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The adapter protein APS associates with the multifunctional docking sites Tyr-568 and Tyr-936 in c-Kit

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The adapter protein APS has previously been shown to be involved in recruiting the ubiquitin E3 ligase c-Cbl to the insulin receptor, the platelet-derived growth factor β -receptor and the erythropoietin receptor, leading to increased degradation of the receptors and inhibition of mitogenesis. Here we demonstrate, by use of immobilized synthetic phosphopeptides corresponding to various autophosphorylated tyrosine residues in the receptor for stem-cell factor (c-Kit), that APS preferentially associates with phosphorylated Tyr-568 and Tyr-936. Tyr-568 has previously been identified as the binding site of the Src family of tyrosine kinases, the Csk-homologous kinase CHK, and the protein

tyrosine phosphatase SHP-2. We have recently demonstrated that Tyr-936 is an autophosphorylation site involved in binding the adapter proteins Grb2 and Grb7. We could further demonstrate that the critical determinant for binding of APS is the presence of either a leucine or an isoleucine residue in the position +3 to the phosphorylated tyrosine. This allowed us to design mutants that selectively failed to associate with APS, while still associating with Src family members, SHP-2 and Grb2, respectively.

Key words: APS, c-Cbl, c-Kit, v-Kit, ubiquitination.

INTRODUCTION

The receptor for stem-cell factor (SCF), the proto-oncogene c-Kit, belongs to the type III family of receptor tyrosine kinases, which also includes the receptors for platelet-derived growth factor (PDGF) and the macrophage colony stimulating factor ('M-CSF') receptor. Mutation in c-Kit have suggested pleiotropic functions of the SCF/c-Kit pathway and include defects in melanogenesis, gametogenesis and haematopoiesis (for reviews, see [1,2]).

Binding of SCF to its receptors leads to receptor dimerization and activation of its intrinsic tyrosine kinase activity. Subsequently, the receptor becomes autophosphorylated on specific tyrosine residues, creating specific recognition motifs for intracellular signal transduction molecules that contain Src homology 2 (SH2) domains. These are conserved stretches of approx. 100 amino acids which bind specifically to phosphotyrosine residues and are found in a variety of intracellular signalling molecules. Thus the activated c-Kit is able to associate with Src family members and SHP-2 through Tyr-568, with phosphoinositide 3-kinase through Tyr-721, with the adapter protein Grb2 through Tyr-703 and Tyr-936, and with the adapter protein Grb7 through Tyr-936 [3–6]. Thus, in some cases, one particular tyrosine residue can serve as the binding site for several signal transduction molecules.

The adapter molecule containing a PH domain and an SH2 domain, APS, was cloned [7] using a yeast two-hybrid system with a constitutively active mutant of c-Kit, D816V, as bait. However, the binding site(s) of APS in c-Kit has so far not been determined. In response to activation of the PDGF β -receptor

[8], the insulin receptor [9], TrkA [10] and the erythropoietin receptor [11], APS associates with the receptors through its SH2 domain and becomes phosphorylated at a single major tyrosine phosphorylation site found at the C-terminus. This phosphorylation creates a binding site for Grb2 and c-Cbl. The physiological function of APS, however, remains to be clarified. By overexpressing wild-type APS in NIH3T3 cells, PDGF-mediated mitogenesis could be inhibited [8]. When overexpressing a mutant of APS lacking the c-Cbl-association site, no such inhibition of PDGF-mediated mitogenesis was seen. This suggests that c-Cbl recruitment to APS brings it in close proximity to the PDGF receptor, allowing it to be ubiquitinated through the action of the E3 ubiquitin ligase activity of c-Cbl [12]. In contrast, APS seems to have a positive role in both TrkA and insulin receptor signalling [10,13,14].

In this study, we identify the two binding sites for APS in c-Kit, Tyr-568 and Tyr-936. Furthermore, we show that the binding specificity of the SH2 domain of APS is determined by a leucine or isoleucine residue in the position +3 to the phosphorylated tyrosine. Mutants were constructed that selectively knock out binding of APS, while leaving the binding of other molecules to these tyrosine residues intact.

