S100 Proteins Modulate Protein Phosphatase 5 Function

A LINK BETWEEN CA\(^{2+}\) SIGNAL TRANSDUCTION AND PROTEIN DEPHOSPHORYLATION*

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Background: The regulation mechanism of PP5 activity remains poorly understood.

Results: Ca\(^{2+}\)/S100 proteins bind the TPR domain of PP5 and lead to activation of its enzyme activity and dissociation of the PP5-client protein interactions.

Conclusion: Ca\(^{2+}\)/S100 proteins are novel PP5 regulators.

Significance: This finding provides a novel Ca\(^{2+}\) signaling pathway via S100 proteins.

PP5 is a unique member of serine/threonine phosphatases comprising a regulatory tetratricopeptide repeat (TPR) domain and functions in signaling pathways that control many cellular responses. We reported previously that Ca\(^{2+}\)/S100 proteins directly associate with several TPR-containing proteins and lead to dissociate the interactions of TPR proteins with their client proteins. Here, we identified protein phosphatase 5 (PP5) as a novel target of S100 proteins. In vitro binding studies demonstrated that S100A1, S100A2, S100A6, and S100B proteins specifically interact with PP5-TPR and inhibited the PP5-Hsp90 interaction. In addition, the S100 proteins activate PP5 by using a synthetic phosphopeptide and a physiological protein substrate, Tau. Overexpression of S100A1 in COS-7 cells induced dephosphorylation of Tau. However, S100A1 and permanently active S100P inhibited the apoptosis signal-regulating kinase 1 (ASK1) and PP5 interaction, resulting in the inhibition of dephosphorylation of phospho-ASK1 by PP5. The association of the S100 proteins with PP5 provides a Ca\(^{2+}\)-dependent regulatory mechanism for the phosphorylation status of intracellular proteins through the regulation of PP5 enzymatic activity or PP5-client protein interaction.

The intracellular Ca\(^{2+}\) signaling cascade is composed of many molecular components, including the large family of EF-hand Ca\(^{2+}\)-binding proteins such as calmodulin, neuronal calcium sensor proteins, and S100 proteins. S100 proteins share two EF-hand motifs, and Ca\(^{2+}\)-binding causes a conformational change, resulting in an interaction with other proteins (1). S100 proteins regulate many physiological processes, such as cell growth and differentiation, phosphorylation, and transcription (2). TPR\(^{2}\) domains consist of repeated and conserved 34 amino acid sequences and interact with varieties of proteins, including Hsp90 anaphase-promoting complex, cell division cycle 37 (cdc37), and immunophilins (3, 4).

Recently, we demonstrated that S100A2 and S100A6 interacted with the TPR domains of Hsp70/Hsp90-organizing protein (Hop), kinesin light chain (KLC) and translocase of outer mitochondrial membrane 70 (Tom70) in a Ca\(^{2+}\)-dependent manner, leading to dissociation of the Hsp90-Hop-Hsp70, KLC-c-Jun N-terminal kinase-interacting protein-1 (JIP-1) and Tom70-Hsps interactions (5). Further studies have revealed an interaction of S100A1 and S100A2 bound to FK506-binding protein 52 (FKBP52) and cyclophilin 40 (Cyp40), which contain a TPR domain, in the presence of Ca\(^{2+}\) that led to inhibition of the Cyp40-Hsp90 and FKBP52-Hsp90 interactions (6).

Among the TPR proteins, PP5 is a member of the phospho-protein phosphatase family of a serine/threonine phosphatase such as PP1, PP2A, and PP2B (7). Structural analysis has shown that PP5 contains a C-terminal catalytic domain and N-terminal three TPRs that are unique in the phosphoprotein phosphatase family (8, 9). PP5 has been isolated from many cells as a complex, including Hsp90 and Hsp70 (10–12). Interaction between PP5 and Hsp90 is mediated by the TPR domain of PP5 and C-terminal EEVD residues of Hsp90 (13, 14). Wild-type PP5 has very low activity against the substrate, but it is activated if the TPR domain is cleaved from the protein. In vitro studies have indicated that PP5 was activated by activators that are known to bind to the TPR domain, such as Hsp90 and arachidonyl CoA (15, 16). A link between PP5 and G protein-coupled signaling has been found. PP5 directly interacts with and is stimulated by G12/G13 (16). Although proteins that interact with PP5 and regulate its activity have been identified, the possibility of its regulation by second messengers remains speculative. For example, there are contradictory reports as to whether or not PP5 is activated by native full-length Hsp90 (15, 17).
S100 Proteins Modulate PP5 Function

