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APS, an adaptor protein containing Pleckstrin homology (PH) and Src homology-2 (SH2) domains inhibits the JAK-STAT pathway in collaboration with c-Cbl

T Wakioka[1,2], A Sasaki[1], K Mitsui[1], M Yokouchi[1,2], A Inoue[2], S Komiya[2] and A Yoshimura[1]

[1]*Institute of Life Science; and* [2]*Department of Orthopaedic Surgery, Faculty of Medicine, Kurume University, Kurume, Japan*

We cloned a novel adaptor protein, APS (adaptor molecule containing Pleckstrin homology (PH) and Src Homology-2 (SH2) domains), which was tyrosine phosphorylated in response to c-kit or B cell receptor stimulation. Here, we report that APS was tyrosine phosphorylated by *Janus* kinase-2 (JAK2) at its C-terminal tyrosine residue and interacted with c-Cbl. Forced expression of APS in an erythropoietin (EPO)-dependent hematopoietic cell line resulted in reduced activation of STAT5 but not cell proliferation in response to EPO. APS bound to the phosphorylated tyrosine residue, Y343 of the erythropoietin receptor cytoplasmic domain. Co-expression of APS and c-Cbl, but not expression of either alone inhibited EPO-dependent STAT5 activation in 293 cells. This required the C-terminal phosphorylation site, as well as PH and SH2 domains of APS. Therefore, one of the major functions of APS is in recruitment of c-Cbl into the receptor/JAK complex, thereby inhibiting JAK signaling activity.
Keywords: APS; cytokine; signal transduction; c-Cbl; JAK; STAT

Introduction

The growth, differentiation and functions of immune and hematopoietic cells are controlled by multiple cytokines, including interleukins (ILs) and colony-stimulating factors (CSFs). Cytokines exert their biological effects through binding to cell-surface receptors that are associated with one or more members of the JAK family of cytoplasmic tyrosine kinases (JAKs). Cytokine-induced receptor dimerization leads to the activation of JAKs, rapid tyrosine phosphorylation of the cytoplasmic domains and subsequent recruitment of various signaling proteins to the receptor complex.[1] Among these proteins are members of the STAT family of transcription factors.[2–4] The tyrosine-phosphorylated STATs form homo- or heterodimers and translocate into the nucleus where they activate target genes.

Regulation of the JAKs is a central component in the control of cytokine signaling. Constitutive, unregulated JAK activity has been implicated in T cell leukemia.[5,6] As a result of the critical role of cytokines in mediating inflammation and immunity, constitutive activation of JAKs and STATs may contribute to hematopoietic disorders, autoimmunity and inflammatory diseases. However, little is known regarding the mechanisms of termination of down-regulation of JAK/STAT activity in cytokine responses. There is evidence that one mechanism involves the recruitment of a tyrosine phosphatase containing an SH2 domain (SHP-1; also referred to as HCP) to receptor complexes resulting in the dephosphorylation of JAKs.[7,8] The potential importance of this mechanism is strongly suggested by the phenotype of *motheaten* (*me/me*) mice lacking SHP-1 that die from a disease with components of autoimmunity and inflammation.[9] However, SHP-1 has been shown to negatively regulate a number of receptor and non-receptor tyrosine kinases including c-kit and ZAP-70.[10,11] Thus, the kinases which are specifically responsible for the phenotype remain to be determined. Degradation of JAK kinases by proteasome pathways has been implicated in down-modulation of JAK pathways.[12–14] Recently, a family of cytokine-inducible SH2 proteins (CISs) have also been proposed to be involved in negative feedback regulation of cytokine signaling.[15,16]

The adaptor molecule c-Cbl has also been demonstrated to be a negative regulator of several tyrosine kinases.[17] In *C. elegans*, the c-Cbl homologue Sli-1 acts as a negative regulator of the Ras homologue Let60.[18,19] Also, several recent studies have demonstrated that mammalian c-Cbl has a negative regulatory function in intracellular signal transduction, such as Syk tyrosine kinase activation in mast cells,[20] T cell receptor-induced ERK2 and AP-1 activation,[21] and Cbl-b, a member of the Sli-1/c-Cbl protein family, inhibits Vav-mediated c-Jun kinase activation.[22,23] Ueno *et al*[24] demonstrated that suppression of c-Cbl expression enhanced the EGF-dependent JAK/STAT pathway, suggesting that c-Cbl is also a regulator of cytokine signaling. Indeed, c-Cbl gene knockout mice exhibited enhanced TCR signaling via Syk and ZAP-70.[25]

