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A role for SHPS-1/SIRPα in Concanavalin A-dependent production of MMP-9

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SHPS-1/SIRPα1 is a transmembrane glycoprotein that belongs to the immunoglobulin (Ig) super family. In the present study, we show that SHPS-1 strongly associates with Concanavalin A (Con A), a plant lectin obtained from jack beans. Further studies with SHPS-1 mutants reveal that the extracellular domain of SHPS-1 containing the Ig sequence is responsible for its association with Con A. Con A treatment induces cross-linking and multimerization of the SHPS-1 protein in the plasma membrane, accompanied by its tyrosine phosphorylation and recruitment of SHP-2. In contrast, Ricinus communis agglutinin (RCA), another lectin obtained from castor bean, does not bind or activate tyrosine phosphorylation of SHPS-1. Moreover, Con A activates Akt in a SHP-2-dependent manner. Treatment of mouse embryonic fibroblasts (MEFs) with Con A induces secretion of matrix metalloproteinase (MMP)-9, a phenomenon that is inhibited in cells expressing YF mutant of SHPS-1, a dominant negative form of Akt or in cells pre-treated with an Akt inhibitor, LY294002 or extracellular-signal regulated kinase (Erk) inhibitor, U0126. In addition, expression of the YF mutant of SHPS-1 inhibits Con A-dependent activation of Akt and Erk kinases. Taken together, our results suggest that SHPS-1 is a receptor for Con A that mediates Con A-dependent MMP-9 secretion through SHP-2-promoted activation of both Akt and Erk pathways.

Introduction

Lectins are a family of proteins which interact with specific terminal sugar residues and cross-link cell surface glycoproteins thereby initiating various cellular responses (Sharon & Lis 1990; Elgavish & Shaanan 1997). Concanavalin A (Con A), a potential multi-receptor cross-linker for TCR and other cell surface receptors, is the most extensively investigated member of the lectin family of plant proteins which displays high affinity for terminal α-d-mannosyl and α-d-glucosyl residues (Gunther et al. 1973). Con A exhibits cell agglutinating and mitogenic activities and induces apoptosis (Tamura et al. 1995; Cribbs et al. 1996; Akhand et al. 1997; Zhao et al. 2002; Amin et al. 2007). In addition, Con A is widely used as a positive control to study T-cell activation and as a model to study the regulatory mechanisms that control secretion and activation of matrix metalloproteinase (MMP)-2 and MMP-9 (Tamura et al. 1995; Sein et al. 2000; Amin et al. 2003a). Despite its significance, the precise mechanism by which Con A promotes its biological responses remains largely unclear.

MMPs, a family of neutral proteinases that catalyze the destruction of the extracellular matrix, are secreted from cells as inactive zymogens and activated by Zn or Ca ion-dependent proteolytic cleavage (Stetler-Stevenson et al. 1989). Among MMPs, MMP-9 (gelatinase B) appears to play an important role in a wide array of pathophysiological processes including development, wound healing, angiogenesis, inflammation, and tumor invasion and metastasis (Deryugina & Quigley 2006; Mon et al. 2006). Increased secretion and activation of MMP-9 is observed in many human cancers (Sato et al. 1992; Davies et al. 1993; Rao et al. 1993). Moreover, stimulation of cells with growth factors, cytokines and lectins including Con A also activate MMP-9 secretion (Samuel et al. 1992; Sein et al. 2000; Hussain et al. 2002; Amin et al. 2003b).
However, the signaling pathways that regulate MMP-9 secretion are not completely well-understood.