MATERIALS AND METHODS

Antibodies, antisera, plasmids and glutathione S-transferase (GST) fusion proteins

Recombinant human SCF was a gift from AMGEN (Thousand Oaks, CA, U.S.A.). The rabbit antiserum Kit-C1, recognizing

Abbreviations used: APS, adapter molecule containing a PH domain and an SH2 domain; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; PLC- γ 1, phospholipase C- γ 1; SCF, stem-cell factor; SH2, Src homology 2.

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the C-terminal tail of c-Kit, was purified as described previously [15]. The PY99 anti-phosphotyrosine antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and monoclonal 9E10 against the myc epitope tag was from Roche. The phospholipase C (PLC)- γ 1 antiserum was raised against a synthetic peptide corresponding to the C-terminus of bovine PLC- γ 1 [16]. The GST fusion proteins containing the SH2 domain of c-Src, SHP-2, Grb2 and Grb7 were gifts from Dr Tony Pawson, Dr Joseph Schlessinger and Dr Ben Margolis, respectively. Myc-tagged murine APS was prepared as described previously [7].

Site-directed mutagenesis

Mutations were introduced using QuikChange™ site-directed mutagenesis system (Stratagen, La Jolla, CA, U.S.A.) according to the manufacturer's instructions. In brief, two complementary oligonucleotides containing the mutation of interest were synthesized and used in the mutagenesis PCR reaction with the wild-type c-Kit cDNA as template. The amplification reaction mixture was then incubated with *Dpn*I to remove template DNA. The non-digested DNA was transformed into *Escherichia coli* strain DH5 α , from which the mutated DNA could be recovered. All mutations were verified by DNA sequencing.

The following oligonucleotides were used: Ile-571 to Ala, 5'-CAATTATGTTTACGCACAGCCAACACAAC-3' and 5'-GT-TGTGTTGGCTGTGCGTAAACATAATTG-3'; Leu-939 to Ala, 5'-GCTGCAGTTTGTGCGTTGGAGTAAATATG-3' and 5'-CATATTTACTCCAACGCAGCAAACACTGCAGC-3'.

Cell culture and transient transfection of COS-7 cells

COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. Transient transfections were performed using LIPOFECTAMINE PLUS reagent (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) according to the manufacturer's protocol. In brief, the day before transfection the cells were plated so that they reached 80% confluence by the day of transfection. The DNA was pre-complexed with the PLUS reagent and then mixed with the LIPOFECTAMINE reagent in serum-free medium. The mixture was then added to the cells. After 3 h, the serum level was increased to 10%. The cells were used for experiment 48 h later.

Synthetic peptides

Peptides were synthesized in an Applied Biosystems peptide synthesizer (model ABI430A) by Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry and phosphorylated according to [17], employing di-*t*-butyl-*N,N*-di-isopropylphosphoramidite, as described [18]. The peptides were purified by reversed-phase chromatography, and peak fractions were analysed by plasma desorption mass spectrometry using an Applied Biosystems Bio Ion 20 instrument. Peptide fractions were freeze-dried and stored under dry conditions. Peptide stock solutions of 1 mM were kept in 20 mM Hepes, pH 7.4, 1 mM dithiothreitol at -20°C . The following tyrosine-phosphorylated peptides were synthesized: CEEINGNNY(p)VYIDPTQ (pY568), CEEINGNNY(p)AYIDPTQ (pY568, A569), CEEINGNNY(p)VAIDPTQ (pY568, A570), CEEINGNNY(p)VYADPTQ (pY568, A571), STNHIY-(p)SNLANCS (pY936), STNHIY(p)ANLANCS (pY936, A937), STNHIY(p)SALANCS (pY936, A938), STNHIY(p)SNAANCS (pY936, A939), CYLQKPMY(p)EVQWKVV (pY553), CEEINGNNYVY(p)IDPTQL (pY570), CEEINGNNY(p)VY(p)IDPTQL (ppY568/570), AEAALY(p)KNLLHSC (pY703), CSDS-TNEY(p)MDMKPGVSY(p)VVPTKA (ppY721/730), CKPG-

VSY(p)VVPTKADK (pY730), CSVRIGSY(p)IERDVT (pY747), CKNDSNY(p)VVKGA (pY823), PNEGNDY(p)-IPLDPK (pY1021 PDGF β R), CGDTY(p)LVLDKWL (pY343 EpoR). The corresponding non-phosphorylated peptides (Y568, Y936 etc.) were also synthesized.

