (10 nM)-induced mRNA expression of those three genes was 25–50% enhanced by PP5 AS compared with PP5 sense or Scr oligonucleotides, without affecting the time course of each gene. The maximal mRNA expression of pS2, c-*myc*, and cyclin D1 detected in 24 h after E_2 stimulation was 25%, 48%, and 36% increased by treatment with PP5 AS, respectively, when compared with Scr oligonucleotide (at 24 h, 2 h, and 6 h after E_2 stimulation for pS2, c-Myc, and CycD1, respectively. $P < 0.05$ for pS2 at 24 h, Scr *vs.* AS; $P < 0.001$ for c-Myc at 2 h, Scr *vs.* AS; $P < 0.05$ for CycD1 at 6 h, Scr *vs.* AS).

Taken together, the data demonstrate that PP5 negatively regulates ER phosphorylation levels, transcriptional activity, and transcriptional induction of estrogen-responsive genes.

DISCUSSION

In the present study, we isolated a serine/threonine phosphatase PP5 as a direct interactant with ERβ through yeast two-hybrid studies. GST pull-down as-

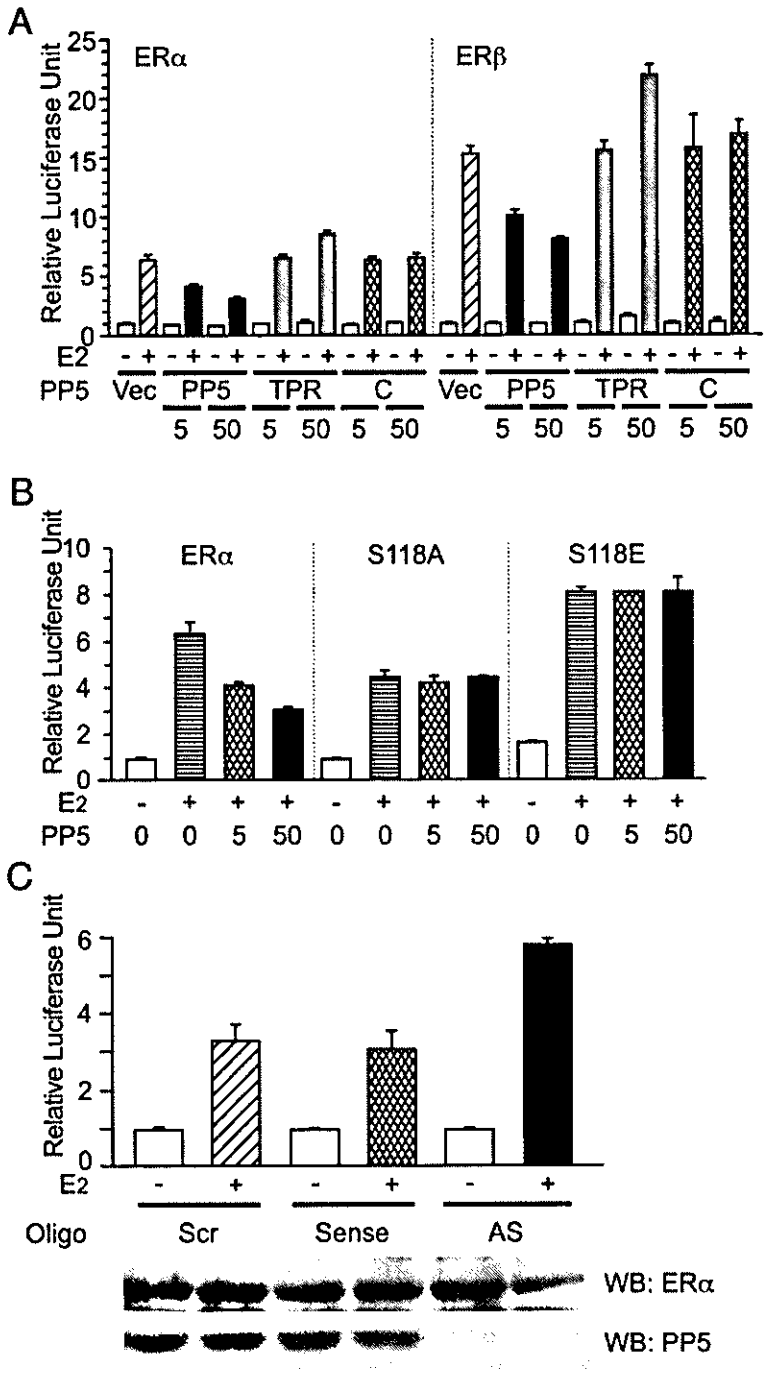

Fig. 6. Inhibition of ER-Mediated Transcription by PP5

A, 293T cells were plated at a density of 1 × 10^4 cells per well on 24-well plates and transfected with 0.8 μg ERE-tk-Luc, 0.7 μg pRL-CMV, 0–50 ng expression vectors for full-length (PP5), TPR domain (TPR), or catalytic domain (C) of PP5, and 5 ng expression vectors for ERα and ERβ. Cells were treated with or without E_2 (10 nM) for 24 h, and luciferase assay were performed. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. B, 293T cells were transfected with 0.8 μg ERE-tk-Luc, 0.7 μg pRL-CMV, 0–50 ng expression vectors for full-length PP5, TPR domain, or catalytic domain (C), and 5 ng expression vestors for wild-type ERα or ERα mutants S118A and S118E. Data are expressed as the mean ± SD of three independent experiments performed in triplicate. C, Effect of PP5 AS oligonucleotide on ER-mediated transcription. Luciferase assays were performed as in panel B except transfection with 0.8 μg ERE-tk-Luc, 0.7 μg pRL-CMV, and 200 ng oligonucleotides into MCF7 cells. *Upper panel* shows the data of three independent experiments performed in triplicate. *Lower panels* show representative data from three independent experiments of Western blots (WB) using the identical cell extracts for luciferase assays. Signals were detected by antibodies against ERα and PP5.