Src homology 2 (SH2)-phosphatase substrate-1 (SHPS-1), also known as SIRPα1, BIT, MFR or p84, is a transmembrane glycoprotein that binds SH2-tyrosine phosphatase (SHP)-1 and SHP-2 and serves as their substrate (Oshima et al. 2002). SHPS-1 belongs to the Ig super family and the putative extracellular region of SHPS-1 has three Ig-like domains and multiple N-linked glycosylation sites. The cytoplasmic region of SHPS-1 contains four YXX(L/V/I) motifs, which serve as putative tyrosine phosphorylation and SH2 domain binding sites. SHPS-1 is tyrosine phosphorylated by various growth factors, cytokines, cell adhesion molecules and mitogens, and subsequently recruits SHP-1/2 (Kharitonenkov et al. 1997; Takada et al. 1998; Amin et al. 2002; Oshima et al. 2002). Both SHPS-1 and SHP-2 are critical components of many signal transduction pathways that lead to cell growth, differentiation, migration and death (Kharitonenkov et al. 1997; Oshima et al. 2002; Neznanov et al. 2003; Feng 2007). However, depending on the type of cells and stimuli, these proteins display both positive and negative regulation of the above processes and their biological importance remains to be elucidated. Moreover, although many people term SHPS-1 as a receptor, its ligands are less well characterized and until now CD47 is the only identified ligand of SHPS-1 (Vernon-Wilson et al. 2000).

In this study, we show for the first time that SHPS-1 is a receptor for Con A. Con A directly binds to the extracellular region of SHPS-1 and this interaction mediates Con A-dependent activation of Akt and secretion of MMP-9. Our study also demonstrates that SHP-2 is recruited to SHPS-1 upon Con A-stimulation which is required for Con A-dependent Akt activation. In addition, our results suggest that activation of both Akt and extracellular-signal regulated kinase (Erk) is required for the increased secretion of MMP-9 by Con A.