In vitro association of GST fusion proteins with synthetic peptides

Synthetic phosphopeptides were immobilized to SulfoLink beads (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions. Synthetic peptide (1 mg) was coupled with 1 ml of packed beads. Beads (25 μ l) were incubated end-over-end with 2 μ g of GST fusion protein in a total volume of 1 ml of lysis buffer [1% (v/v) Triton X-100, 10% (v/v) glycerol, 0.15 M NaCl, 20 mM Hepes, pH 7.4, 5 mM EDTA, 1 μ g/ml leupeptin, 1% Trasyolol and 1 mM PMSF] for 1 h at 4°C . After three washes with lysis buffer, bound proteins were separated by SDS/PAGE (10% gel), electrotransferred to Immobilon-P filters (Millipore, Bedford, MA, U.S.A.) and probed with a polyclonal antibody directed against GST.

In vitro association of APS and synthetic peptides

Synthetic peptides were covalently coupled to SulfoLink beads (Pierce) according to the manufacturer's instructions. COS-7 cells expressing myc-tagged APS were lysed in buffer containing 1% (v/v) Triton X-100, 20 mM Hepes, pH 7.4, 0.15 M NaCl, 5 mM EDTA, 1 mM Na_3VO_4 , 1% Trasyolol, 1 μ g/ml leupeptin, 1 mM PMSF. Peptides conjugated to matrix were added to the lysates and incubated end-over-end for 2 h. After washing three times with lysis buffer, the samples were subjected to SDS/PAGE and immunoblotted with monoclonal 9E10 (Roche) to detect myc-tagged APS.

Immunoprecipitation and Western blotting

Immunoprecipitations and Western blotting were performed essentially according to Blume-Jensen et al. [15]. Immobilon-P membranes (Millipore) were used in all experiments.

RESULTS

SCF stimulation leads to phosphorylation of APS and association of APS with c-Kit

COS-7 cells were transiently transfected with wild-type c-Kit and myc-tagged murine APS [7]. Cells were starved overnight and stimulated with 100 ng/ml SCF for 5 min at 37°C . After lysis and clarification of the lysate by centrifugation, lysates were subjected to immunoprecipitation with either Kit-C1 antibodies (immunoprecipitation of c-Kit) or myc-tag antibody (immunoprecipitation of APS). After extensive washing, immunoprecipitated proteins were resolved by SDS/PAGE (7% gel), transferred to Immobilon P membrane and probed with either anti-c-Kit antibody (Kit-C1), anti-phosphotyrosine antibodies (PY99) or myc-tagged antibody (APS; Figure 1). Ligand-induced phosphorylation of c-Kit as well as of APS was seen. Furthermore, c-Kit could be co-immunoprecipitated with APS, indicating a physical interaction.

Phosphorylated Tyr-568 and Tyr-936 in c-Kit constitute high-affinity-binding sites for APS

A panel of phosphopeptides corresponding to all known tyrosine phosphorylation sites in c-Kit were synthesized (Tyr-553, Tyr-568, Tyr-570, Tyr-703, Tyr-721, Tyr-730, Tyr-747, Tyr-823, and Tyr-936). Tyr-1021 of the PDGF β -receptor and Tyr-343 of the