Previously, we cloned and characterized a novel adaptor molecule containing a Pleckstrin homology (PH) domain and Src homology-2 (SH2) domain, APS.[26] APS was cloned using the yeast two-hybrid system with the oncogenic c-kit kinase domain as bait. This protein forms a new subfamily of SH2 proteins with Lnk and SH2-B. APS is tyrosine phosphorylated in response to c-kit or B cell receptor stimulation, and a single major tyrosine phosphorylation site was found at its C-terminus which is highly conserved in this family. Phosphorylation of the C-terminus creates a binding site for Grb2 and c-Cbl. However, its physiological function remained to be clarified. Among the hematopoietic cell lines we examined to date, APS is exclusively expressed in B lymphoma cells, and Lnk as well as SH2-B have also been suggested to be involved in T cell receptor and Fc receptor signaling, respectively. Therefore, we and others proposed that these proteins are involved in immunoreceptor signaling.[26] However, APS, Lnk and SH2-B were found to be expressed not only in lymphocytic tissues but also in a wide variety of tissues.[26–29] Therefore, this family may be linked to tyrosine kinases in addition to immunoreceptor-coupled tyrosine-kinases. Recently, an isoform of SH2-B (SH2-Bβ) was shown to be a good substrate for JAK2; it was phosphorylated in response to gamma interferon (IFNγ) and growth hormone (GH).[30] SH2-B has also been shown to bind to the insulin receptor and IGF-1 receptor.[31,32] These results suggested that this family may have a function in a wide variety of cytokine and growth factor signaling pathways in addition to immunoreceptor signaling.

In this study, we found that APS was highly phosphorylated in response to the cytokine stimulation and suppressed erythropoietin (EPO)-induced STAT5 activation. Using the 293 cell reconstitution systems, we showed that APS and c-Cbl

Correspondence: A Yoshimura, Institute of Life Science, Kurume University, Aikawamachi 2432-3, Kurume 839-0861, Japan; Fax: 81 942 31 5212
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synergistically inhibit cytokine-dependent STAT5 activation. Therefore, APS was suggested to function as a negative regulator of cytokine signaling in collaboration with c-Cbl.

Materials and methods

Cells

Saos-2 and 293 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). Daudi cells were maintained in RPMI with 10% FCS. Murine IL3-dependent lymphoid Ba/F3 cells were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10% conditioned medium from the WEHI-3B cell line as a source of IL3 as described.[33] NIH-3T3 cells stably expressing APS will be described elsewhere.[43]

Northern blotting analysis

For Northern blotting, total RNA (5 μg) was separated on 1.0% agarose/2.4% formaldehyde gels, then transferred on to positively charged nylon membranes (Pharmacia-Amersham Biotech, Tokyo, Japan). After fixation under calibrated UV irradiation, these membranes were hybridized with DIG-labeled riboprobes prepared using a DIG-RNA labeling kit (Boehringer Mannheim, Tokyo, Japan). The blots were visualized using alkaline phosphatase-labeled anti-DIG antibody and a chemiluminescent substrate according to the manufacturer's instructions. Probes were described previously.[33]

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously.[26]

Generation of Ba/F3 cells stably expressing APS

Ba/F3 transformants expressing the EPO receptor (BF-ER) were described previously.[33] A full-length APS cDNA (codon 2–632) with Myc-epitope tag at the N-terminus was subcloned into the pLXSN or pcDNA3 mammalian expression vectors. These plasmids were introduced into BF-ER cells by electroporation and stable transformants were selected with 1.0 mg/ml G418. Expression of APS in transformants was detected by immunoblotting with anti-Myc or anti-APS. Transformants were maintained in the presence of 0.5 units/ml EPO and 0.5 mg/ml G418.

In vitro binding assay

A GST fusion protein with the APS SH2 domain (GST-SH2; codon 397–551) was prepared for the *in vitro* binding experiments as described previously.[26] APS-C-terminus (RAVENQpYSFY) and the EPO receptor (GDTpYLVLDKWL) phosphopeptides were synthesized and conjugated to Affigel-10 (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. A polypeptide substrate, GST-EPOR cytoplasmic domain (codon 325–430) containing two tyrosine residues (YY) or tyrosine to phenylalanine substitution mutants (FY, YF and FF) at Y343 or Y401 was produced in TKB-1 bacteria (Stratagene, La Jolla, CA, USA) and purified on GSH-Sepharose (Pharmacia-Amersham Biotech). The purified protein was dialyzed against 10 mm Tris-buffered saline (TBS) and concentrated to 2 mg/ml.

Myc-tagged APS in pcDNA3 (5 μg/transfection) was transiently expressed in 293 cells grown in 10-cm dishes. Cells were lysed in 1 ml of lysis buffer A (20 mm Hepes buffer, pH 7.3, 150 mm NaCl, 0.5% NP-40, 50 mm NaF, 10 mm sodium pyrophosphate, 1 mm sodium vanadate, 1 mm phenylmethylsulfonyl fluoride and 1% aprotinin), then centrifuged. Before the precipitation experiments, bovine serum albumin (BSA) was added to a final concentration of 1 mg/ml. Cell extracts were incubated with 20 ml (50% v/v) of peptide- or GST-EPO receptor-conjugated Sepharose, then washed extensively with phosphate buffered saline (PBS) containing 0.5% NP-40, and analyzed by immunoblotting with anti-Myc (9E10) and anti-c-Cbl (Santa Cruz Biotechnology, CA, USA) antibodies.