In this study, we show that Ca\(^{2+}\)/S100 proteins modulate PP5 function via two different mechanisms. First, binding of S100A1, S100A2, S100A6 and S100B, which interact with the TPR domain of PP5, leads to a full activation of the enzyme. PP5 activated by the S100 proteins increased the dephosphorylation of Tau, known as a physiological substrate of PP5, in vitro and in vivo. Second, S100A1 and S100P inhibit the interaction between PP5 and ASK1 and inhibit the dephosphorylation of ASK1 by PP5. These observations indicate that Ca\(^{2+}\)/S100 proteins could modulate PP5 function by activating its phosphatase activity or inhibiting the interaction between PP5 and its binding partners.

EXPERIMENTAL PROCEDURES

Materials—Glutathione-Sepharose and Nickel-nitritotriacetic acid-agarose were purchased from GE Healthcare and Qiagen, respectively. S100 antibodies were obtained as follows: anti-S100A1 (R&D Systems), anti-S100A2 (Sigma), anti-S100A6 (Sigma), and anti-S100B (QED, San Diego, CA). Anti-S100A12 antibody was prepared as described previously (18). Anti-Hsp90 antibody was purchased from Stressgen (Victoria, Canada), and anti-PP5 antibody was purchased from BD Transduction Laboratories. Anti-pSer396 Tau antibody was purchased from Abcam, and anti-pSer409 and anti-Tau antibodies were purchased from Cell Signaling Technology. HRP-labeled anti-mouse and anti-rabbit IgG were purchased from Cell Signaling Technology. FuGENE6 or HD transfection reagent according to the manufacturer’s protocol (Invitrogen). COS-7 cells were purchased from Japanese Collection of Research Bioresources and maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a humidified 95% O\(_2\), 5% CO\(_2\) incubator. Transfection was performed using FuGENE6 or HD transfection reagent according to the manufacturer’s protocol (Invitrogen). To prepare COS-7 cells stably expressing Tau protein, cells were incubated with 500 ng/ml of the CM5 chip. His-PP5 (100 g/ml) were used for amine coupling of PP5 to the dextran surface of the CM5 chip. His-PP5 (100 g/ml) was immobilized in 20 mM ammonium acetate (pH 4.2) until 1377 (0.16 pmol) or 2600 (0.3 pmol) response units were bound and a stable base line was obtained. For all procedures, 20 mM HEPES (pH 7.4), 150 mM NaCl, and 0.005% Tween 20 were used at a flow rate of 20 ml/min. Recombinant S100A1, S100A2, S100A6, and S100B were injected at various concentrations (1.25 mM, 625 nM, 313 nM, 156 nM). The His-PP5-coupled sensor chip was regenerated between protein injections with a brief (60-s) wash with 50 mM NaOH until the response unit base line returned to its preinjection level. Response curves were prepared for fitting by subtraction of the signal generated simultaneously on the control flow cell. Biacore sensogram curves were evaluated in BIAevaluation 3.0 using a 1:1 Langmuir model.

Surface Plasmon Resonance (SPR)—Protein binding interactions were performed using a SPR Biacore 2000 system (Biacore). CM5 chip, N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide, N-hydroxysuccinimide, and ethanalamine-HCl (Biacore) were used for amine coupling of PP5 to the dextran surface of the CM5 chip. His-PP5 (100 g/ml, 140 ml) was immobilized in 20 mM ammonium acetate (pH 4.2) until 1377 (0.16 pmol) or 2600 (0.3 pmol) response units were bound and a stable base line was obtained. For all procedures, 20 mM HEPES (pH 7.4), 150 mM NaCl, and 0.005% Tween 20 with 1 mM CaCl\(_2\) or 1 mM EGTA were used at a flow rate of 20 ml/min. Recombinant S100A1, S100A2, S100A6, and S100B were injected at various concentrations (1.25 mM, 625 nM, 313 nM, 156 nM). The His-PP5-coupled sensor chip was regenerated between protein injections with a brief (60-s) wash with 50 mM NaOH until the response unit base line returned to its preinjection level. Response curves were prepared for fitting by subtraction of the signal generated simultaneously on the control flow cell. Biacore sensogram curves were evaluated in BIAevaluation 3.0 using a 1:1 Langmuir model.