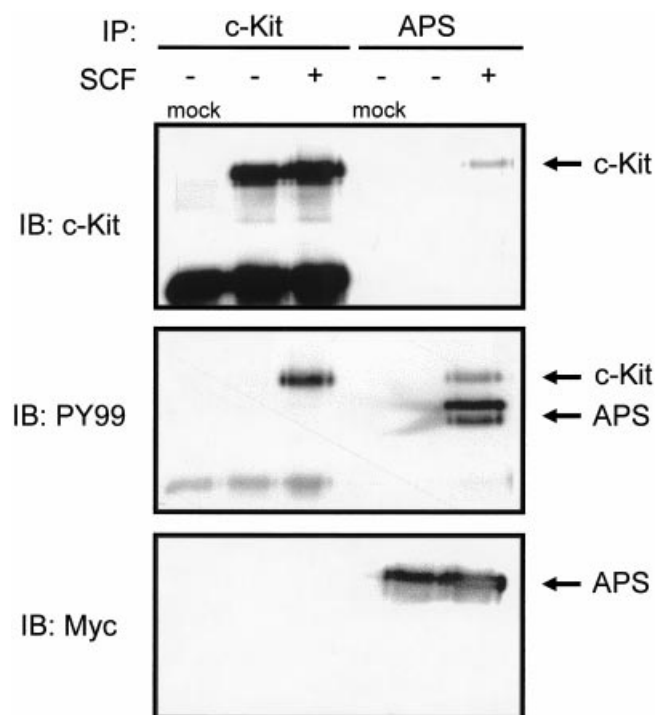


Figure 1 APS becomes tyrosine phosphorylated and forms a complex with c-Kit upon ligand stimulation

COS-7 cells were transiently transfected with wild-type human c-Kit and with a myc-tagged version of murine APS. Following overnight starvation, cells were stimulated (+) or not stimulated (–) with 100 ng/ml SCF for 5 min at 37 °C, lysed and subjected to immunoprecipitation with either Kit-C1 antibodies (c-Kit) or the 9E10 anti-myc monoclonal (Myc). Immunoprecipitated proteins were separated on a SDS/PAGE (7% gel), electrotransferred to Immobilon-P and probed for expression of c-Kit, with anti-phosphotyrosine antibody (PY99) or with the 9E10 antibody (Myc). IP, immunoprecipitation; IB, immunoblot.

erythropoietin receptor, which had been demonstrated previously to bind APS [8,11], were chosen as positive controls. Peptides were covalently coupled to Sulfolink beads through an N-terminally added cysteine residue. COS-7 cells were transiently transfected with myc-tagged murine APS, cell lysates were prepared and incubated with the immobilized peptides. The beads were extensively washed, and bound material was separated by SDS/PAGE (7% gel). Separated proteins were electrotransferred to Immobilon-P membrane and probed with anti-myc antibody for APS.

It could be seen that APS bound to immobilized Tyr-334 peptide of the erythropoietin receptor in a phosphorylation-dependent manner. Surprisingly, the second positive control, Tyr-1021 of the PDGF β -receptor, did not associate with APS. As a control, the filter was stripped and probed with an antibody against PLC- γ 1, which is known to associate with Tyr-1021 [19]. As can be seen in Figure 2, PLC- γ 1 bound in a phosphorylation-dependent manner to the Tyr-1021 phosphopeptide. The Tyr-568 and the Tyr-936 peptides of c-Kit both associated with APS. When comparing the sequences surrounding Tyr-568 and Tyr-936 in c-Kit with that of Tyr-334 of the erythropoietin receptor, it becomes clear that these peptides all contain either a leucine or isoleucine residue in position +3 (Tyr-568, YVYIDPT; Tyr-936, YSNLANC; Tyr-334, YLVLDKW). It was also evident that Tyr-1021 of the PDGF β -receptor does not have a leucine or isoleucine residue in position +3 (YIIPLPD). However, it is known that APS binds to the PDGF β -receptor [8]. When