Luciferase assay

A luciferase gene construct containing the APRE promoter and β-gal gene construct, EPO receptor construct, STAT5 construct and/or full-length APS construct in pLXSN (Myc-APS; codon 2–632), or deletion mutants in pLXSN, or HA-tagged c-Cbl gene construct[20] were co-transfected into 293 cells using the calcium-phosphate method. After transfection, the cells were factor depleted for 12 h, then stimulated with 10 unit/ml EPO for 12 h. Cell extracts were prepared and luciferase and control β-galactosidase activities were measured as described previously.[33]

Results

Cytokine/JAKs-induced tyrosine phosphorylation of APS

Recently we found that APS was highly expressed not only in B cell lymphoma Raji and Daudi cells but also in an osteosarcoma cell line, Saos-2. To investigate whether cytokines activate APS, we stimulated cells with various cytokines and examined phosphorylation of APS in Saos-2 in response to interferon-γ (IFNγ). Phosphorylation of exogenous APS was also examined in transformants expressing Myc-tagged APS in NIH-3T3 fibroblasts as well as Ba/F3 cells expressing the EPO receptor (BF-ER) in response to leukemia inhibitory factor (LIF) and EPO. As shown in Figure 1a, endogenous and exogenous APS was tyrosine phosphorylated in response to stimulation with these various cytokines. In the 293 cell reconstitution system, APS was phosphorylated by JAK2 activation by the EPO receptor (Figure 1b). C-terminal truncated APS (Myc-ΔC; lacking C-terminal 113 amino acids) was not phosphorylated, suggesting that the C-terminal tyrosine residue conserved among this family is the phosphorylation site.

Binding of APS to the phosphorylated EPO receptor

To investigate whether APS binds to the EPO receptor and/or JAK2, we performed *in vitro* and *in vivo* binding assays. To obtain better signals, we used the 293 cell reconstitution sys-

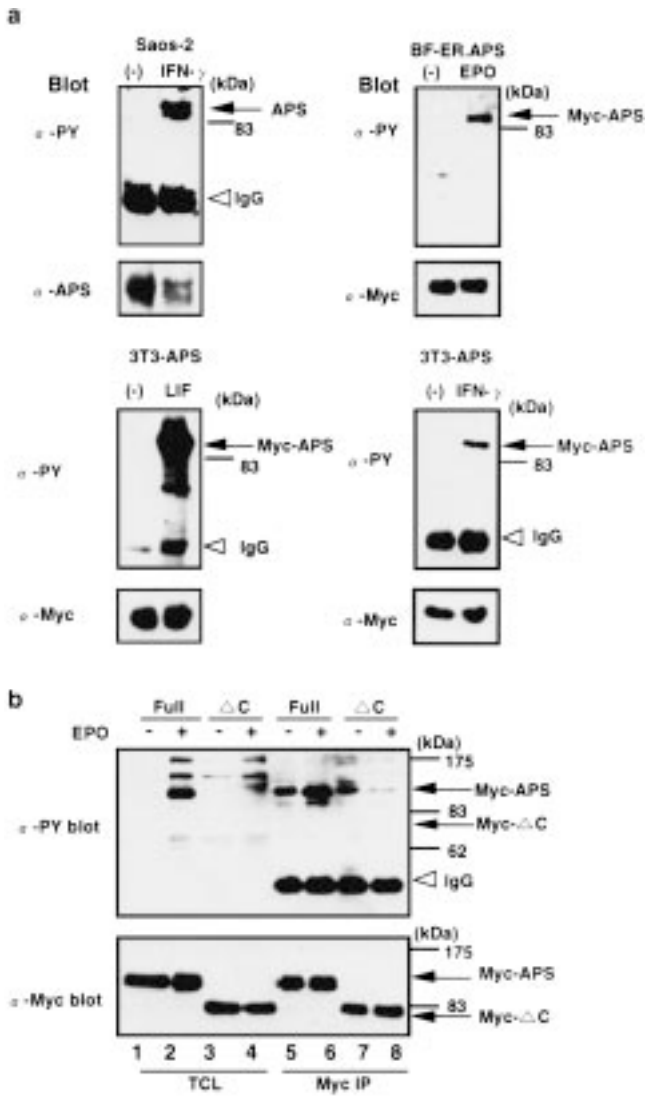

Figure 1 (a) Tyrosine phosphorylation of APS with various cytokines. After starvation for 6 h, Saos-2 cells, Ba/F3 cells expressing the EPO receptor and APS (BF-ER.APS) and NIH3T3 cells stably transformed with APS (3T3-APS) were stimulated with the indicated cytokines (1000 unit/ml IFNγ, 10 unit/ml EPO, and 10 ng/ml LIF) at 37°C for 15 min, then immunoprecipitated with anti-APS. Anti-APS immune complexes were resolved on 7% SDS-PAGE gels, then immunoblotted with anti-phosphotyrosine (α-PY), anti-Myc (α-Myc) or anti-APS (α-APS). (b) The C-terminal tyrosine residue of APS is the site for phosphorylation. The EPO receptor cDNA, Flag-tagged JAK2 cDNA and Myc-APS or Myc-ΔC cDNA were transfected into 293 cells. Cells were stimulated with EPO (10 units/ml) for 15 min, then lysed and immunoprecipitated with anti-Myc antibody (α-Myc). The total cell lysates or immune complexes adsorbed with protein A-Sepharose were immunoblotted with anti-PY (α-PY) or anti-Myc (α-Myc).