Results
Con A reversibly binds to SHPS-1 via its extracellular domain

As a potential multi-receptor cross-linker for various cell surface receptors, we hypothesized that Con A might bind to SHPS-1. To test this hypothesis, we prepared various SHPS-1 mutants along with the wild-type one. In the YF mutant, all four tyrosine-residues at the C-terminal cytoplasmic end of SHPS-1 were mutated to phenylalanine by site directed mutagenesis. In the IC mutant, the extracellular domain was deleted. Both the YF and IC mutants were myc-tagged at the C-terminal end. Wild-type and YF constructs were stably transfected into mouse embryonic fibroblasts (MEFs) (Fig. 1A). SHPS-1 and Con A interactions were studied as described in the Experimental procedures. As shown in Fig. 1B, both wild-type and YF mutant SHPS-1 protein was able to associate with Con A, indicating that the C-terminal...
tyrosine residues of SHPS-1 are not important for binding of Con A. To examine whether the extracellular domain of SHPS-1 was important for this association, we transiently expressed the IC mutant in Cos7 cells and RIPA lysates were incubated with Con A-agarose beads and immunoblotted with anti-SHPS-1 and anti-myc. As shown in Fig. 1C, Con A could not bind to the IC mutant, indicating that the association occurs through the extracellular domain. To establish SHPS-1 as a “receptor” for Con A, we next examined whether Con A displayed specific, reversible and saturated binding to SHPS-1. To rule out the possibility of indirect association and establish specific binding between the two molecules, SHPS-1 protein was immunoprecipitated from RIPA lysate and transferred to PVDF membrane after SDS-PAGE. The membrane was incubated with HRP-conjugated Con A and stained with ECL. As shown in Fig. 2A, SHPS-1 was clearly detectable in both wild-type and YF-SHPS-1 expressing cells after incubation of the membrane with HRP-Con A. In contrast, HRP-conjugated p53 antibody efficiently bound to p53 protein, but not with SHPS-1 protein (data not shown). These results confirm the association of Con A with SHPS-1 to be specific between the two molecules. We next tested saturated binding between SHPS-1 and Con A by incubating different concentrations of total protein (50, 100, 200, 400 and 600 µg) with the same amount of Con A-agarose beads (20 µL) and measured the binding of SHPS-1 to Con A as in Fig. 1B. Supernatants obtained after binding were also examined for SHPS-1. As shown in Fig. 2B, upper panel, the amount of bound SHPS-1 protein initially increased with increasing concentrations of total protein (between 50 and 200 µg), but eventually became saturated at 200 µg of total protein. This was also evidenced by the appearance of SHPS-1 in the supernatant at 200 µg or more protein (lower panel). To further confirm the specific and saturated binding between the two molecules, we incubated RIPA-lysate with Con A-agarose for 30 min and after brief spinning, collected the beads as sample one. The supernatant was again incubated with Con A-agarose and similarly, the beads were collected as sample two. In the same manner, a third sample was collected and the supernatant was treated as sample four. The samples were subsequently immunoblotted with anti-SHPS-1 and anti-Erk-2. As shown in Fig. 2C, upper panel, all the SHPS-1 protein was associated with
Con A during the first incubation and no SHPS-1 was detected in the subsequent steps, indicating that SHPS-1 becomes saturated. In contrast, Erk-2 did not associate with Con A-agarose (lower panel), suggesting that Con A specifically binds to SHPS-1. We next tested whether this binding of Con A with SHPS-1 is reversible or irreversible. We incubated total cell lysates with Con A-agarose beads. After washing the beads with lysis buffer 3 times, we re-incubated the beads with 0.3 mM methyl-\(\alpha\)-d-glucoside (has specific binding affinity to Con A) and 0.3 mM lactose monohydrate (has no affinity to Con A) for 5 min on ice. The supernatants obtained after brief spinning of the beads and the washes were subjected to immunoblotting with anti-SHPS-1. As shown in Fig. 2D, most of the bound SHPS-1 was recovered by incubating the beads with 0.3 mM methyl-\(\alpha\)-d-glucoside (upper panel, lane 5), but not by lactose monohydrate (lower panel). This result indicates that the interaction between Con A and SHPS-1 is reversible and occurs specifically between Con A and SHPS-1. We next examined whether Con A occupation caused cross-linking of the receptor resulting in the formation of dimer or multimer. Con A-untreated and -treated MEFs over-expressing wild-type SHPS-1 were lysed with extraction buffer (Wong et al. 1999), which contained only Triton-X100 as a detergent and the lysates were immunoprecipitated and immunoblotted with anti-SHPS-1. As shown in Fig. 2E, upper panel, Con A-treatment caused depletion of SHPS-1 from the lysate, probably due to the formation of a multimeric complex. We next dissolved the pellet obtained after centrifugation of the lysates before immunoprecipitation with X2 ME (+) sample buffer, resolved them by SDS-PAGE and immunoblotted with anti-SHPS-1. As shown in the lower panel, SHPS-1 protein remained in the pellet in Con A-treated cells. In contrast, most of the SHPS-1 protein was dissolved with the extraction buffer in untreated cells. This result suggests that occupation of SHPS-1 with Con A causes cross-linking of SHPS-1 with the formation of a multimeric complex.

**Con A induces tyrosine phosphorylation of SHPS-1 and recruitment of SHP-2**

Stimulation of cells with growth factors, cytokines and mitogens induces tyrosine phosphorylation of SHPS-1. Being a ligand of SHPS-1, we next examined whether Con A also induced tyrosine phosphorylation of SHPS-1. Cells over-expressing wild-type SHPS-1 were treated with 15 µg/mL Con A for the indicated time periods and RIPA lysates were immunoprecipitated with anti-SHPS-1, and immunoblotted with anti-phosphotyrosine. As shown in Fig. 3A, Con A-treatment induced tyrosine phosphorylation of SHPS-1 in a time-dependent manner. Initially, there was very little tyrosine phosphorylation of SHPS-1 without Con A-treatment. Con A-treatment induced efficient tyrosine phosphorylation of SHPS-1 in 5–15 min, which then declined to the basal level at 30–60 min. In many instances, tyrosine phosphorylation of SHPS-1 recruits SHP-2. To examine SHPS-1–SHP-2 complex formation by Con A, SHPS-1 immunoprecipitates from Con A-untreated and -treated cells were probed with anti-SHP-2. As expected, Con A also induced recruitment of SHP-2 with a similar kinetics to SHPS-1 tyrosine phosphorylation (Fig. 3B). Initially, there was little association of SHP-2 with SHPS-1 which was increased after Con A-treatment up to 15 min, followed by declining

