



APS, an adaptor protein containing PH and SH2 domains, is associated with the PDGF receptor and c-Cbl and inhibits PDGF-induced mitogenesis

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Previously we cloned a novel adaptor protein, APS (adaptor molecules containing PH and SH2 domains) which was tyrosine phosphorylated in response to *c-kit* or B cell receptor stimulation. Here we report that APS was expressed in some human osteosarcoma cell lines, markedly so in SaOS-2 cells, and was tyrosine-phosphorylated in response to several growth factors, including platelet derived growth factor (PDGF), insulin-like growth factor (IGF), and granulocyte-macrophage colony stimulating factor (GM-CSF). Ectopic expression of the wild type APS, but not C-terminal truncated APS, in NIH3T3 fibroblasts suppressed PDGF-induced MAP kinase (Erk2) activation, *c-fos* and *c-myc* induction as well as cell proliferation. *In vitro* binding experiments suggest that APS bound to the β type PDGF receptor, mainly via phosphotyrosine 1021 (pY1021). Indeed, tyrosine phosphorylation of PLC- γ , which has been demonstrated to bind to pY1021, but not that of PI3 kinase and associated proteins, was reduced in APS transformants. PDGF induced phosphorylation of the tyrosine residue of APS close to the C-terminal end. *In vitro* and *in vivo* binding experiments indicate that the tyrosine phosphorylated C-terminal region of APS bound to c-Cbl, which has been shown to be a negative regulator of tyrosine kinases. Since coexpression of c-Cbl with wild type APS, but not C-terminal truncated APS, synergistically inhibited PDGF-induced *c-fos* promoter activation, c-Cbl could be a mechanism of inhibitory action of APS on PDGF receptor signaling.

Keywords: adaptor molecules containing PH and SH2 domains (APS); platelet derived growth factor receptor (PDGFR); signal transduction; molecular biology

Introduction

Receptor protein tyrosine kinases are activated by binding of a ligand to the extracellular region of the receptor, following the initiation of multiple biological actions in cells (Ullrich and Schlessinger, 1990; Pawson and Gish, 1992). Platelet-derived growth factor (PDGF) also regulates various cellular responses such as development (Stephenson *et al.*, 1991; Morrison *et al.*, 1990), proliferation (Gazit *et al.*, 1984; Ferns *et al.*,

1991), differentiation (Alimandi *et al.*, 1997), cell survival (Barres *et al.*, 1992) and cellular transformation (Waterfield *et al.*, 1983; Gazit *et al.*, 1984). Interaction of PDGF with its receptor induces dimerization of two distinct receptor subunits, resulting in the activation of intracellular tyrosine kinase domain and creating binding sites for several signal transducing molecules containing SH2 domain or PTB domain (Cantley *et al.*, 1991). These proteins include Nck, phosphatidylinositol 3 kinase (PI3K), phospholipase C- γ (PLC- γ), protein-tyrosine-phosphatase SHP-2 (SHPTP2/Syp/PTP-1D/PTP2C) and the GTPase-activating protein of Ras (RasGAP). Each molecule binds to a specific phosphorylated tyrosine residue of the PDGF receptor, then is activated and transmits signals that ultimately lead to biological responses. However their regulatory mechanism as well as downstream signals are still not completely understood, and several lines of evidence suggest that unidentified proteins are involved in PDGF receptor signaling (Arvidsson *et al.*, 1994; Kazlauskas, 1994; Yokote *et al.*, 1994).

Previously, we cloned and characterized a noble adaptor molecule containing Pleckstrin homology (PH) domain and Src homology-2 (SH2) domain, APS (Yokouchi *et al.*, 1997). APS was cloned using a yeast two-hybrid system with 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 is found at the C-terminus, which is highly conserved in this family. Phosphorylation of C-terminus creates a binding site for Grb2 and c-Cbl, however the physiological function of APS remains to be clarified. Since APS is exclusively expressed in B lymphoma cells among hematopoietic cell lines and Lnk as well as SH2-B are also suggested to be involved in T cell receptor and Fc receptor signaling respectively, we speculated that these proteins are involved in immunoreceptor signaling. However, APS, Lnk and SH2-B were found to be expressed not only in lymphocytic but a wide variety of tissues (Yokouchi *et al.*, 1997; Huang *et al.*, 1995; Takaki *et al.*, 1997; Osborne *et al.*, 1995, 1996). Therefore this family may be linked to tyrosine kinases in addition to immunoreceptor-coupled tyrosine kinases. Recently an isoform of SH2-B (SH2-B β) was shown to be a good substrate of JAK2; it was phosphorylated in response to gamma interferon (IFN- γ) and growth hormone (GH) (Rui *et al.*, 1997). SH2-B has been shown to bind to insulin receptor and IGF-1 receptor (Wang and Riedel, 1998).

These results suggests that this family has functions in addition to immunoreceptor signaling.

In this study we found that APS was highly phosphorylated in response to PDGF and bound to the PDGF receptor. The experiments presented here focus on the response of APS activated by PDGF. The binding site of APS SH2 domain was probably pY1021 of the PDGF receptor β . Moreover, forced expression of APS in NIH3T3 cells resulted in suppression of PDGF-induced Erk2 activation, *c-myc* expression as well as cell growth. Since c-Cbl associated with the phosphorylated C-terminal region of APS and these two genes synergistically inhibited PDGF-induced *c-fos* promoter activation in 293 cells, c-Cbl may be involved in suppression of PDGF signaling.

Results

Expression and tyrosine phosphorylation of APS in SaOS-2

In a previous study, we have shown that APS was expressed exclusively in B cells among hematopoietic cell lines but not in solid tumor cell lines. We continued to search for cell lines expressing APS, however, we found that expression of APS in solid tumor cells was very rare. Among 20 solid tumor cell lines we examined, only one, human osteosarcoma cell line, SaOS-2, expressed APS by Northern blotting (Figure 1a). In this line, the expression level of APS was approximately fivefold higher than that in human

B cell line, Raji. The APS mRNA was slightly larger in SaOS-2 than Raji cells. However by Western blotting, we could not find any difference in molecular size of APS between SaOS-2 and Raji (data not shown). We also detected APS protein in several human osteosarcoma cell lines at very low levels although APS mRNA was undetectable by Northern blotting (Figure 1b).