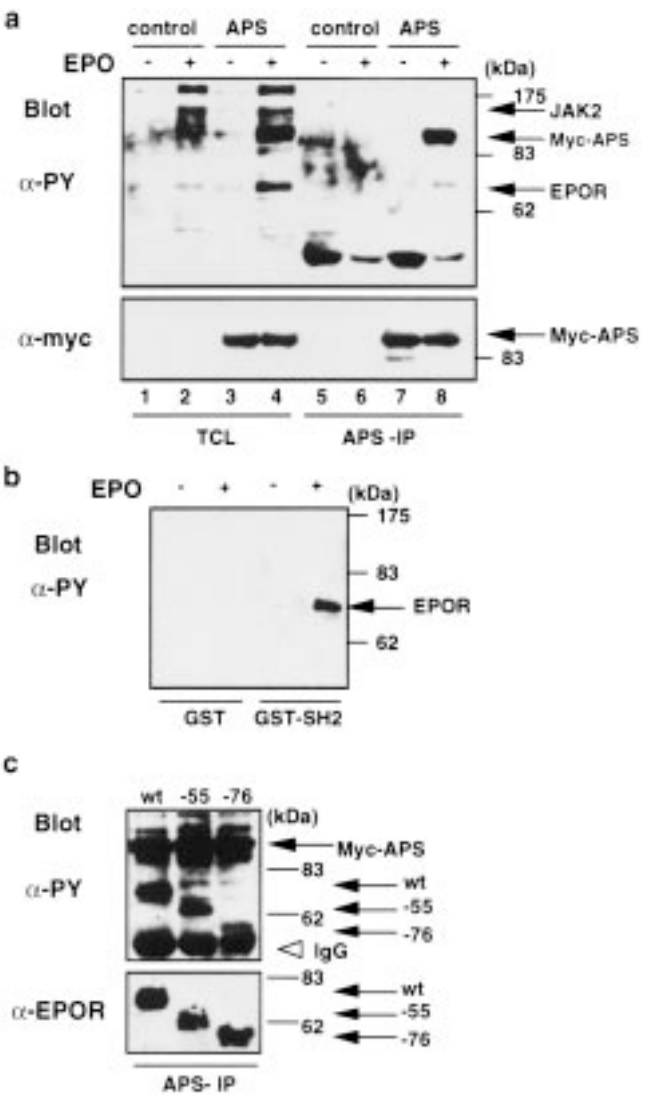

Figure 2 APS binds to the EPO receptor. In (a), wild-type EPO receptor (EPOR) cDNA, Flag-JAK2 cDNA and plasmids carrying Myc-APS or empty vector (pcDNA3) were co-transfected into 293 cells. Cells were stimulated with or without EPO (10 units/ml) for 5 min, then lysed and immunoprecipitated with anti-APS antibody. The immunoprecipitates were immunoblotted with anti-PY (α-PY) or anti-Myc antibody (α-Myc). In (b), extracts of EPO-treated or untreated 293 cells expressing EPOR and JAK2 were incubated with 5 μg of immobilized GST or GST fused with the SH2 domain of APS (GST-SH2) at 4°C for 1 h. The bound materials were analyzed with immunoblotting with anti-PY (α-PY). In (c), the 293 cells expressing Myc-APS, JAK2 and wild-type (WT) or C-terminal truncated (–55 and –76) EPO receptor mutants were treated with EPO (10 units/ml), then lysed and immunoprecipitated with anti-APS. The immune complex was blotted with anti-PY (α-PY) or anti-EPO receptor antibody (α-EPOR). Positions for the EPO receptor and Myc-APS are indicated.

tem. First, we examined whether APS binds to the EPO receptor or JAK2. As shown in Figure 2a, the EPO receptor but not JAK2 was co-precipitated with APS. The tyrosine phosphorylated EPO receptor, but not JAK2 and STAT5, was precipitated with the SH2 domain of APS fused to GST *in vitro* (Figure 2b). A truncated EPO receptor containing Y343 and Y401 still bound to APS, suggesting that the APS binding site overlaps the STAT5 binding sites (Figure 2c). To clarify which tyrosine residue is recognized by the APS SH2 domain, we performed *in vitro* binding assay using GST-fusion protein with the cytoplasmic domain of the EPOR (GST-YY, FY, YF, and FF) or phosphopeptides coupled to agarose (Figure 3). GST fusion proteins were expressed and tyrosine phosphorylated in the TKB-1 strain which carries active tyrosine kinase (Figure 3a, α-PY and CBB). As shown in Figure 3a top panel, Myc-APS bound to GST-YY and GST-YF, but not to GST-FY and GST-FF (α-Myc). While STAT5 bound equally to GST-YF and GST-FY as reported previously (α-STAT5). Therefore, phosphorylated Y343 is a major binding site for APS. The specificity was also confirmed by using phosphopeptides (Figure 3b,c). APS

bound to the EPO receptor phosphopeptide containing Y343 'GDTpYLVLDKWL' but not to the phosphopeptides of the C-terminal region of APS, or phosphopeptides of JAK2 (Figure 3b,c). These results indicated that the SH2 domain of APS specifically recognizes Y343 of the EPO receptor.