Competitive Binding Assay in Vivo—For the PP5 binding assay, pME18S-S100 and pME18S-HA-PP5 plasmids were cotransfected to COS-7 cells. After 48 h, the cells were washed once with PBS and lysed in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, and 0.5% Nonidet P-40 with protease inhibitor mixture (Roche). Samples were sonicated and centrifuged at 14,000 rpm for 10 min. Supernatants were incubated with 30 ml of anti-HA antibody-agarose in the presence of 1 mM CaCl\(_2\) or EGTA for 1 h at room temperature. After washing, the samples were analyzed by Western blotting with anti-Hsp90, anti-HA-HRP, or anti-S100 protein antibody. For the ASK1 binding assay, the pME18S-FLAG-PP5, pME18S-cMyc-ASK1, and pME18S-S100A1, or pME18S-S100P, or pME18S-S100P-PA plasmid was cotrans-
fect. The proteins were purified and incubated with 30 μl of anti-FLAG antibody-agarose. The samples were analyzed by Western blotting with anti-PP5 or anti-c-Myc-HRP antibody.

**In Vitro Phosphatase Assay**—The phosphatase activity of PP5-WT or truncated PP5 (PP5-CD) was measured by Ser/Thr phosphatase assay kit (Upstate) following the manufacturer’s protocol. The phosphopeptide (KRpTIRR, 100 μM) was incubated with 250 ng of His-PP5-WT or His-PP5-CD in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 0.01% Tween 20 either with 1 mM CaCl₂ or EGTA in a volume of 50 μl. Various concentrations (0 to 50 μM) of the S100 proteins or Hsp90 (Hsp90 and HspC90) were added and incubated for 10 min at 37°C. After an addition of 100 μl of malachite green solution (0.034% malachite green, 10 mM ammonium molybdate, 0.01% Tween 20) and incubated with 100 ng of His-PP5-WT or His-PP5-CD in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 0.01% Tween 20 either with 1 mM CaCl₂ or EGTA in a volume of 50 μl. Various concentrations (0 to 50 μM) of the S100 proteins or Hsp90 (Hsp90 and HspC90) were added and incubated for 10 min at 37°C. After an addition of 100 μl of malachite green solution (0.034% malachite green, 10 mM ammonium molybdate, 1 mM HCl, 3.4% ethanol, and 0.01% Tween 20), the absorbance of samples at 630 nm was measured by a microplate reader. The amount of released phosphate was calculated using a phosphate standard curve prepared from a known amount of phosphate.

**Tau Dephosphorylation in Vitro**—Recombinant Tau441 was phosphorylated with GSK-3β or PKA. For the phosphorylation of Ser-396, 10 μg of Tau protein was incubated with 1 μg of GSK-3β in a buffer consisting of 40 mM HEPES (pH 7.5), 10 mM MgCl₂, 0.5 mM DTT, and 0.2 mM ATP for 90 min at 30°C. For the Ser-409 phosphorylation of Tau, the protein was incubated with 250 units of protein kinase A in a buffer consisting of 40 mM HEPES (pH 6.8), 10 mM β-mercaptoethanol, 10 mM MgCl₂, and 0.2 mM ATP for 90 min at 30°C. After the phosphorylation reaction, samples were cleaned up with the Amicon ultra spin column (Millipore) and washed with 500 μl of 20 mM Tris-HCl (pH 7.5) and 0.01% Tween 20. Phosphorylated Tau was incubated with 100 ng of His-PP5 in a buffer consisting of 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 0.01% Tween 20 either with 1 mM CaCl₂ or EGTA. S100A1, S100A2, S100A6, or S100A12 protein was added to the reaction mixture and incubated for 10 min at 37°C. The samples were separated by SDS-PAGE and analyzed by Western blotting with anti-pSer-396, pSer-409, or anti-Tau antibody.