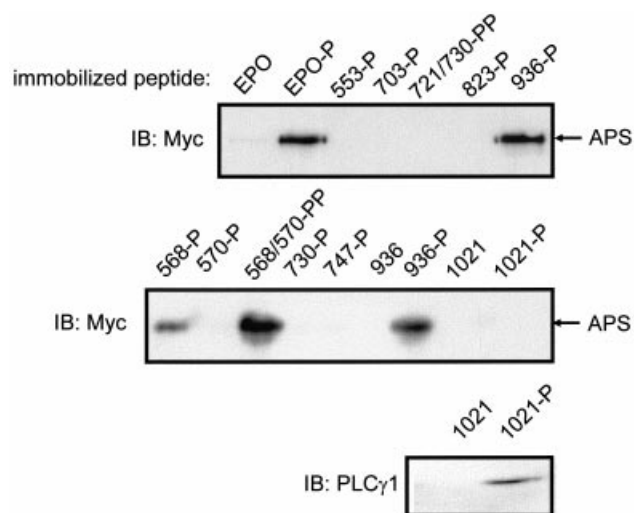


Figure 2 APS binds to synthetic phosphopeptides corresponding to Tyr-568 and Tyr-936 in c-Kit

COS-7 cells were transiently transfected with wild-type human c-Kit and with a myc-tagged version of murine APS. Cell lysates were prepared and allowed to incubate with synthetic phosphopeptides (as indicated above each lane in the Figure) immobilized to Sulfolink beads. After washing, bound material was separated by SDS/PAGE, electrotransferred to Immobilon-P and probed with 9E10 (Myc) for APS detection. As a control, the filter was stripped and probed with an antibody against PLC- γ 1 (PLC γ 1). IB, immunoblot.

scrutinizing the PDGF β -receptor for tyrosine residues that are followed by a leucine or isoleucine residue in position +3, we found that Tyr-763 fulfils this requirement (YADIESS). Tyr-763 was recently identified as an autophosphorylation site and a binding site for the protein tyrosine phosphatase SHP-2 in the PDGF β -receptor [20].

To determine the specificity in binding of Tyr-568, Tyr-763, Tyr-936 and Tyr-1021 to APS, lysates from COS-7 cells transiently transfected with myc-tagged APS were incubated with immobilized phosphorylated or non-phosphorylated peptide Tyr-568, Tyr-763, Tyr-936 and Tyr-1021 (Figure 3). It could be demonstrated that none of the non-phosphorylated peptides were able to compete for binding to the SH2 domain of APS, while Tyr-568, Tyr-763 and Tyr-936 phosphopeptides competed binding efficiently. As expected, neither the non-phosphorylated nor the

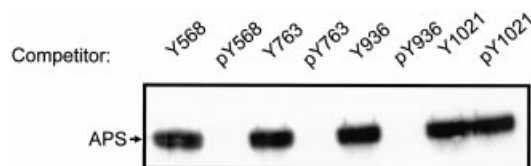


Figure 3 Binding of APS to Tyr-936 can be competed with phosphopeptides corresponding to Tyr-568 and Tyr-936 in c-Kit, and with Tyr-763 in the PDGF β -receptor

COS-7 cells were transiently transfected with a myc-tagged version of murine APS. Cell lysates were prepared and allowed to incubate with synthetic phosphopeptides immobilized to Sulfolink beads in the presence of phosphorylated or non-phosphorylated synthetic peptides (as indicated above each lane) in solution. After washing, bound material was separated by SDS/PAGE, electrotransferred to Immobilon-P and probed with 9E10 for APS detection.

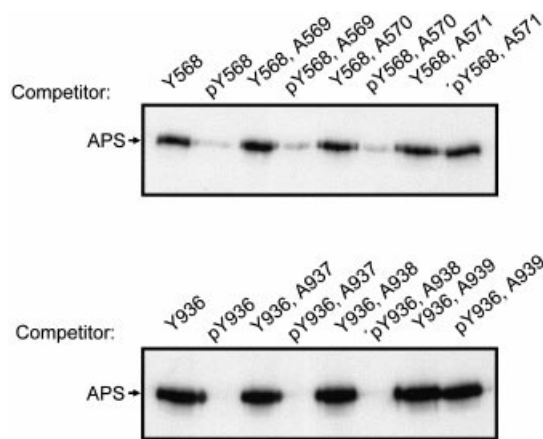


Figure 4 Binding of Tyr-568 and Tyr-936 to APS is dependent on a isoleucine or leucine residue, respectively, in position +3

COS-7 cells were transiently transfected with a myc-tagged version of murine APS. Cell lysates were prepared and allowed to incubate with synthetic phosphopeptides (as indicated above each lane) immobilized to Sulfolink beads. After washing, bound material was separated by SDS/PAGE, electrotransferred to Immobilon-P and probed with 9E10 for APS detection.

phosphorylated Tyr-1021 peptide had any effect on binding of APS to Tyr-936.