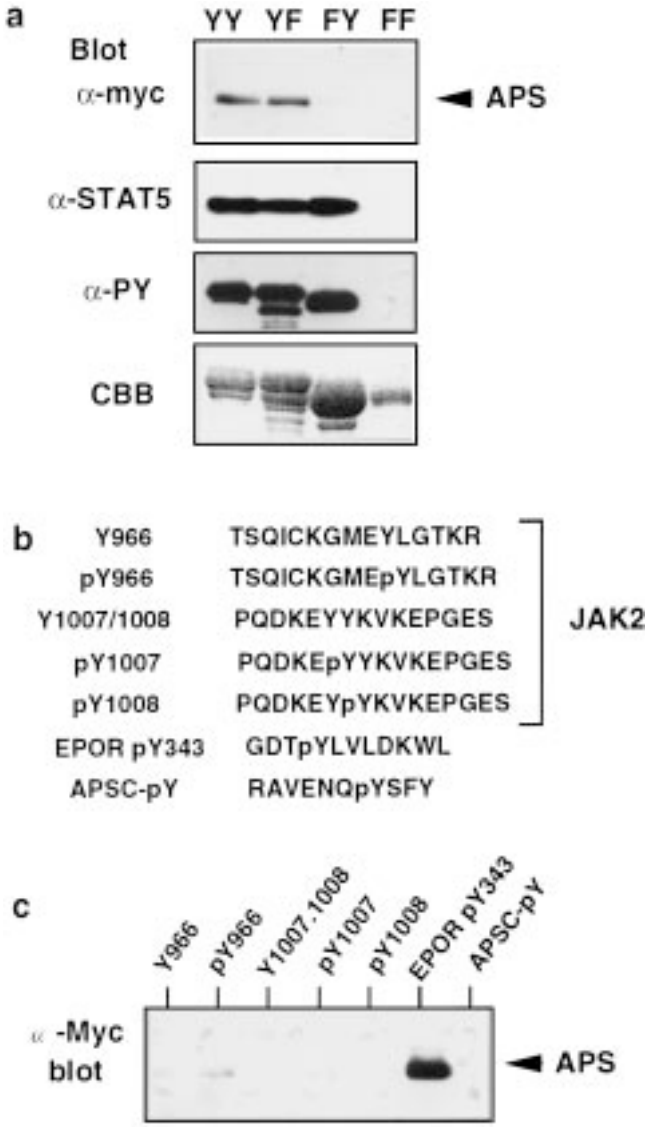

Figure 3 APS binds to pY343 of the EPO receptor. (a) Expression of the GST fusion proteins with the cytoplasmic domain of the EPO receptor containing Y343 and Y401 and their substitution mutants (YY; wild-type, YF; Y401F, FY; Y343F, FF; Y343F, Y401F). These GST fusion proteins were expressed in TKB-1 and stained with Coomassie brilliant blue (CBB) or immunoblotted with anti-PY antibody (α-PY). Extracts of the 293 cells expressing Myc-APS were incubated with 5 μg of immobilized GST-fusion peptides at 4°C for 1 h. The complex with GST-fusion peptides was resolved by 7% SDS-PAGE, then immunoblotted with anti-Myc (α-Myc) or anti-STAT5 (α-STAT5) antibodies. (b) Sequences of peptides used for *in vitro* binding assay. (c) Extracts from the 293 cells expressing Myc-SH2C (the SH2 domain and C-terminal region of APS) were incubated with approximately 5 μl of agarose conjugated with indicated phosphopeptides or non-phosphopeptides, then precipitates were analyzed by immunoblotting with anti-Myc antibody (α-Myc). The amino acid sequences of the peptides are listed.

c-Cbl binds to C-terminal tyrosine residues of APS in vitro and inhibits EPO-dependent STAT5 activation synergistically with APS

To examine the effects of APS on EPO signaling at the molecular level, we screened for proteins capable of binding to APS using an *in vitro* binding assay. Cell extracts from Raji, Ba/F3 and 293 cells were incubated with a phosphopeptide containing a C-terminal tyrosine residue (RAVENQpYSFY). As a control, the EPO receptor phosphopeptide containing Y343 (GDTpYLVLDKWL) was used. We found that c-Cbl interacted with phosphorylated APS C-terminal peptide but not with the EPO receptor phosphopeptide (Figure 4a), while PLCγ bound to the EPO receptor phosphopeptide but not to the APS phosphopeptide (data not shown).

Since c-Cbl has been shown to be a negative regulator of various tyrosine kinases, this molecule may play an important role in the function of APS. To determine the biological effects of the interaction between APS and c-Cbl, we measured EPO-induced STAT5 activation in the presence of APS and/or c-Cbl using the 293 cell transient expression system. As shown in Figure 4b, although forced expression of 0.5 μg of full-