![Figure 3](image_url)

**Figure 3** Con A induces tyrosine phosphorylation of SHPS-1 and recruitment of SHP-2. (A) Upper panel, MEFs were serum starved for over-night, treated with 15 µg/mL Con A for the indicated time period or left untreated (NT), lysed with RIPA buffer and immunoprecipitated with anti-SHPS-1, followed by immunoblotting with anti-phospho tyrosine. Lower panel, the same membrane was stripped and reprobed with anti-SHPS-1. (B) Overnight serum starved cells were stimulated with 15 µg/mL of Con A for the indicated times or left untreated (NT). RIPA lysates were immunoprecipitated with anti-SHPS-1 and immunoblotted with anti-SHP-2 (upper panel) and reprobed with anti-SHPS-1 (lower panel). (C) Cells were treated with Con A as in Fig. 3B, RIPA lysates were immunoprecipitated with anti-SHP-2 and immunoblotted with anti-SHPS-1 (upper panel) and reprobed with anti-SHP-2 (lower panel).
interactions which reached the basal level at 60 min of Con A treatment. To further study the interaction of SHPS-1–SHP-2 after Con A-treatment, we immunoprecipitated SHP-2 protein by SHP-2 polyclonal antibody and probed the blot with anti-SHPS-1. As shown in Fig. 3C, SHPS-1–SHP-2 interaction again showed a similar kinetics of SHPS-1 tyrosine phosphorylation.

*Ricinus communis* agglutinin (RCA) does not bind to SHPS-1

We next examined whether the activation of SHPS-1 was specific for Con A or occurred upon stimulation with other lectins. To test this, we incubated total cell lysates with Con A-agarose or RCA-agarose beads and examined the interaction as in Fig. 1B. As shown in Fig. 4A, Con A efficiently associated with SHPS-1 (lane 1); however, RCA failed to associate with SHPS-1 (lane 2). We also treated cells with 15 µg/mL RCA for different times, immunoprecipitated SHPS-1 protein with anti-SHPS-1 and probed the blot with phosphotyrosine antibody. As shown in Fig. 4B, RCA did not induce efficient tyrosine phosphorylation of SHPS-1.

Con A activates Akt in a SHP-2-dependent manner

We have previously reported that SHP-2 is required for Con A-induced activation of both Erk and p38 MAP kinases (Amin *et al.* 2003a). We next examined the activation of Akt downstream of the SHPS-1–SHP-2 complex. The activation of Akt was examined in cells expressing either wild-type SHP-2 or a truncated SHP-2 which is functionally inactive. As shown in Fig. 5A,B, Con A-treatment induced activation of Akt, as measured by its phosphorylation level, in a time- and dose-dependent manner in SHP-2 wild-type cells. In contrast, this Con A-dependent activation of Akt was severely impaired in SHP-2 mutant cells. These results suggest that SHP-2 must be functional for Con A-dependent Akt activation. Phosphorylation of Akt became obvious after 15 min of Con A-treatment and then increased with time up to 1 h, whereas 5 µg/mL was able to activate Akt and 15–20 µg/mL exhibited optimal activation. To further confirm that the defective Akt activation in SHP-2 mutant cells is due to lack of functional SHP-2, we reintroduced wild-type SHP-2 into SHP-2 mutant cells (Fig. 5C, C7 and C10). As shown in Fig. 5D, expression of wild-type SHP-2 in SHP-2 mutant cells rescued the Con A-dependent Akt activation in two independent clones. We next expressed a dominant negative mutant of SHP-2 (CS) in Cos7 cells along with the empty vector (Fig. 5E). As shown in Fig. 5F, expression of dominant negative SHP-2 inhibited Con A-dependent activation of Akt, whereas the vector had no effect. These results further confirm a critical role of SHP-2 in Con A-dependent Akt activation.