Binding of APS to phosphorylated tyrosine residues is determined by a leucine or isoleucine residue in position +3

In order to determine the amino acid sequence requirement for association to APS, a series of synthetic phosphopeptides were generated where individual amino acids were replaced with alanine residues [CEEINGNNY(p)VYIDPTQ (pY568); CEEINGNNY(p)AYIDPTQ (pY568, A569); CEEINGNNY(p)VAIDPTQ (pY568, A570); CEEINGNNY(p)VYADPTQ (pY568, A571); STNHIIY(p)SNLANCS (pY936); STNHIIY(p)ANLANCS (pY936, A937); STNHIIY(p)SALANCS (pY936, A938); STNHIIY(p)SNAANCS (pY936, A939)]. Phosphorylated Tyr-568 or Tyr-936 peptides were coupled to SulfoLink, and the beads were incubated with lysates from COS-7 cells transiently transfected with myc-tagged APS in the presence of the indicated phosphopeptide or reference peptide. It could be shown that binding of APS could be competed by all phosphopeptides (Figure 4), except when a leucine or isoleucine residue in position +3 was replaced with an alanine residue (pY568, A571) and (pY568, A939), respectively.

To test whether the same amino acid substitution had any effect on the association of Src and SHP-2 to Tyr-568 [3,21] or of Grb2 and Grb7 to Tyr-936 [6], immobilized phosphopeptides were incubated with GST fusion proteins of the Src SH2 domain, the SHP-2 SH2 domains, the Grb2 SH2 domain or the Grb7 SH2 domain. It could be demonstrated that the pY568, A571 peptide, which failed to associate with APS, bound as efficiently as pY568 peptide to the SH2 domains of Src and SHP-2 (Figure 5). Likewise, the pY936, A939 peptide that failed to associate with APS, bound as efficiently as the pY936 to the SH2 domains of Grb2 and Grb7, respectively (Figure 6). As expected, the pY936, A938 peptide, affecting an asparagine residue in position +2 which is a prerequisite for binding of Grb2 to tyrosine residues, failed to associate with the SH2 domain of Grb2.

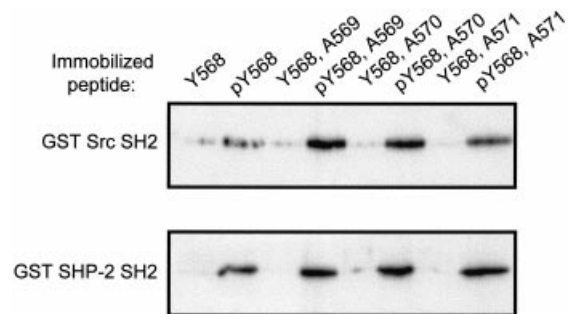


Figure 5 Association of the SH2 domains of Src or SHP2 to Tyr-568 is not dependent on an isoleucine residue in position +3

GST fusion proteins (GST Src SH2 or GST SHP-2 SH2) were incubated with synthetic peptides (as indicated above each lane) immobilized to Sulfolink beads. The beads were washed, and bound material was separated by SDS/PAGE, followed by electrotransfer to Immobilon-P. The filter was probed with a polyclonal antiserum against GST.