To investigate what kind of signal activates APS in SaOS-2 cells, we stimulated SaOS-2 using various growth factors or cytokines and examined phosphorylation of APS (Figure 1c). Endogenous APS was tyrosine phosphorylated in response to PDGF-BB, insulin-like growth factor-1 and -II (IGF-I,-II) and granulocyte-macrophage colony stimulating factor (GM-CSF). In this study, PDGF-BB was used as PDGF. In PDGF and IGF-I stimulation, a phosphorylated faint p190 band and strong p145 band were detected in immunoprecipitates with anti-APS respectively (arrowheads). The phosphorylated p190 and p145 are probably PDGF and IGF-1 receptors respectively, as judged by their molecular size.

Effect of forced expression of APS on cell proliferation

The lack of expression of APS in most tumor cell lines but high level of expression in normal tissues suggest that a high level of APS expression is not preferable for transformed phenotype or cell growth. To assess the effect of APS expression on cell proliferation, we created NIH3T3 transformants overexpressing full length APS (Full) as well as those expressing C-terminal truncated APS (Δ C). Parental 3T3 cells did

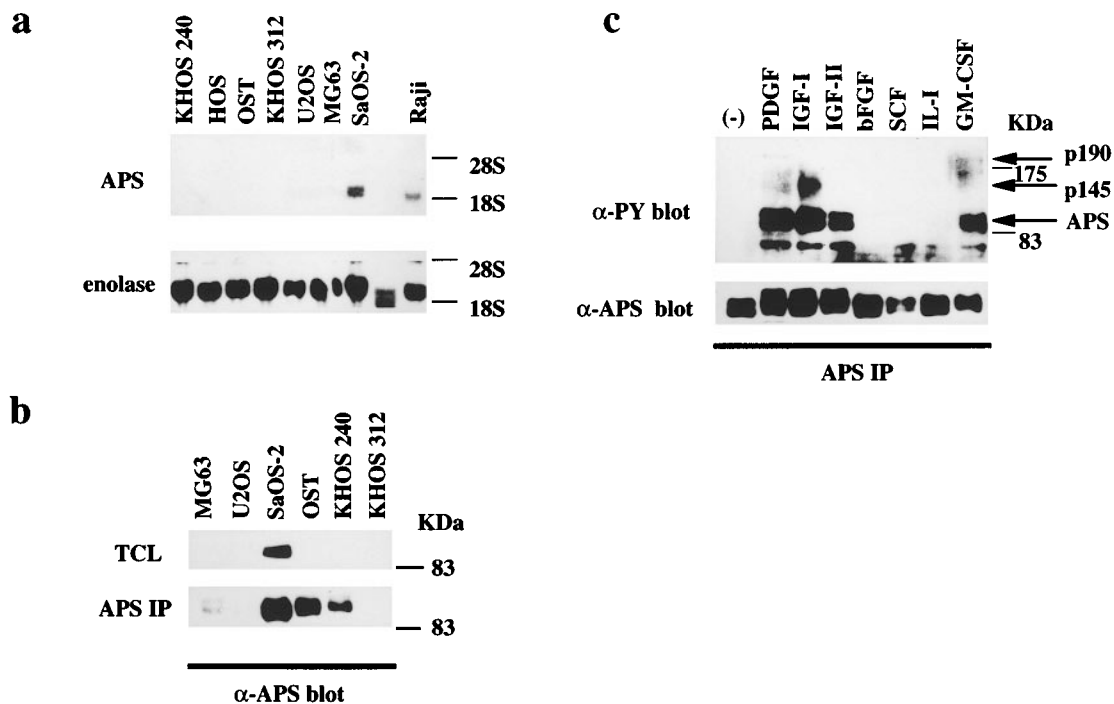


Figure 1 Expression of APS in several osteosarcoma cell lines and tyrosine phosphorylation of APS in Saos-2 cells. (a) Hybridization was carried out for membranes carrying total RNAs from various human osteosarcoma cell lines and B cell line Raji ($5 \mu\text{g}/\text{lane}$) using the human APS probe or control enolase probe. (b) Various human osteosarcoma cells ($1 \times 10^7/\text{sample}$) were lysed and immunoprecipitated with anti-APS. The immunoprecipitates were blotted with the same antibody and peroxidase-conjugated protein A. (c) After starvation for 12 h, Saos-2 cells ($1 \times 10^7/\text{sample}$) were stimulated with indicated growth factors or cytokines at 37°C for 5 min, then lysed and immunoprecipitated with anti-APS. The total cell extracts (TCL) or anti-APS immunocomplex was resolved on an 8% SDS-PAGE gel, then immunoblotted with antiphosphotyrosine (α -PY) or anti-APS (α -APS). Arrowheads indicate phosphorylated p190 and p145

not express endogenous APS (data not shown). Morphologically, transformants appeared to be larger and flatter than the parental 3T3 cells. As shown in Figure 2a, although serum-dependent cell proliferation of APS transformants and parental 3T3 cells was almost equal, PDGF-dependent cell proliferation (Figure 2b) as well as DNA synthesis (Figure 2c) was markedly reduced in transformants expressing wild type APS (Full), compared to parental 3T3 cells (control) or transformants expressing C-terminal truncated APS (Δ C). Next, we examined PDGF-induced *c-myc* and *c-fos* induction and Erk activation (Figure 3), which are shown to be closely related to mitogenesis. Erk activation was measured by in gel kinase assay after immunoprecipitation with Erk specific antibody (Figure 3b). PDGF-induced *c-myc* induction was evident after 1 h stimulation in parental 3T3 (control) as well as Δ C transformant, while *c-myc* induction at 1 h was largely reduced in the full length APS transformant (Figure 3a). PDGF also transiently induced *c-fos*, however, the period of *c-fos* expression was shorter in APS transformants than in parental 3T3 cells or Δ C transformants (compare at 30 min stimulation in Figure 3a). PDGF activates Erk2 kinase activity as strong as FCS in parental 3T3 or Δ C transformant, while PDGF-dependent Erk2 kinase activity was reduced in APS transformants (Figure 3b). PDGF-induced phosphorylation of Erk2 was also reduced in APS transformants compared with parental 3T3 cells or Δ C transformants (data not shown). These

results indicate that APS suppresses PDGF-induced mitogenic responses.