**Tyrosine phosphorylation of SHPS-1 is required for Con A-induced MMP-9 secretion and Akt activation**

One of the major biological effects of Con A is to induce secretion of MMP-9. In order to study the role of SHPS-1 in Con A-dependent MMP-9 secretion, we examined MMP-9 secretion in MEFs by gelatin zymography. As shown in Fig. 6A,B, Con A induced the secretion of MMP-9 in a dose- and time-dependent manner. A dose of 15–20 µg/mL was required to induce efficient MMP-9 secretion at 24 h of stimulation. We also measured the Con A-dependent secretion of MMP-9 in cells expressing YF mutant of SHPS-1 and compared these with that of the mock cells. As shown in Fig. 7A, expression of YF mutant strongly inhibited Con A-dependent MMP-9 secretion. Similarly, the YF mutant strongly suppressed Con A-dependent Akt activation (Fig. 7B), but only slightly inhibited Erk phosphorylation (Fig. 7C). These results suggest that tyrosine phosphorylation of SHPS-1 is important for the secretion of MMP-9 and activation of Akt by Con A.
production were inhibited (Fig. 8B), suggesting that PI3 K-dependent activation of Akt is critical for MMP-9 production by Con A. To further confirm the involvement of Akt activation in Con A-induced MMP-9 production, we expressed a dominant negative form of Akt in MEFs by stable transfection (Fig. 8C). As shown in Fig. 8D,E, expression of dominant negative Akt also strongly suppressed Con A-induced Akt activation and MMP-9 production, confirming the importance of Akt activation in Con A-dependent MMP-9 production. We have previously reported that both Akt and Erk activations are required for IL-1β-dependent MMP-9 secretion in Balb 3T3 cells (Amin et al. 2003b). We next examined the involvement of Erk signaling in Con A-dependent MMP-9 production using an inhibitor, U0126, specific for MEK1, the immediate upstream kinase of Erk activation. As shown in Fig. 9A,B, pre-treatment of cells with U0126 strongly inhibited Con A-dependent Erk activation and MMP-9 production, indicating that activation of MEK-1/Erk signaling is also critical for Con A-dependent MMP-9 production.

**Discussion**

In the present study, we demonstrate that the transmembrane glycoprotein, SHPS-1, is a receptor for Con A and
regulates Con A-induced Akt and Erk activation, and MMP-9 secretion. Our results suggest that Con A directly binds to SHPS-1 via its extracellular domain. The association of Con A cross-links SHPS-1 resulting in the formation of an insoluble multimeric complex. This is similar to the phenomenon by which growth factors and cytokines activate their receptors. The most compelling evidence for the direct association between SHPS-1 and Con A comes from the fact that the SHPS-1 protein is specifically detected after incubation of an SDS-PAGE transferred membrane with HRP-conjugated Con A. Moreover, the interaction of Con A and SHPS-1 is reversible and becomes saturated. Methyl-α-d-glucoside (has specific binding affinity to Con A), but not lactose monohydrate (has no affinity to Con A), was enough to release SHPS-1 from Con A. Therefore, it is fair to say that SHPS-1 is a receptor for Con A. Occupation of SHPS-1 by Con A-induced tyrosine phosphorylation of SHPS-1, although the YF mutant could also efficiently bind to Con A. These results suggest that association of the two molecules is the early event, followed by tyrosine phosphorylation of SHPS-1, which also strengthens our speculation that Con A is a ligand for SHPS-1.

As a receptor for Con A, SHPS-1 must have some role in Con A-induced cell signaling. Indeed, our studies indicate that cross-linking of SHPS-1 by Con A induces tyrosine phosphorylation of SHPS-1 and recruitment of SHP-2. Expression of a YF-SHPS-1 mutant which has dominant negative effect on SHPS-1-dependent signaling (Amin et al. 2002) strongly inhibit the Con A-dependent secretion of MMP-9, activation of Akt and to some extent Erk, suggesting that SHPS-1 is a receptor for Con A that regulates activation of Akt and secretion of MMP-9.