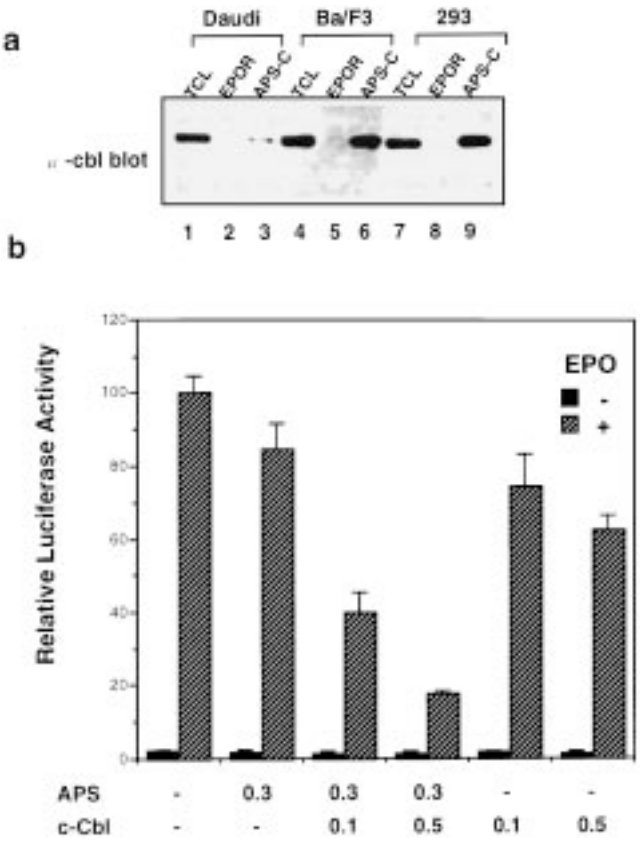

Figure 4 APS is associated with c-Cbl *in vitro* and synergistically inhibits EPO-dependent STAT5 activation. (a) Extracts from Raji, Ba/F3, 293 cells were incubated with approximately 5 μg of agarose conjugated with EPO receptor phosphopeptide (EPOR) or APS phosphopeptide (APS-C) at 4°C for 1 h, then precipitates were analyzed by immunoblotting with anti-c-Cbl antibody (α-cbl). (b) 293 Cells were transfected with STAT5-responsive APRE promoter-luciferase reporter constructs, the EPO receptor cDNA and the STAT5 cDNA in combination with indicated amounts (μg plasmids) of APS or c-Cbl. Cells were stimulated with EPO (10 units/ml) for 12 h and luciferase activity was measured. Data shown are the means of two independent determinations.

length APS cDNA alone had no clear effect on EPO-induced STAT5 activation, co-expression with c-Cbl synergistically inhibited EPO-induced STAT5 promoter activation (Figure 4b).

To assess which domains of APS were responsible for c-Cbl-dependent suppression of STAT5, we examined the effects of various truncation mutants of APS (Figure 5a). As shown in Figure 5b, C-terminal truncated APS, or deletion mutants lacking N-terminal and PH domains did not exhibit c-Cbl-dependent STAT5 inhibition. In contrast, deletion of the N-terminal proline-rich region exhibited no effect. These results suggest that binding of c-Cbl to the C-terminal phosphorylation site of APS is important for the negative regulatory effect of APS against EPO-induced STAT5 signaling, and N-terminal region, PH domain and SH2 domain of APS are essential for the STAT5 inhibition.

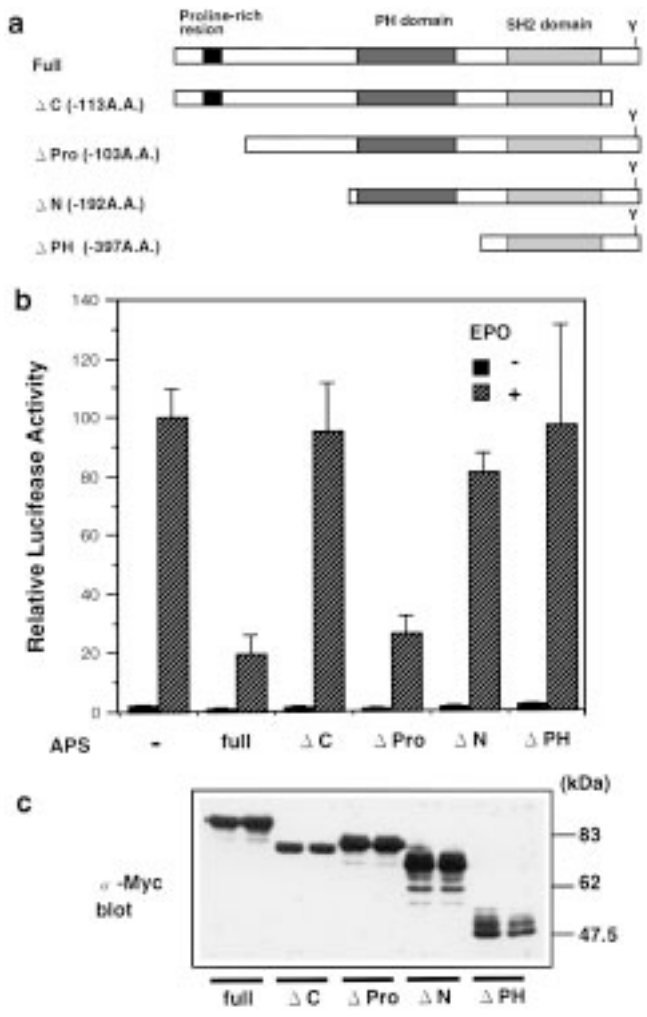

Figure 5 The C-terminal phosphorylation site, N-terminal region and PH domain of APS are essential for inhibition of EPO-induced STAT5 signaling. (a) Structure of full-length and truncated mutants of APS. The mutants are named according to the deleted region. full, codon 2–632; ΔC codon 2–519; ΔPro, codon 104–632; ΔN, codon 193–632; ΔPH, codon 402–632. (b) 293 Cells were transfected with plasmids carrying the EPO receptor, STAT5 reporter gene, c-Cbl (0.5 μg) and full-length APS or truncated mutants (0.3 μg). Cells were stimulated with EPO (10 units/ml) for 12 h and luciferase activity was measured. Data shown are the means of two independent determinations. (c) Expression level of each APS mutant was examined by immunoblotting with anti-Myc (α-Myc).