PDGF-induced tyrosine phosphorylation of APS and binding to tyrosine - 1021 of the receptor

To clarify the mechanism by which APS inhibits PDGF-mediated mitogenesis, we investigated the interaction between PDGF receptor and APS. First, we compared tyrosine phosphorylation of APS in 3T3 transformants in response to PDGF-AA, PDGF-BB, IGF-I, IGF-II and bFGF (Figure 4a). PDGF-AA and PDGF-BB equally induced tyrosine phosphorylation of APS, while bFGF only weakly induced APS phosphorylation. IGF-I and IGF-II did not induce phosphorylated APS in 3T3 cells. Figure 4b shows the time course of tyrosine phosphorylation of APS and its associated proteins. Tyrosine phosphorylation of APS was detectable after 1 min stimulation with PDGF and increased gradually with time. The mechanism of such a slow phosphorylation is not clear at present. In the anti-APS immunoprecipitates (Figure 4c), unidentified 190 kDa phosphoprotein (p190), p145, p75 and p65 were seen on early stimulation (p65 was seen even before stimulation), and a strong association of p190 (probably the PDGF receptor judged by molecular size) and p145 was seen after 5–10 min stimulation.

The distribution of the ectopically expressed full length APS was examined by indirect immunofluorescence staining using anti-Myc antibody (Figure 5).

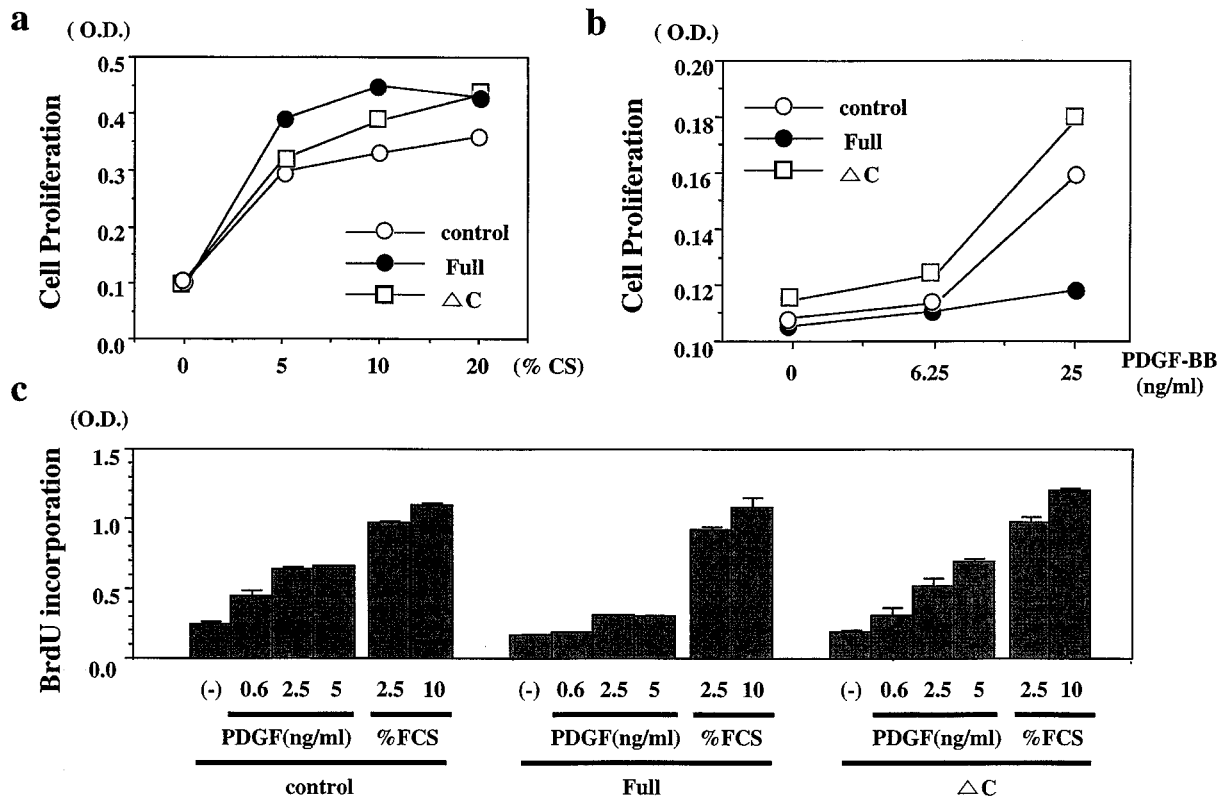


Figure 2 Effect of APS expression on the growth of NIH3T3 cells. In (a) and (b), parental 3T3 cells (control) and transformants expressing wild type APS (Full) or C-terminal truncated APS (Δ C) were spread on 96 well plates (1×10^3 /well) in DMEM with the indicated concentration of calf serum (CS) (a) or PDGF (b). After 3 days, the cells were enumerated by a colorimetric method. In (c), parental 3T3 cells (control) or transformants (Full and Δ C) were spread on 96 well plates (1×10^4 /well) in DMEM with 10% CS. After starvation, cells were stimulated with the indicated concentration of CS or PDGF for 24 h. DNA synthesis was measured by BrdU incorporation. The mean value with s.d. of two independent determinations is shown