**In Vivo Dephosphorylation Study**—For the dephosphorylation of Tau, COS-7 cells stably expressing Tau were transfected with the pME18S or pME18S-S100A1 expression plasmid. After 48 h, 48 h, the protein was purified, and samples were analyzed by Western blotting with anti-pThr-845 ASK1 antibody.

**Statistics**—Data are presented as the mean ± S.D. of at least three independent experiments. Data are analyzed by one-way analysis of variance with Bonferroni post hoc analysis. p < 0.05 was considered to indicate statistical significance.
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FIGURE 1. Interaction of PP5 and S100 proteins. A, SDS-PAGE of S100 proteins used in the binding assays (2.5 μg/lane). B, GST pull-down assays were carried out using GST-PP5 and the S100 proteins. GST-PP5 (50 μg) and S100 proteins (50 μg) were incubated with glutathione-Sepharose beads in the presence of 1 mM CaCl₂ or EGTA for 1 h. Beads were washed, and samples were separated with 10% Tricine SDS-PAGE and stained with CBB. Molecular weight markers are indicated on the left. C, analysis of PP5 and S100 protein binding by SPR. Recombinant His-PP5 was immobilized to the dextran surface of the CM5 chip in 20 mM ammonium acetate (pH 4.2) until 1377 (0.16 pmol) or 2600 response units (0.3 pmol) were bound and a stable baseline was obtained. Upper panel, recombinant S100A1, S100A2, S100A6, and S100B (1.25 μM each) were injected in the presence of 1 mM CaCl₂ (solid line) or EGTA (broken line). Response curves were prepared for fitting by subtraction of the signal generated simultaneously on the control flow cell. BIAevaluation 3.0 using a 1:1 Langmuir model. Lower panel, recombinant S100A1, S100A2, S100A6, and S100B were injected at various concentrations (1.25 μM, 625 nM, 313 nM, 156 nM), and response curves were prepared. RU, resonance units. D, the calculated kinetic parameters of S100 protein binding to PP5.

| Protein | Ka(1/Ms) | Kd(1/s) | KD | chi² | Rmax(RU) |
|---------|----------|----------|-----|------|----------|
| S100A1  | 5.68x10⁴ | 3.02x10⁻³ | 5.32x10⁻⁸ | 6.29 | 87.6     |
| S100A2  | 9.88x10⁴ | 1.80x10⁻² | 1.82x10⁻⁷ | 25.1 | 456      |
| S100A6  | 6.89x10⁴ | 1.32x10⁻² | 1.92x10⁻⁷ | 13.7 | 165      |
| S100B   | 5.11x10⁴ | 4.83x10⁻³ | 9.45x10⁻⁸ | 14.1 | 45.7     |
with lysates of transfected COS-7 cells. Therefore, COS-7 cultures were cotransfected with HA-PP5 and S100A1, S100A2, or S100A12 expression vector. The coprecipitated endogenous Hsp90 with HA-PP5 was detected by Western blot analysis with an anti-Hsp90 antibody. As expected from an in vitro binding study (Fig. 3A), overexpression of S100A1 or S100A2 inhibited the PP5/Hsp90 interaction (B). No clear inhibition was seen when S100A12, a negative control, was overexpressed.

**S100 Proteins but not Hsp90 Bind to the Carboxylate Clamp Mutants of PP5**—Hsp90 binds to a motif called a carboxylate clamp in the TPR domain of PP5. It consists of four basic amino acids residues: Lys-32, Arg-74, Lys-97, and Arg-101 (13, 14). As S100A1, S100A2, and S100A6 inhibited the binding of PP5 and Hsp90, we assumed that these S100 proteins bind competitively to one of these four amino acids in the carboxylate clamp. Alanine substitution mutants of the carboxylate clamp were constructed and incubated with S100A1, S100A2, S100A6, or Hsp90 in the presence of CaCl₂ and pulled down with glutathione-Sepharose beads. All four carboxylate clamp mutants of PP5 failed to bind Hsp90 (Fig. 4A), which is consistent with earlier reports (13, 14). However, all of the S100 proteins bound to the carboxylate clamp mutants of PP5, and no inhibition was observed (Fig. 4B). On the basis of these findings, we conclude that the point mutations of the carboxylate clamp within PP5 had no effect on S100 protein binding.