Effects of forced expression of APS on EPO-dependent signaling in a hematopoietic cell line

To confirm the negative effect of APS expression on EPO/STAT5 signaling *in vivo*, we transfected full-length APS into BF-ER cells, which grow in response to IL3 and EPO. Parental BF-ER cells did not express endogenous APS (data not shown). BF-ER transformant expressing APS (BF-ER.APS) grew as fast as the parental BF-ER cells in response to both IL3 and EPO (data not shown). To examine the effect of APS on STAT5 activity, we examined induction of CIS and oncostatin M (OSM) genes. We have previously shown that these two genes are targets of STAT5.[33,34] As shown in Figure 6a, EPO-dependent induction of CIS and OSM genes, but not c-myc gene expression, were decreased in BF-ER.APS cells, especially at the early stage of stimulation (30 min). As shown in Figure 6b, EPO-dependent phosphorylation of STAT5 was partially suppressed compared with BF-ER cells. These findings suggested that APS partially suppresses EPO-dependent activation of STAT5 in hematopoietic cells.

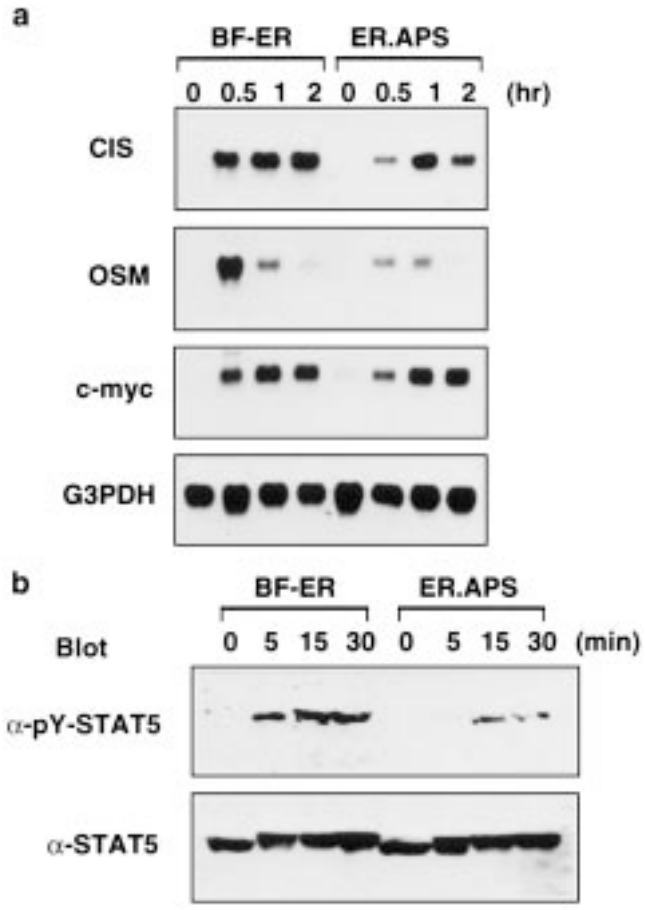

Figure 6 Inhibitory effect of APS on EPO-dependent STAT5 activation. (a) Ba/F3 cells expressing EPO receptor (ER) or cells expressing both the EPO receptor and APS (ER.APS) were stimulated with EPO (10 units/ml) for the indicated periods. Membranes carrying total RNA from each cell line (5 μg/lane) were hybridized with the mouse CIS, OSM, c-myc or control G3PDH probe. (b) Ba/F3 transformants (ER and ER.APS) were stimulated with EPO (10 units/ml) for the indicated periods. Cells were lysed and total cell extracts were resolved by 7% SDS-PAGE, then immunoblotted with anti-phospho STAT5 (α-pY-STAT5) or anti-STAT5 antibody (α-STAT5).

Discussion

In this study, we identified APS as a novel substrate of JAK kinases. Rui *et al*[30] suggested that growth hormone (GH)-induced activation and phosphorylation of JAK2 recruits SH2-Bβ and its associated signaling molecules into a GH receptor-JAK2 complex, thereby initiating some as yet unidentified signal transduction pathways. However, the physiological significance of phosphorylation of SH2-B has not yet been clarified. We also demonstrated that APS binds to the EPO receptor, and overexpression of full-length APS in Ba/F3 cells expressing the EPO receptor resulted in partial suppression of EPO-induced STAT5 activation. Since EPO- or IL3-dependent cell growth in APS transformants was almost equivalent to that in parental cells (data not shown), the inhibitory effect of APS may be specific to the STAT5 pathway. We found that APS bound to Y343 of the phosphorylated EPO receptor, which is also one of the STAT5 binding sites. Therefore, part of the mechanism of STAT5 inhibition by APS may be due to a masking effect of APS on docking sites of STAT5 in the EPO receptor. In addition, in the 293 cell reconstitution system we found that APS inhibited STAT5 activation in collaboration with c-Cbl. Since APS bound to c-Cbl through its C-terminal phosphorylation site, c-Cbl may be an important effector protein of APS. This model is illustrated in Figure 7. Precious mechanism of inhibition of STAT5 activation by APS-c-Cbl complex will be addressed in the future study.