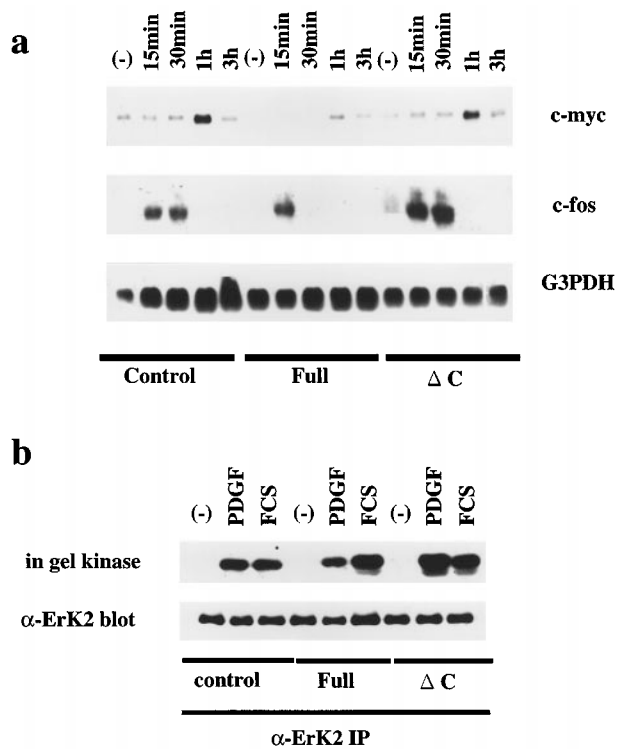


Figure 3 APS suppressed PDGF receptor-induced mitogenic signals. Parental (3T3) cells and transformants expressing wild type APS (Full) or C-terminal truncated APS (Δ C) were stimulated with PDGF (100 ng/ml) for the indicated periods. In (a) membranes carrying total RNA from each cell (5 μ g/lane) were hybridized with the mouse *c-myc*, *c-fos* or control G3PDH probe. In (b), total cell extracts were immunoprecipitated with anti-Erk2 then subjected to in gel kinase assay (in gel kinase) and immunoblotting with anti-Erk2 (α -Erk2 blot)

Analysis of the fluorescence staining patterns by confocal microscopy revealed that APS was present throughout the cytoplasm before stimulation (Figure 5a). However, after stimulation with PDGF for 5 min, part of the APS protein was localized in the cell membrane and peripheral region (Figure 5b). APS was especially concentrated in the ruffling region at the periphery (arrowheads).

It is very difficult to identify p190 phosphoprotein co-immunoprecipitated with APS as the PDGF receptor in NIH3T3 transformants using anti-PDGF receptor antibody, because the level of p190 is very low. Thus, to assess whether APS actually interacts with the PDGF receptor, the human PDGF receptor β chain and Myc tagged full length APS (Myc-APS) or APS SH2 domain (Myc-SH2) were transiently expressed in 293 cell, then the cells were stimulated with PDGF (Figure 6a). The tyrosine phosphorylated PDGF receptor β was co-immunoprecipitated with Myc-APS or Myc-SH2 only after stimulation, although the amount of the receptor co-precipitated with wild type APS was much less than that coprecipitated with SH2 domain. This suggests a rapid dissociation of full length APS from the PDGF receptor as in the case of *c-kit* (Yokouchi *et al.*, 1997). Full length APS (Myc-APS) but not SH2 was tyrosine phosphorylated by PDGF (lanes 10 and 12, α PY blot in Figure 6a). As shown in the case of *c-kit* (Yokouchi *et al.*, 1997), tyrosine phosphorylation also occurred at the C-

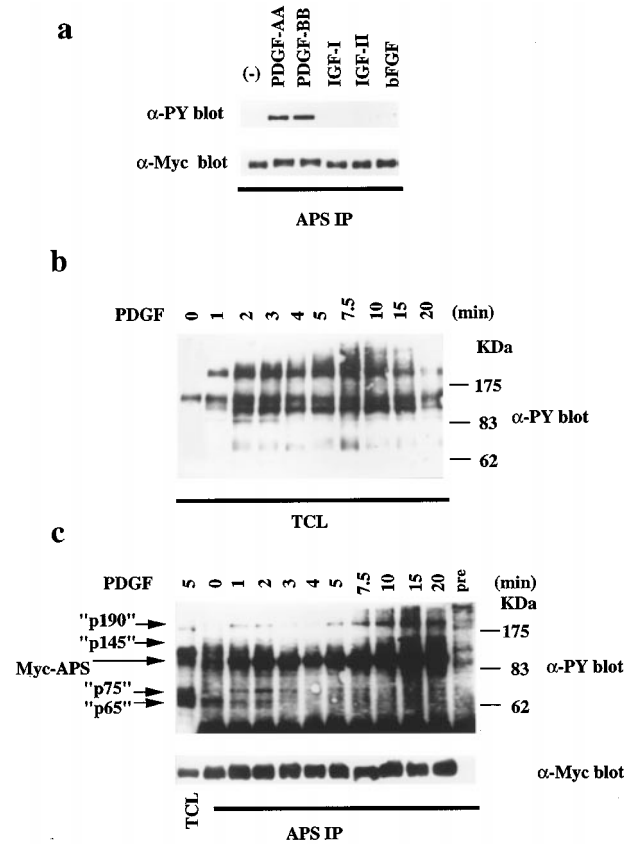


Figure 4 Tyrosine phosphorylation of APS and its associated proteins in 3T3 cells. After starvation, wild type APS transformants were stimulated with indicated growth factors (100 ng/ml) for 5 min (a), and with 100 ng/ml of PDGF for indicated periods (b, c). Then cells were lysed and immunoprecipitated with anti-APS. The total cell extracts (TCL) and the immunoprecipitates with anti-APS (APS IP) were resolved on an 8% SDS-PAGE gel, then immunoblotted with anti-phosphotyrosine (α -PY blot) or anti-Myc (α -Myc blot). Arrowheads indicate Myc-tagged APS (Myc-APS) and its associated proteins

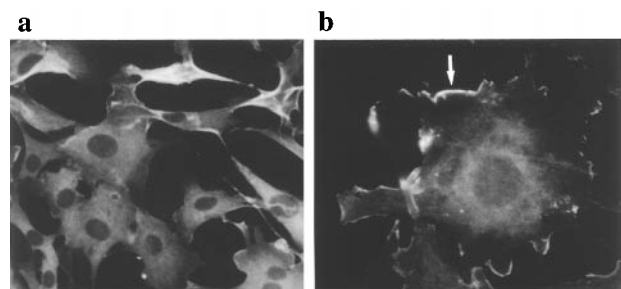


Figure 5 Cellular distribution of APS in 3T3 transformants. 3T3 cells stably expressing APS were plated on coverslips and incubated with anti-Myc antibody then subjected to immunofluorescence detection. After serum starvation for 12 h, cells were stimulated without (a) or with PDGF (100 ng/ml) for 5 min (b). Arrowhead indicates that APS was especially concentrated in the ruffling region at the periphery

terminal end, because C-terminal truncated APS was not phosphorylated in this assay (data not shown).