Our results demonstrate a critical role of SHP-2 for Con A-dependent Akt activation. The role of SHP-2 in signal transduction is highly complicated. This phosphatase acts to promote mitogenic stimulation of Erk activity (Shi et al. 1998). It is also a positive regulator for Akt (Wu et al. 2001; Amin et al. 2002) or JNK activation (Fukunaga et al. 2000), while being a negative regulator for JNK activation by cellular stress (Shi et al. 1998) or IFN-stimulated activation of JAK/STAT pathway (You et al. 1999). Moreover, it mediates cytokine-induced NF-κB activation independently of all these pathways (You et al. 2001). Previously, we reported that SHP-2 is critical for Con A-stimulated Ras-Erk and Ras-p38 MAPK activation (Amin et al. 2003a). Our current results suggest SHP-2 as a positive regulator for Con A-induced activation of Akt also. However, activation of Akt by Con A is not Ras-dependent since pre-treatment with the Ras inhibitor FTI is unable to inhibit Con A-dependent Akt activation (data not shown) although this inhibitor efficiently inhibits Con A-dependent Erk and p38 phosphorylation (Amin et al. 2003a).

Despite its biological importance, the regulatory mechanism for MMP-9 secretion remains largely unclear. Secretion of MMP-9 by FGF requires activation of MEK1-Erk signaling in breast cancer cell line, MCF-7 (Suyama et al. 2002). In contrast, secretion of MMP-9 by EGF is dependent on the activation of Erk, p38 and

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**Figure 7** Tyrosine phosphorylation of SHPS-1 is critical for Con A-dependent secretion of MMP-9 and phosphorylation of Akt and Erk. (A) Overnight serum starved mock and YF-SHPS-1 transfected MEFs were treated with 15 μg/mL Con A for 24 h (Con A, +) or left untreated (Con A, –). Conditioned media were subjected to gelatin zymography for MMP-9 activity. (B, C) Mock and YF-SHPS-1 transfected cells were treated with 15 μg/mL Con A for 1 h for phospho Akt and 15 min for phospho Erk (Con A, +) or left untreated (Con A, –). Upper panels, TCL were probed with anti-phospho Akt (B) or anti-phospho Erk (C). Lower panels, the same membranes were reprobed with anti-Akt (B) or anti-Erk-2 (C).
PI3K in ovarian cancer cells, OVCA429 (Ellerbroek et al. 2001), whereas in a breast cancer cell line (SKBr3) this is Erk-dependent but PI3K-independent (Reddy et al. 1999). Yao et al. (2001) reported that secretion of MMP-9 by heregulin–β1 in human breast cancer cells (SKBr3) was PKC-, p38- and Erk-dependent, but Akt independent. It seems that the signaling required for MMP-9 secretion might differ, in part, depending on the type of cell or stimuli. In this report, we show for the first time that the dual signaling pathways, the PI3K-Akt and the MEK1-Erk, are required for the activation of MMP-9 secretion by Con A. We found that inhibition of either of these pathways by their specific inhibitors or by dominant negative protein expression resulted in drastic inhibition of MMP-9 secretion. We have previously reported that secretion of MMP-9 by the proinflammatory cytokine IL-1β and fibronectin also require dual signaling, through the Akt and Erk (Thant et al. 2001; Amin et al. 2003b). Results presented in this paper confirm and extend the previous observations and suggest that the MEK1-Erk and the PI3K-Akt pathways are common and essential signals for the activation of MMP-9 by different types of stimuli.