The I571A and L939A mutants of c-Kit fail to associate with APS

The information from the peptide-association experiments allowed us to design mutants of c-kit that would selectively knock out binding of APS without affecting association of other molecules to Tyr-568 and Tyr-936, respectively. The I571A and L939A mutants were generated singly or in combination. COS-7 cells were transiently transfected with either wild-type c-Kit or the I571A mutant, the L939A mutant or the I571A/L939A double mutant, together with myc-tagged APS. Cells were starved overnight and stimulated with 100 ng/ml SCF for 5 min at 37 °C. After lysis, APS was immunoprecipitated with myc antibodies. Following separation by SDS/PAGE (7% gel), proteins were

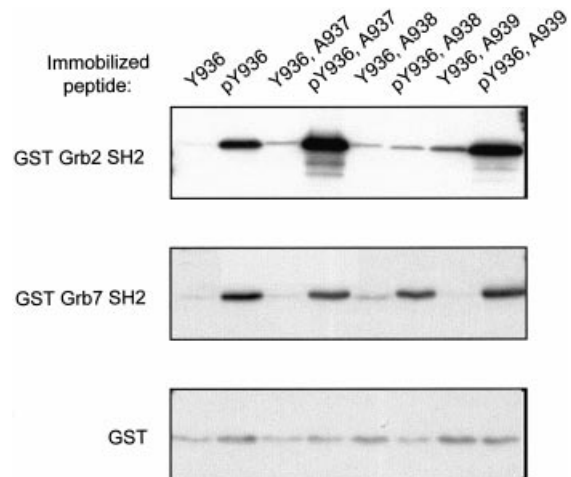


Figure 6 Association of the SH2 domains of Grb2 or Grb7 to Tyr-936 is not dependent on a leucine residue in position +3

GST fusion proteins (GST Grb2 SH2 or GST Grb7 SH2) were incubated with synthetic peptides immobilized to Sulfolink beads. The beads were washed, and bound material was separated by SDS/PAGE followed by electrotransfer to Immobilon-P. The filter was probed with a polyclonal antiserum against GST.

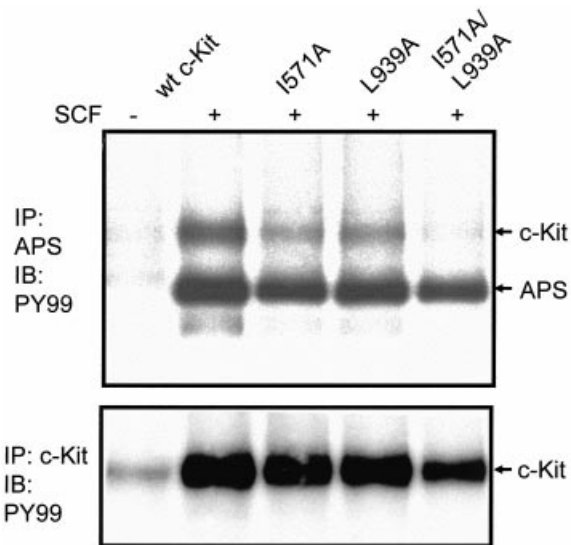


Figure 7 SCF-dependent association of APS with c-Kit is lost in the I571A/L939A mutant

COS-7 cells were transiently transfected with wild-type c-Kit (wt c-Kit), I571A mutant c-Kit (I571A), L939A mutant c-Kit (L939A) or the double mutant I571A/L939A c-Kit (I571A/L939A). After starvation overnight, cells were stimulated with 100 ng/ml SCF for 5 min at 37 °C. After lysis samples were subjected to immunoprecipitation with the 9E10 monoclonal or anti-Kit-C1, followed by SDS/PAGE and transfer to Immobilon P membrane. Filters were probed with anti-phosphotyrosine antibodies (PY99). IP, immobilized peptide; IB, immunoblot.

electrotransferred to Immobilon-P membrane and probed with anti-phosphotyrosine antibodies (PY99; Figure 7). It could be seen that SCF stimulation led to tyrosine phosphorylation of APS as well as to physical complex formation between c-Kit and APS. The I571A and the L939A mutants each associated more weakly than wild-type receptors, while tyrosine phosphorylation of APS remained unaffected. The double mutant I571A/L939A completely failed to associate with APS. To verify equal loading in each lane, filters were stripped and reprobed with 9E10 monoclonal to detect APS, or anti-Kit-C1 to detect c-Kit (results not shown).