To clarify which tyrosine residue is recognized by APS-SH2 domain, we performed *in vitro* binding assay using various PDGF receptor tyrosine mutants

expressed in HepG2 cells (Valius *et al.*, 1993, 1995). Transformants expressed wild type PDGF receptor (wt), PDGF receptor lacking five major phosphorylation sites (F5; all of Y740, Y751, Y771, Y1009, and Y1021 were substituted for phenylalanine), and 'add-back' mutants to F5 containing Y740, and Y751, Y771, Y1009 or Y1021. After stimulation with PDGF, cell extracts were incubated with recombinant SH2 domain of APS fused to GST (Yokouchi *et al.*, 1997). As shown in Figure 6b, tyrosine phosphorylated wild type PDGF receptor as well as Y1021 mutant bound to the APS SH2 domain.

Since Y1021 has been shown to be the binding site for PLC γ , we compared tyrosine phosphorylation of PLC γ in parental NIH3T3 cells, APS transformants and Δ C transformants (Figure 7a). As expected, tyrosine phosphorylation of PLC γ in wild type APS transformants was reduced compared with parental cells or Δ C transformants. However, phosphorylation of p85 P13 kinase subunit and its associated proteins

(p120 and p190) differed little (Figure 7b). Thus, APS probably competes with PLC γ for the binding to the receptor. In addition, this result suggests that a part of the negative regulatory mechanism of APS against PDGF-induced mitogenic signaling results from this competitive reaction.

c-Cbl is a single phosphoprotein bound to C-terminal tyrosine residues of APS in vitro

To further clarify the molecular events in the negative effects of APS on PDGF signaling, we searched for the protein binding with APS using *in vitro* binding assay. Parental 3T3 cells were stimulated with or without PDGF for 5 min then cell extracts were incubated with phosphopeptide containing C-terminal tyrosine residue (RAVENQpYSFY). As a control, the EPO receptor phosphopeptide containing Y343 (GDTpYLVDL-KWL) was used. As shown in Figure 8a, c-Cbl and Grb2 interacted with phosphorylated APS C-terminal peptide but not with the EPO receptor phosphopeptide, while PLC γ bound to the EPO receptor phosphopeptide but not to the APS phosphopeptide. c-Cbl is a major tyrosine phosphorylated protein that binds to the APS phosphopeptide, suggesting that c-Cbl plays an important role in the regulatory function of APS.

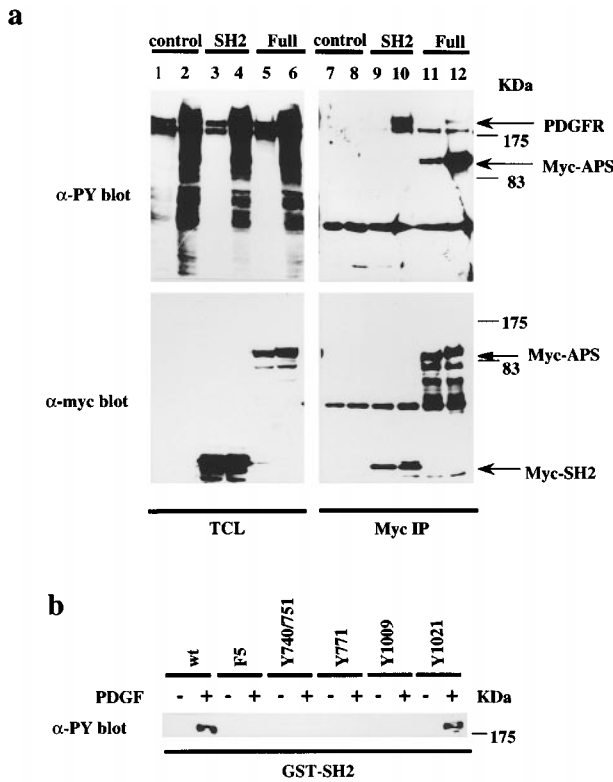


Figure 6 APS bound to pY1021 of the PDGF receptor. In (a), wild type PDGF receptor- β cDNA and plasmids carrying Myc-SH2 (SH2; lanes 3,4,9 and 10), Myc-APS (full; lanes 5,6,11 and 12), or empty vector (control; lanes 1,2,7 and 8) were co-transfected into 293 cells. After starvation, cells were stimulated without (lanes 1,3,5,7,9 and 11) or with PDGF (100 ng/ml) (lanes 2,4,6,8,10 and 12) for 5 min, then lysed and immunoprecipitated with anti-Myc. In (b), extracts of the PDGF-treated HepG2 cells (1×10^7 /sample) expressing the PDGF receptor with tyrosine mutations were incubated with 5 μ g of immobilized GST-SH2 at 4°C for 1 h. The total cell extracts (TCL), anti-Myc immunocomplex on agarose beads (a), or the complex with GST-SH2 (b) were resolved on a 8% SDS-PAGE gel, then immunoblotted with anti-pY (α -pY) or anti-Myc (α -Myc). In a, positions for PDGF receptor, Myc-APS and Myc-SH2 are given. In b, wt indicates wild type PDGF receptor, and F5 is the receptor containing substitution of tyrosines Y740, Y751, Y771, Y1009 and Y1021 by phenylalanines. Y740/751, Y771, Y1009 and Y1021 are 'add back' mutants changing phenylalanine to tyrosine at the indicated positions in F5