In summary, we demonstrated for the first time that SHPS-1/SIRPα works as a functional receptor for Con A to activate MMP-9 production via the Akt and MAPK pathways. It should be noted that Con A is a member of the Con A-like lectins/glucanases superfamily and Yagita et al. (1992) reported the presence of Con A-like molecule on NK-sensitive target cells in mice. Signaling based on the Con A-SHPS-1 interaction reported in this paper might represent other important biological responses of mammalian cells which are not yet revealed.
Experimental procedures

Materials
Con A, Con A-conjugated agarose beads and HRP-conjugated Con A were purchased from Honen (Tokyo, Japan); U0126 from Promega Corporation (Madison, WI); LY294002 from Cayman Chemical (Ann Arbor, MI). Phospho-Akt, Akt and phospho-p42/44 MAPK antibodies were purchased from Cell Signaling (Beverly, MA), anti-Erk-2 and anti-SHP-2 from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-SHPS-1 polyclonal and anti-Myc (9E10) monoclonal antibodies were prepared as described (Hamaguchi et al. 1988; Machida et al. 2000).

Cell culture, plasmid preparation and cell transfection
MEFs expressing wild-type and a truncated form of SHP-2 in which 65 amino acids (46–110) at the N-SH2 domain were deleted by gene targeting making SHP-2 functionally inactive were generated, immortalized and maintained as described previously (Shi et al. 1998). Cos7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% FBS in a CO2 incubator at 37 °C. HA-tagged wild-type and dominant negative SHP-2 plasmids were gifts from Akito Maeda (Institute for Liver Research, Kansai Medical University, Japan). Transfections were performed using Gene porter transfection reagent according to the manufacturer’s instruction.

Treatment of cells with Con A
Before treatment, cells were trypsinized, counted and 1×10^6 cells were plated in 60 mm culture plate in DMEM containing FBS. After 24 h, DMEM was replaced with serum free DMEM and incubated overnight. Just before treatment, the old serum free media were replaced with fresh serum free media and treated with the indicated concentration of Con A for the indicated time. Culture plate containing same number of cells and maintained identically with the treatment plates except Con A-stimulation was used as negative control.

Con A-SHPS-1 binding assay
Cells maintained in DMEM containing FBS were harvested with RIPA buffer (10 mM Tris–HCl, pH 8.0, 1% Triton X-100, 1% DOC, 0.1% SDS, 1 mM EDTA, 0.5 mM Na2VO4 and 1 mM PMSF) and the lysates were clarified by centrifugation at 2×15 000 r.p.m. for 30 min. The supernatants were incubated with Con A-agarose beads with continuous rotation for 2 h at 4 °C. The beads were washed several times with RIPA buffer to remove unbound proteins. Finally, 30 µL sample buffer containing 2-mercaptoethanol was added to the beads, vortex briefly, centrifuged and the supernatant was subjected to Western blotting.

Immunoprecipitation, SDS-PAGE and immunoblotting
Immunoprecipitation, SDS-PAGE and immunoblotting were performed as described elsewhere (Hamaguchi et al. 1993). Briefly, proteins were resolved on SDS-10% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% non-fat skimmed milk and incubated with the respective antibody, followed by incubation with the corresponding secondary antibody. Proteins were visualized by enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham Pharmacia Biotech). In some instances, immunoprecipitation of specific protein was done before SDS-PAGE. For this purpose, cells were lysed with the corresponding lysis buffer and the lysates were clarified by centrifugation at 15 000 r.p.m. for 30 min. The supernatants were then incubated with the corresponding antibody for 2 h followed by 1 h incubation with protein A-Sepharose beads.

Assay of MMP-9 activity by gelatin zymography
MMP-9 activity in conditioned media was measured by zymography as described previously (Hamaguchi et al. 1995). Briefly, conditioned media were subjected to gel electrophoresis containing 0.03% gelatin. Gels were then washed twice and incubated overnight at 37 °C in the reaction buffer (50 mM Tris–HCl, pH 7.4, 10 mM CaCl2) and stained with Coomassie Brilliant Blue.

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Con A as a new ligand for SHPS-1


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