**Activation of PP5 by S100 Proteins in a Ca²⁺-dependent Manner**—As S100 proteins bind to the TPR domain of PP5 (Fig. 2), we next examined the effect of S100 proteins on the phos-

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**FIGURE 2. Interactions between PP5, its deletion mutants, and S100 proteins.** A, schematics of PP5 deletion mutants. The number refers to amino acid positions of PP5. TPR, catalytic domain deletion mutant; ΔTPR, TPR domain deletion mutant; GST, GST tag only. B, a GST pull-down assay was performed using WT and PP5 deletion mutants. A GST tag was used as a control. These proteins (25 μg) were incubated with glutathione-Sepharose beads and S100A1, S100A2, S100A6, or S100B protein in a presence of 1 mM CaCl₂ or EGTA. After agitation at 25 °C for 1 h, the beads were washed and then eluted with SDS sample buffer. The eluted samples were analyzed by 10% Tricine SDS-PAGE. The gel was stained with CBB. Molecular weight markers are indicated on the right.
phosphatase activity of PP5. His-PP5 was incubated with a synthetic phosphopeptide (KRpTIRR) as a substrate with various concentrations of S100 proteins in the presence of 1 mM CaCl₂ or EGTA. As shown in Fig. 5, the basal phosphatase activity of PP5 is ~10 nmol/min/mg protein. S100A1, S100A2, S100A6, and S100B strongly activated PP5 activity in a Ca²⁺-dependent manner (Fig. 5A). Among these, S100B activated PP5 the most. After the addition of 10 μM of each S100 protein, the activity reached about 834 (59-fold) and 627 (49-fold) nmol/min/mg protein with S100B and S100A1, respectively. This activation was also observed with S100A2 (590 nmol/min/mg protein; 46-fold) and S100A6 (259 nmol/min/mg protein, 18-fold), but...
S100A12, used as a negative control, had no clear activation. The TPR domain shields the catalytic unit of PP5 from activation and removal of the TPR domain activated PP5 (22). We created a mutant His-PP5 by removing the N-terminal TPR domains and leaving the C-terminal catalytic domain (CD). The removal of the TPR domain resulted in a constitutively active form of the enzyme (≈650 nmol/min/mg protein). The activity of the mutant was ≈65 times higher than the basal activity of the wild-type enzyme. The S100 proteins did not stimulate the PP5 (CD) (Fig. 5B). Next, we tested whether full-length Hsp90 and HspC90 (a 12kDa C-terminal domain of Hsp90) were capable of stimulating PP5 activity. Fig. 5C shows the dose-dependent activation of His-PP5 by Hsp90 and HspC90. In comparison with the S100 proteins, Hsp90 is a weak activator of PP5, inducing only 6.5-fold stimulation of the enzyme, whereas HspC90 induces a 17.3-fold activation. These results concur with the previous findings that HspC90 promoted a 10-fold stimulation of PP5 (15).

Activation of PP5 with S100 Proteins and Tau Dephosphorylation in Vitro and in Vivo—PP2A, PP2B, and PP5 play a key role in the dephosphorylation of Tau, whose hyperphosphorylation contributes to Alzheimer’s disease (23–29). Therefore, we confirmed the activation of PP5 by Ca²⁺/S100 using phosphorylated Tau as a physiological protein substrate. Multiple sites of Tau were phosphorylated byTau kinases such as PAK, GSK-3β, Cdk5, and CaMKII in normal and abnormal conditions (30, 31). For the in vitro PP5 phosphatase assay, we used GSK-3β and protein kinase A to phosphorylate Ser-396 and Ser-409 of Tau, respectively. Phosphorylated Tau was incubated with His-PP5 and S100A1, S100A2, or S100B in the presence of CaCl₂ or EGTA. The phosphorylation status of Tau was monitored by Western blot analysis with an anti-pSer-396 or a pSer-409 Tau antibody (Fig. 6A). S100A12 was used as a negative control. S100A1, S100A2, and S100B activated PP5 and increased the dephosphorylation of pSer-396 Tau. S100A12 protein had no effect, as it showed no interaction with PP5 (Fig. 6A). As well as pSer-396, these S100 proteins increased the dephosphorylation of the pSer-409 Tau. These observations indicate that S100A1, S100A2, and S100B can activate PP5 and increase the dephosphorylation of a physiological protein substrate Tau as well as a synthetic peptide substrate.