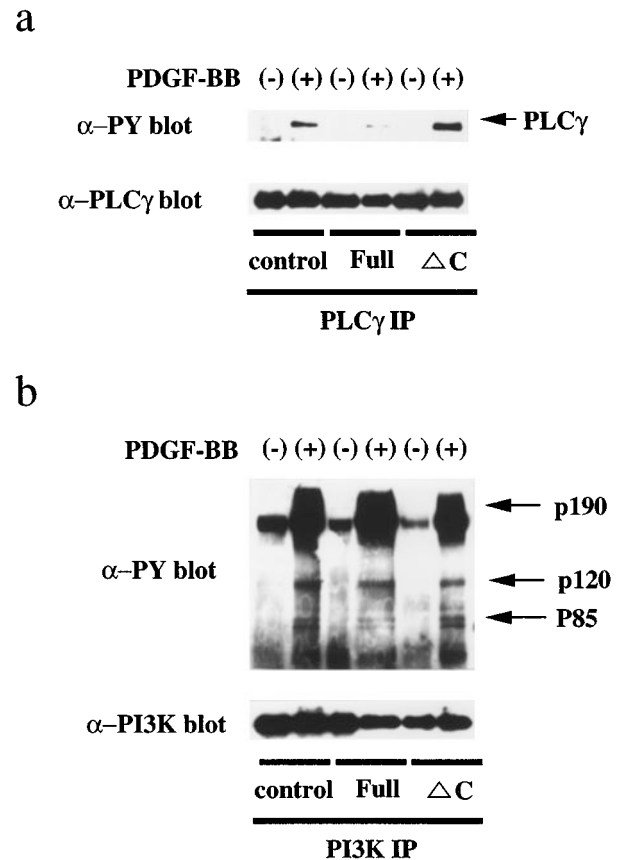


Figure 7 Tyrosine phosphorylation of PLC γ and PI3K in transformants. Parental or transformant cells were stimulated with 100 ng/ml of PDGF-BB at 37°C for 5 min, then lysed and immunoprecipitated with anti-PLC γ (a) or anti-PI3K (b). The immunocomplex on agarose beads was resolved on a 7% SDS-PAGE gel, then immunoblotted with anti-pY (α -pY), anti-PLC γ (α -PLC γ) (a), or anti-PI3K (α -PI3K) (b). Positions for PLC γ , p190, p120 and p85 are indicated

To confirm the association between APS and c-Cbl in cells, the PDGF receptor, HA tagged full length c-Cbl, and Myc tagged full length (Myc-APS) or C-terminally truncated APS (Myc- Δ C) were transiently expressed in 293 cell. After PDGF stimulation, immunoprecipitation with anti-Myc or anti-c-Cbl antibody was performed (Figure 8b). c-Cbl was co-immunoprecipitated with full length APS but not C-terminal truncated APS.

To elucidate the biological effect of APS and c-Cbl interaction, we measured PDGF-induced *c-fos* promoter activation in the presence of APS and/or c-Cbl using 293 cell transient expression assay system. 293 cells endogenously expressed c-Cbl (data not shown). As shown in Figure 9a, wild type APS (APS full) suppressed PDGF-induced *c-fos* promoter activation in a dose-dependent manner. C-terminal truncated APS

(APS Δ C) had little effect. Transient expression of c-Cbl alone also inhibited PDGF-induced *c-fos* activation (data not shown). When c-Cbl was co-expressed at a concentration where c-Cbl alone did not exhibit marked effect on *c-fos* activation, the inhibitory effect of APS was enhanced. No such synergistic effect between APS and c-Cbl was seen when c-Cbl was co-expressed with APS Δ C. These results raised the possibility that c-Cbl which interacts with the C-terminal region of APS is involved in the negative regulatory effect of APS against PDGF-induced mitogenic signaling.

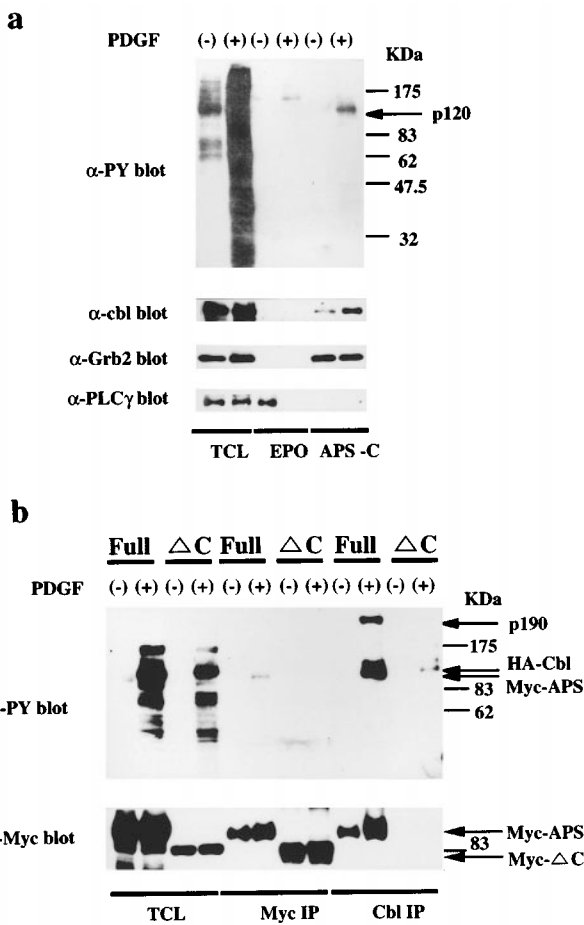


Figure 8 APS is associated with c-Cbl *in vitro* and *in vivo*. In (a), extracts from unstimulated (-) or PDGF (100 ng/ml) stimulated (+) 3T3 cells (1×10^7 /sample) were incubated with approximately 5 μ g of agarose conjugated with the EPO receptor phosphopeptide (EPO) or APS phosphopeptide (APS-C) at 4°C for 4 h, then precipitates were analysed by immunoblotting with anti-pY (α -pY), anti-Cbl (α -Cbl), anti-Grb2 (α -Grb2) and anti-PLC γ (α -PLC γ). The arrowhead indicates a p120 phosphoprotein bound to APS phosphopeptide. In (b), the PDGF receptor β subunit cDNA, HA-tagged c-Cbl cDNA and Myc-APS (Full) or Myc- Δ C (Δ C) were cotransfected into 293 cells. After 12 h starvation, cells were stimulated with PDGF (100 ng/ml) for 5 min, then lysed and immunoprecipitated with anti-Myc (α -Myc) or anti-c-Cbl (α -Cbl). The total cell lysates (TCL) or immune complexes adsorbed with protein A-Sepharose were immunoblotted with anti-pY (α -pY) or anti-Myc (α -Myc)