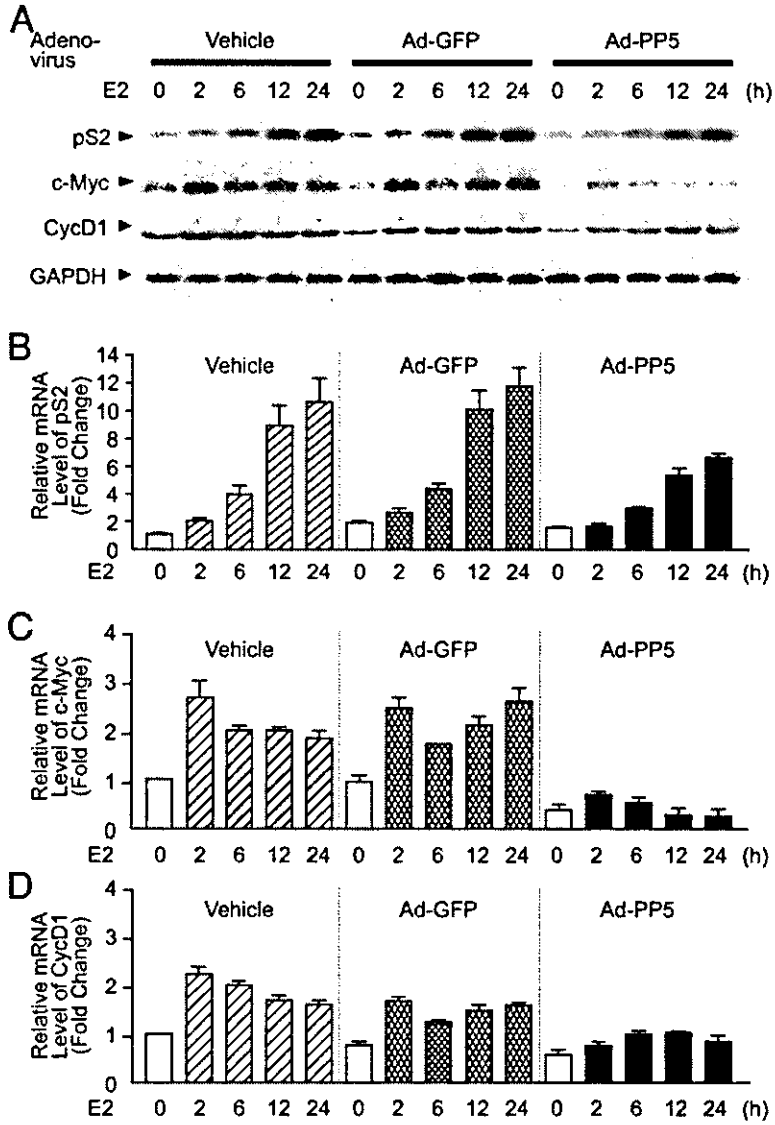

Fig. 7. PP5 Attenuates E_2-Induced Transcription of Estrogen-Responsive Genes in MCF7 Cells

MCF7 cells were plated at a density of 1 × 10^6 cells per well on 10-cm plates and infected with recombinant adenoviruses expressing PP5 (Ad-PP5) and GFP (Ad-GFP), or transfected with vehicle (Vehicle) for 12 h at a multiplicity of infection of 10. Cells were serum starved for 24 h after the infection and treated with E_2 (10 nM) for indicated times. Northern blot analysis was carried out using 20 μg total RNA and hybridized with ^{32}P-labeled cDNAs for pS2, c-*myc*, cyclin D1 (CycD1), and GAPDH. A, The representative result of three independent experiments of Northern blotting. B, C, and D, Quantification of mRNA levels of estrogen-responsive genes including pS2 (B), c-*myc* (C), and CycD1 (D) after normalization to GAPDH mRNA levels in MCF7 cells after E_2 treatment. Data are expressed as the mean ± SD values of fold change over control from three independent experiments. Data for cells treated with vehicle and harvested at the starting point of E_2 treatments are used as control mRNA levels.

says, mammalian two-hybrid assays, and immunoprecipitation studies show that PP5 interacts with both ERα and ERβ through its TPR domains. We demonstrated that PP5 negatively regulates the degree of both estrogen-dependent and EGF-dependent ERα phosphorylation on S118, leading to the suppression of ER transcriptional activity and the reduction of E_2-induced expression of estrogen-responsive genes including pS2, c-*myc*, and cyclin D1. We thus present the first evidence that PP5 acts as an inhibitory regulator of ER signaling pathway in a negative direction.

The status of ER phosphorylation can be regulated by phosphatases, as okadaic acid treatment enhances ERα phosphorylation and ERα transactivation (29). While preparing this manuscript, another member of the PPP family PP2A has been found to interact with ERα and dephosphorylate S118 of the receptor (30). Here we have shown the evidence that ER phosphorylation and function can be modulated by PP5. There are several differences between PP5 and PP2A regarding the interactions with ERs. In our results, the TPR domains of PP5 are the sites responsible for