The activation of PP5 by S100A1 and the dephosphorylation of Tau were also studied in living cells. Because Tau was not abundantly expressed in COS-7 cells, we established stably transfected COS-7 cell lines expressing high levels of Tau441. Compared with that of the control, the ionomycin treatment of the Tau-expressed cells showed no clear change of Tau phosphorylation state at pSer-396 (control, 106.0 ± 14.9; S100A1, 106.0 ± 17.3%; n = 3, p < 0.05) (Fig. 6B). As well as pSer-396, these S100 proteins increased the dephosphorylation of the pSer-409 Tau. These observations indicate that S100A1, S100A2, and S100B can activate PP5 and increase the dephosphorylation of a physiological protein substrate Tau as well as a synthetic peptide substrate.

S100 Proteins Inhibit PP5 and ASK1 Interaction in Vivo—ASK1 is a MAP3K that activates the JNK and p38 MAP kinase cascades and is activated in response to oxidative stress such as H₂O₂ (32). The activation of ASK1 is associated with autophosphorylation at Thr-845, which is located in the kinase

**FIGURE 4. S100 proteins but not Hsp90 bind to carboxylate clamp mutants of PP5.** WT and alanine mutant of the carboxylate clamp of GST-PP5 (K32A, R74A, K97A, and R101A) were incubated with S100A1, S100A2, S100A6, or Hsp90 proteins. The protein complex was precipitated with glutathione-Sepharose beads in the presence of 1 mM CaCl₂. After agitation at 25 °C for 1 h, the beads were washed and then eluted with SDS sample buffer. The eluted samples were analyzed by 10% Laemmli SDS-PAGE (A) or 10% Tricine SDS-PAGE (B) and stained with CBB. Molecular weight markers are indicated on the left. A, binding of WT or PP5 mutants and Hsp90. B, binding of WT or PP5 mutants and S100 proteins.
domain. Following the activation of ASK-1 by oxidative stress, PP5 binds to and dephosphorylates ASK-1 to function as a negative feedback inhibitor (33). To examine the role of the S100 proteins, increasing amounts of S100A1 expression plasmid were transfected to COS-7 with the PP5 and ASK1 expression plasmid (Fig. 7A). Ionomycin treatment of the cells inhibited the binding of PP5 and ASK1, as revealed by Western blotting. No clear inhibition was observed without ionomycin stimu-
FIGURE 6. S100 proteins activate PP5 and increase the dephosphorylation of Tau in vitro and in vivo. A, pSer-396 or pSer-409 Tau was incubated with His-PP5 and S100A1 (lanes 4 and 5), S100A2 (lanes 6 and 7), S100B (lanes 8 and 9), or S100A12 (lanes 10 and 11) in the presence of 1 mM CaCl$_2$ or EGTA for 10 min at 37 °C. Samples were separated with SDS-PAGE and analyzed by Western blotting (WB) with anti-pSer-396 Tau, anti-pSer-409 Tau, or anti-Tau antibody. A detailed description can be found in the experimental procedures. Lane 1, non phosphorylated TAU; lanes 2-11, phosphorylated Tau; lane 3, PP5 treatment only.