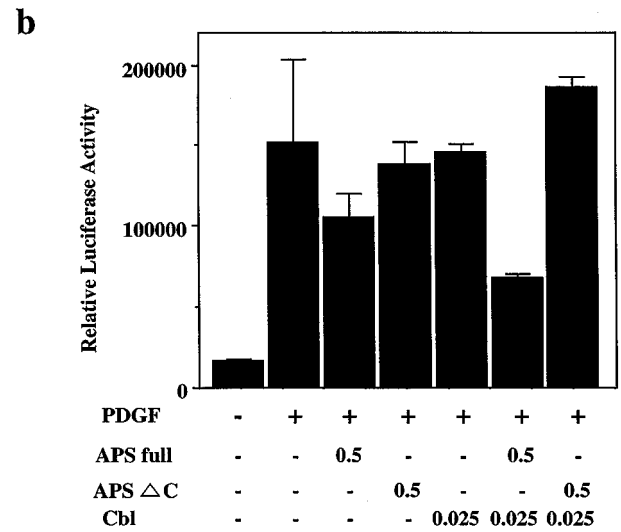
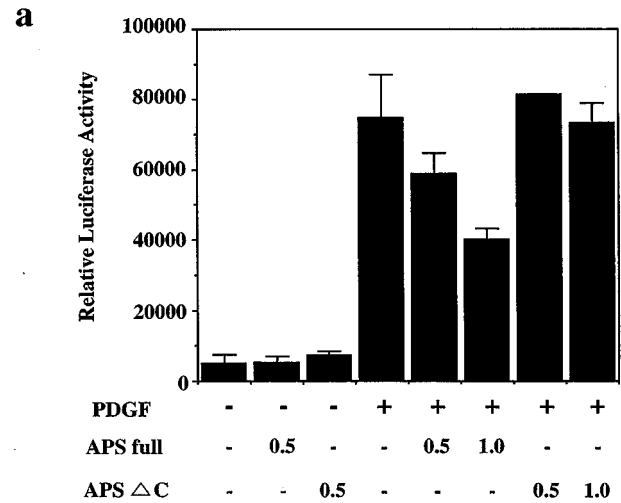


Figure 9 APS and c-Cbl synergistically inhibited PDGF-induced *c-fos* promoter activation. In (a), 293 cells were transfected with *c-fos* promoter-luciferase reporter constructs in combination with the PDGF receptor β cDNA and indicated amount of plasmids of full length APS (APS full) or C-terminal truncated APS (APS Δ C). In (b), 293 cells were transfected with the PDGF receptor β and reporter genes in combination with the indicated amount of c-Cbl (Cbl), APS full or APS Δ C. After serum starvation for 20 h, cells were stimulated with 50 ng/ml of PDGF for 12 h and luciferase activity was measured. Shown is the mean with s.d. of two independent determinations

Discussion

In this study, we found that APS is a substrate of PDGF receptor. Overexpression of full length APS in 3T3 cells resulted in a suppression of PDGF-induced DNA synthesis and cell growth. Since serum- or bFGF-dependent cell growth in transformants was almost equal to that in parental 3T3 cells (Figure 2 and data not shown), the inhibitory effect of APS may be specific to the PDGF signaling. Moreover, forced expression of APS resulted in suppression of PDGF-induced Erk2 activation, and *c-myc* expression. These results suggested that APS negatively regulates PDGF-mediated mitogenic signaling.

Negative regulatory function of APS against growth factor-induced mitogenesis has been suggested by the fact that APS was not expressed in most of the tumor cell lines, but highly expressed in a wide variety of normal tissues. Only the B-cell Burkitt lymphoma cell lines expressed APS among the hematopoietic cell lines we examined. We also examined 20 solid tumor cell lines including ones from esophagus, colon and lung carcinomas, and kidney tumors. We found that only Saos-2 expressed a high level of APS by Northern blotting, and very low level APS expression was seen in the other osteosarcoma cell lines by immunoblotting. Saos-2 has been shown to lack p53 and Rb (Rodan *et al.*, 1987; Masuda *et al.*, 1987), suggesting that this cell line is already extensively transformed and may not require any growth factor stimulation. Indeed, Saos-2 cells exhibited significant levels of DNA synthesis even after 24 h serum starvation (Yokouchi M unpublished data). Thus, high level of APS may not affect growth of Saos-2 cells. Alternatively, we found that tyrosine phosphorylation of APS was weaker in Saos-2 cells than in B cells stimulated with anti-IgM or in NIH3T3 transformants stimulated with PDGF. In Saos-2 cells, activation of APS may be suppressed by an unknown mechanism.

We identified tyrosine-1021 (Y1021) of the PDGF receptor where PLC γ binds as a major binding site for APS. Indeed, overexpression of APS partially inhibits tyrosine phosphorylation of PLC γ , probably because of competition for the same binding site. The amino acid sequence around Y1021 of the PDGF receptor is DNDYIPL. We found that APS also binds to the EPO receptor phosphopeptide containing pY343 (QDTYLVL) (Wakioka T, unpublished data), which PLC γ also binds to. Thus, the SH2 domain of APS may recognize two hydrophobic amino acids at positions +1 and +2 right after the phosphotyrosine residue. However, since very little information is available at present, further study is needed to find a consensus motif which APS recognizes. In PDGF receptor signaling, the association between receptor and PLC γ is partly required for PDGF-dependent DNA synthesis (Valius *et al.*, 1993; Claesson-Welsh, 1994; Alimandi *et al.*, 1997), therefore it has been postulated that PLC γ plays a pivotal role in PDGF-mediated mitogenic response. It is suggested that the suppression of PDGF-induced DNA synthesis caused by overexpression of APS is partly due to a lower level of activation of PLC γ .

Basic FGF marginally induced tyrosine-phosphorylation of APS in NIH3T3 cells (Figure 4a), and overexpression of APS had little effect on bFGF-

induced mitogenesis (YM; unpublished data). C-terminal truncated APS which lacks the tyrosine phosphorylation site also had no effect on PDGF-induced mitogenic signals (Figures 2 and 3). These results strongly suggest that tyrosine phosphorylation of the C-terminal region is important for suppression of PDGF-induced mitogenic signals. In our present and previous study, we demonstrated that c-Cbl binds to the tyrosine phosphorylated C-terminal region of APS but not to the unphosphorylated counterpart (Figure 7 and Yokouchi *et al.*, 1997). Since c-Cbl has shown to negatively regulate signals of tyrosine kinases, c-Cbl is an interesting candidate for mediating the negative effect of APS on PDGF signaling. A recent study revealed that D(N/D)XpY is a binding motif for the Cbl-PTB domain (Lupher *et al.*, 1997). The tyrosine phosphorylation site of APS contains ENQpY, whereas those of Lnk and of SH2B, both members of the APS family, also contain DNQpY and NNQpY respectively. Thus, the C-terminal tyrosine residue of APS family members seems to be a good candidate for Cbl-PTB domain binding sites. Furthermore, interestingly, tyrosine-1021 (Y1021) of the PDGF receptor also has D(N/D)XpY motif, DNDpY. Although it has not been demonstrated that c-Cbl binds to PDGF receptor β , pY1021 is a potential c-Cbl binding site. Since the receptor forms an oligomer after stimulation, binding to the same phosphotyrosine residue on the receptor may facilitate further interaction between APS and c-Cbl. Further study is underway to find the binding sites of c-Cbl on the receptor.