We identified tyrosine-343 (Y343) but not Y401 of the EPO receptor as a major binding site for APS. The amino acid sequence around Y343 of the EPO receptor is QDTYLVL and that of Y401 is FEYTILD. Recently, we found that APS SH2 domain bound to Y1021 of the PDGF receptor (DNAYIGVT) (YM unpublished data). Thus, the SH2 domain of APS may recognize hydrophobic amino acids at positions +1 after the phosphotyrosine residue. However, since available information is very limited at present, further studies are necessary to find a consensus APS recognition motif.

In the present and our previous studies, we demonstrated that c-Cbl binds to the tyrosine phosphorylated C-terminal region of APS but not to the unphosphorylated counterpart (Figure 5 and Ref. 26). A recent study revealed that D(N/D)XpY is a binding motif for the Cbl-PTB domain.[35] The tyrosine phosphorylation site of APS contains ENQpY and those of Lnk and SH2-B, which are members of the APS family, also contain DNQpY and NNQpY, respectively. These observations suggest that the C-terminal tyrosine residues of members of the APS family are good candidates for Cbl-PTB domain binding sites. It has been demonstrated that c-Cbl is tyrosine phosphorylated in response to stimulation with various cytokines including EPO, although the role of c-Cbl in EPO signaling has not been clarified.[36,37] Recent studies have demonstrated that c-Cbl has a negative function in intracellular signal transduction, such as Syk tyrosine kinase activation in mast cells and T cell receptor signaling.[20,25] Since it has been shown that proteasome-dependent PDGF receptor degradation is accelerated by c-Cbl binding, c-Cbl may be involved in ubiquitin-dependent degradation of the bound protein.[38,39] Although the precise mechanism remains to be clarified, it is possible that the negative regulatory function of APS was enhanced by association with phosphorylated c-Cbl. It is possible that APS is involved in c-Cbl function as an upstream or downstream mediator. For example, APS may facilitate anchoring of c-Cbl to the membrane, since APS is retained at the membrane after stimulation probably because APS has a PH domain which has been shown to interact with phospholipids. APS-c-Cbl complex may desensitize the receptor/JAK complexes, to which c-Cbl alone cannot bind. The physiological significance of the negative regulatory function of APS will be defined in future studies by generating transgenic or gene-disrupted mice.

Recently, negative regulators of tyrosine kinase signaling have attracted a great deal of attention. We have shown that the JAK binding proteins JAB and CIS3 inhibit JAK kinase activity by direct binding to the catalytic domain.[40] CIS1 partially inhibits EPO-dependent STAT5 activation by binding to the receptor.[33] APS may be another type of signal inhibitor of cytokine signaling. The other SH2 proteins Grb10 and Grb14 have been shown to contain a second novel domain that interacts with the insulin receptor and insulin-like growth factor receptor in an activation loop-dependent manner.[41,42] These studies suggested that Grb10 and Grb14 may interact within or near the activation loop of the insulin receptor kinase domain, thereby physically blocking tyrosine kinase activity and ultimately inhibiting the action of insulin. Grb7, Grb10 and Grb14 comprise another SH2 protein subfamily containing PH and SH2 domains and a proline-rich sequence, similar to the APS family. Although there is little sequence similarity between APS and Grb7/10/14, it would be interesting to determine whether other tyrosine kinases possess their own specific inhibitory SH2 proteins. Elucidation of the precise inhibitory mechanism of tyrosine kinases or cytokine signaling could provide a novel basis for developing methods for inhibiting tyrosine kinases.

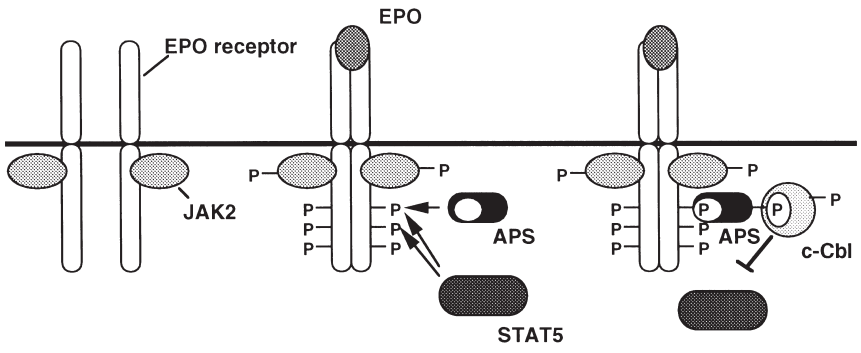

Figure 7 A mode of inhibitory action of APS on the EPO receptor/STAT5 signaling pathway. After stimulation, the EPO receptor is tyrosine phosphorylated by JAK2. The first (Y343) and the second (Y401) phosphotyrosine resides are major binding sites for STAT5, while APS mainly binds to the first one. 'P' indicates phosphorylated tyrosine residues. Phosphorylated APS recruits c-Cbl, thereby inhibiting STAT5 activation.

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