B, COS-7 cells stably expressing Tau were either transfected with pME18S vector only (Mock) or S100A1 expression vector (S100A1) or not transfected (Control). After 48 h, cells were stimulated with 5 μM ionomycin for 15 min or 60 min. Lysates were prepared and analyzed by Western blotting with anti-pSer-396 Tau, anti-PP5 antibody. The density of each band was measured using Image J software (National Institutes of Health) and the % ratio of pSer-396 Tau against total Tau was calculated. Each column represents the mean ± S.D. calculated from three to five independent experiments.
tion. This suggests that an increase of intracellular Ca\textsuperscript{2+} concentration by ionomycin stimulated the S100A1 binding to PP5, thus inhibiting the PP5-ASK1 interaction. However, calcium ionophores, such as ionomycin and A23187, and thapsigargin are known to generate reactive oxygen species (34) and could influence the PP5-ASK1 binding. To overcome this problem, “permanently active S100P” (S100P-PA) was used, and the use of ionophore or thapsigargin was avoided. Followed the report of Austermann et al. (19), we constructed S100P-PA that contained mutations in the two EF-hand loops predicted to lock the protein in a permanently active state. Similar to S100A1, S100A2, S100A6, and S100B (Fig. 1B), wild-type S100P bound to GST-PP5 \textit{in vitro} (Fig. 7B). When wild-type S100P or S100P-PA was transfected into COS-7 with PP5 and ASK1, S100P-PA inhibited the binding of PP5 and ASK1, whereas wild-type S100P had no clear effect. This inhibition caused the increase of phosphorylated pThr-845 of ASK1 (Fig. 7C), indicating that S100P-PA disturbed the dephosphorylation by dissociating PP5 from ASK1.

**DISCUSSION**

PP5 is involved in wide-ranging cellular processes, including DNA damage repair, cell growth and differentiation, cell cycle arrest, and cellular heat shock response (35). The discoveries that PP5 forms complexes with glucocorticoid receptors (10) and ASK1 (33) implicates the phosphatase as a regulator of signaling networks initiated by glucocorticoids and oxidative stress. Partial proteolysis of PP5 led to concomitant removal of the TPR domain and up to a 50-fold increase in phosphatase activity. PP5 is presumably not regulated by proteolysis in intact cells. Thus, identification of the natural activator of PP5 is of great significance.

In this study, we have shown the two distinct functions of S100 proteins against PP5. Certain members of the S100 proteins, such as S100A1, S100A2, S100A6 and S100B, specifically bind to the TPR domain of PP5 in a Ca\textsuperscript{2+}-dependent manner and result in the activation of the enzyme. The low basal activity of PP5 is a consequence of the autoinhibitory role of its TPR domain (22, 37). Long-chain fatty acyl-CoA esters (15) and Hsp90 are potential activators of the enzyme. Despite these reports, the physiological activator of PP5 remains to be identified. The S100 proteins greatly activated the PP5 phosphatase activity and dephosphorylated a synthetic phosphopeptide (Fig. 5A). In the brain, Tau is phosphorylated normally or abnormally at more than 30 sites by Tau kinases such as Cdk5, protein kinase A, and GSK-3β (31). We studied the effect of S100 pro-
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teins on the dephosphorylation of pSer-396 and pSer-409 Tau as glycophorin protein substrates. S100A1, S100A2, and S100B greatly increased the dephosphorylation of both sites by PP5 in vitro in a Ca\(^{2+}\)-dependent manner (Fig. 6A). Until now, there have been few reports, some controversial, on the Hsp90-activating effects. Ramsey et al. (15) reported that full-length Hsp90 had no effect on the PP5 activity and that the C-terminal domain of Hsp90 (HspC90) activated the enzyme substantially. Later, Yang et al. (17) reported that full-length Hsp90 was a moderate activator of PP5. Our study has shown that full-length-Hsp90 weakly (6.5-fold) and HspC90 moderately (17.3-fold) activated PP5 (Fig. 5C). However, it was still very low potency compared with those of S100 proteins. S100 proteins could be novel strong physiological activators of PP5. It is interesting that the S100 proteins function as PP5 activators in intact cells. As such, PP5 could dephosphorylate its physiological substrates effectively. Using COS-7 cells stably expressing Tau441, the overexpression of S100A1 with ionomycin treatments showed a significant decrease of pSer-396 Tau. The result indicates that intracellular Ca\(^{2+}\) increases by the ionomycin treatment stimulated the binding of S100A1 and PP5 and activates its phosphatase activity in vivo. Recently, attention has been focused on the importance of PP5 in Alzheimer’s disease (38). PP5 is highly expressed in the brain and neurons, and the reduction of PP5 expression increases the susceptibility of the neuron to \(\alpha\Bb\) toxicity. The overexpression of PP5 prevented MAPK phosphorylation and neurotoxicity induced by \(\alpha\Bb\) (36).