In *C. elegans*, a Cbl homologue, Sli-1 acts as a negative regulator of the Ras homologue, Let60 (Jongeward *et al.*, 1995; Yoon *et al.*, 1995). Also several recent studies have demonstrated that mammalian c-Cbl has a negative function in intracellular signal transduction. c-Cbl inhibits Syk tyrosine kinase activation in mast cell (Ota and Samelson, 1997) and T cell receptor-induced Erk2 and AP-1 activation (Rellahant *et al.*, 1997). Moreover, Cbl-b, a member of the Sli-1/c-Cbl protein family (Keane *et al.*, 1995), inhibits Vav-mediated *c-jun* kinase activation (Bustelo *et al.*, 1997). Although these mechanisms remain to be clarified, it is possible that negative regulatory function of APS was enhanced by association with phosphorylated c-Cbl. In addition, two recent studies showed that overexpression of c-Cbl in fibroblast lead to a decrease in EGF receptor phosphorylation without affecting MAP kinase activity (Ueno *et al.*, 1997; Bowtell and Langdon, 1995). 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 it has the PH domain which has been shown to interact with phospholipids. The physiological meaning of the negative regulatory function of APS will be clarified by creating transgenic or gene-disrupted mice.

Materials and methods

Cells

HepG2, 293 and SaOS-2 cell line were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10%

fetal calf serum (FCS). NIH3T3 cells were maintained in DMEM with 10% calf serum (CS).

Northern blot analysis

For Northern blotting, total RNA (5 µg) was separated on 1.0% agarose/2.4% formaldehyde gel, then transferred on to positively charged nylon membranes (Amersham). After fixation under calibrated UV irradiation, these membranes were hybridized with DIG-labeled riboprobes prepared using a DIG-RNA labeling kit (Boehringer). The blot was visualized using alkaline-phosphatase labeled anti-DIG antibody and a chemiluminescent substrate according to the manufacture's instructions. Wild type NIH3T3 cells or cells stably expressing APS were cultured for 12 h in DMEM with 0.3% FCS and then stimulated with an amount of PDGF-BB (100 ng/ml) for 15 min, 30 min, 1 h or 3 h. Northern blot analysis was performed as mentioned above.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously (Yokouchi *et al.*, 1997). In gel kinase assay for Erk2 (MAP kinase) using myelin basic protein (MBP) as substrate was performed as described (Goto *et al.*, 1990).

Generation of NIH3T3 cells stably expressing APS

Myc-tagged version of full length APS (APS Full; codon 2–632) or deletion mutant lacking the C-terminal region (APS ΔC; codon 2–519) cDNAs were created by PCR then subcloned into the pLXSN expression vector. These plasmids were introduced into NIH3T3 fibroblast cells by a calcium phosphate method and stable transformants were selected with 0.7 mg/ml G418. Expression of APS in transformants was detected by immunoblotting with anti-Myc or anti-APS. A similar level of expression was observed in APS Full transformants and ΔC transformants. Growth of transformants was measured by a colorimetric method as described using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium as a substrate. BrdU incorporation assay was performed by a colorimetric method using a cell proliferation ELISA kit (Boehringer Mannheim). In both experiments, before stimulation, cells were cultured for 24 h in DMEM with 0.3% FCS.

Immunofluorescence analysis

For immunofluorescence analysis, cells were seeded onto glass coverslips in 60 mm dishes. Cells grown to 50–70% confluency were washed three times with phosphate-buffered saline (PBS; 140 mM NaCl, 2.68 mM KCl, 10 mM Na₂HPO₄, 1.47 mM KH₂PO₄ [pH 7.4]), then fixed with 4% formaldehyde for 10 min at room temperature

and washed three times with PBS. Then cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature and washed three times with PBS. Cells were incubated with a 1:1000 dilution of anti-Myc antibody (9E10) in PBS with 10% goat serum for 1 h at room temperature. They were washed three times (15 min each) with PBS and incubated for 1 h with Texas red-conjugated goat anti-rabbit IgG antibodies (Cappel) at 1:100 dilution, or cy3-conjugated affinity purified secondary antibody (CHEMICON) at 1:100 dilution in PBS. Cells were washed three times in PBS for 15 min each and mounted on glass slides. Samples were analysed with an insight confocal laser scanning microscope.

In vitro binding assay

GST fusion protein of the APS SH2 domain (GST-SH2; codon 397–551) was freshly prepared for the *in vitro* binding experiments as described previously (Yokouchi *et al.*, 1997). APS or the EPO receptor phosphopeptide was synthesized and conjugated to Affigel-10 (BioRad) according to the manufacture's instructions. Before PDGF receptor stimulation, the cells were cultured for 12 h at 37°C in DMEM with 0.3% FCS. GSH-Sepharose beads (Pharmacia) carrying approximately 5 µg fusion protein or Affigel 10 (BioRad) carrying approximately 5 µg phosphopeptide were incubated in cell lysates at 4°C for 1 h or 4 h respectively, washed three times with 0.5% Triton X-100/PBS, then analysed by immunoblotting.

Luciferase assay

Luciferase gene construct containing the *c-fos* promoter and β-gal gene construct was described previously (Endo *et al.*, 1997). PDGF receptor β gene construct and HA-tagged c-Cbl gene construct were described previously (Satoch *et al.*, 1993; Ota and Samelson, 1997). These genes were co-transfected into 293 cell line grown in six well plates using a calcium-phosphate method. After transfection, the cells were serum depleted for 20 h, then stimulated with 50 ng/ml PDGF for 12 h. Cell extracts were prepared and luciferase and control β-galactosidase activity was measured as described previously (Matsumoto *et al.*, 1997).

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