PP5 is a major component of Hsp90-steroid hormone receptor complexes. The enzyme has been implicated as a modulator of steroid receptor function through its association with Hsp90 (11). Among the S100 proteins we examined, S100A2 bound most abundantly to PP5 in vitro, followed by S100A6, S100A1, and S100B. In addition, S100A1 and S100A2 lead to a Ca\(^{2+}\)-dependent inhibition of the Hsp90-PP5 interaction. Less displacement was seen with S100A6 (Fig. 3A). The charged residues in the PP5-TPR domains, predicted to form the so-called two-carboxylate clamp form salt bridges with the EEVD of HSP90, and the point mutations of carboxylate clamp diminished the binding (13, 14). As S100 proteins interfered with the Hsp90 binding to PP5, we assumed that they bound to the amino acid residues composing the carboxylate clamp. However, S100 proteins bound to the alanine mutants of the carboxylate clamp, although Hsp90 binding was clearly inhibited (Fig. 4, A and B). The results suggest that the mode of interaction of the S100 proteins with PP5 is different from the PP5-Hsp90 electrostatic interaction. It is possible that the binding site of S100 proteins may be close enough to physically interfere with the Hsp90 binding to PP5. We have shown previously that S100 proteins bind to the TPR domains of Hop, KLC, Tom70, Cyp40, and FKBPs2 and result in inhibition of the ligand-TPR protein interactions (5, 6). Interestingly, a preference of S100 protein binding exists among the TPR proteins. For example, S100A2 bound to KLC, FKBPs2, and PP5 most tightly, whereas S100A6 bound to Hop, Cyp40, and Tom70 more effectively than S100A2. The reasons for the selectivity of S100 protein binding to TPR proteins are not fully understood. It is not yet possible to predict the residues important for the S100-TPR protein interactions. In vitro binding studies were confirmed in vivo using COS-7 cells overexpressing PP5 and S100 proteins (Fig. 3B).

Endogenous Hsp90 binding to PP5 was in competition with S100A1 and S100A2 proteins. From these observations, it is apparent that S100 proteins could modulate the function of Hsp90 by competing with the binding of Hsp90 and PP5 in intact cells.

ASK1, similar to Hsp90, associates with PP5 through its TPR domains, and thereby, PP5 dephosphorylates the essential pThr-845 in the kinase domain of ASK1, enabling inactivation of ASK1 by negative feedback (32). In our study, S100A1 dose-dependently inhibited the interaction between PP5 and ASK1 in vivo by ionomycin stimulation (Fig. 7A). Because reactive oxygen species production could be stimulated by calcium ionophores and thapsigargin, and because H2O2 can cause a calcium flux in cultured cells, the discrimination of the reactive oxygen species signal pathway and the calcium signal pathway by these pharmacological tools is difficult. We used permanently active S100P in in vivo analyses. Overexpression of permanently active S100P inhibited the PP5 and ASK1 binding and thus inhibited the dephosphorylation of pThr-845 of ASK1 by PP5 (Fig. 7C). S100 proteins such as S100A1 and S100P could modulate the phosphorylation level of ASK1 by PP5 and may exert influence on the stress response. However, further study will be necessary. These findings indicate that the S100 proteins could modulate PP5 function through its interaction between PP5 and its binding partner proteins responding to intracellular Ca\(^{2+}\) signals.

In conclusion, our results strongly suggest that the Ca\(^{2+}\)-dependent interactions of the S100 proteins with PP5, via its TPR domain, activates its enzyme activity and in vivo as well as in vitro. Furthermore, the association of the S100 proteins with PP5 provides a Ca\(^{2+}\)-dependent regulatory mechanism for the phosphorylation status of intracellular proteins through the regulation of PP5-client protein interaction. The findings suggest a new intracellular Ca\(^{2+}\) signaling pathway via S100 protein-TPR motif interactions.

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