DISCUSSION

In this study we demonstrated the presence of two association sites for APS in c-Kit, Tyr-568 and Tyr-936. These two tyrosine residues have previously been identified as autophosphorylation sites. Tyr-568 has been demonstrated to associate with the Src family of tyrosine kinases, the Csk-homologous kinase CHK and with the protein tyrosine phosphatase SHP-2 [3,21,22]. Tyr-936 has recently been demonstrated to constitute a docking site for the adapter proteins Grb2 and Grb7 [6]. Thus, both Tyr-568 and Tyr-936 constitute multifunctional docking sites for signal transduction molecules.

APS is an adapter protein containing a PH domain and an SH2 domain. It was originally cloned using a yeast two-hybrid system with the constitutively active oncogenic mutant D816V of c-Kit as bait. APS forms a new subfamily of SH2 protein with Lnk and SH2-B. Targeted deletion of Lnk leads to enhanced haematopoiesis of haematopoietic progenitor cells [23], underscoring its function as a negative regulator of haematopoiesis. SH2B has been shown to be of importance for both female and

male reproduction [24]. Stimulation of the B-cell receptor, c-Kit [7], the PDGF β -receptor [8], the Epo receptor [11], TrkA [10] or the insulin receptor [9] leads to tyrosine phosphorylation of APS and its association with the receptors. Tyrosine phosphorylation of APS occurs on a single major tyrosine-phosphorylation site found in its C-terminus. This creates a binding site for Grb2 and c-Cbl. Overexpression of full-length APS has been shown to inhibit PDGF-induced mitogenicity [8], while overexpression of a mutant APS lacking the c-Cbl association site failed to inhibit PDGF-induced mitogenicity. Since the sequence surrounding the C-terminal tyrosine of APS is not a consensus site for binding of Grb2 (YXN), it is likely that association with Grb2 might occur indirectly through binding to c-Cbl. Grb2 has been demonstrated to associate to c-Cbl through one of its SH3 domains [25].

While APS has been shown to promote a positive signalling response, leading to enhanced mitogenicity in the case of the insulin receptor and TrkA, in the case of the type III receptors, namely the PDGF β -receptor and c-Kit, it transduces a negative signal leading to degradation of the receptors. The reason for this difference in the mode that the receptors use APS to signal remains obscure. In the case of the insulin receptor, a positive effect of APS on insulin-receptor signalling was demonstrated in transfected CHO cells in terms of receptor, as well as downstream extracellular-signal-regulated kinase (ERK) phosphorylation [14]. In contrast, in 3T3-L1 adipocytes, expression of APS does not seem to have an effect on insulin-receptor stability or ERK signalling, while positively affecting Glut4 activation [13]. Also, in the case of TrkA (the receptor for nerve growth factor), Qian and Ginty [26] demonstrated a positive effect of APS on signalling. In contrast, overexpression of APS led to decreased mitogenicity and increased degradation of the PDGF β -receptor [8].

A possible explanation for the striking difference in APS-mediated signalling between the different receptor tyrosine kinases, could lie in the mode of interaction with the respective receptor. In the case of the insulin receptor and TrkA, where APS mediates a positive response, APS interacts with phosphorylated tyrosine residues in the activation loop of the tyrosine kinase domain. Ahmed et al. [9] demonstrated that phosphorylation of Tyr-1158 and Tyr-1162 in the activation loop of the insulin receptor is necessary for interaction with APS; furthermore, the activation loop of TrkA was identified as the site of interaction with APS [10]. The amino acid sequence surrounding the activation-loop tyrosine residues are quite similar, but quite different from that of both the erythropoietin receptor, the PDGF receptor and c-Kit. The sequence surrounding the activation-loop tyrosine residues is YETDYY-RKGG in the case of the human insulin receptor, while in TrkA the corresponding sequence is YSTDYYRVGG. Thus, in contrast with the tyrosine residues responsible for association with APS in the PDGF β -receptor, c-Kit and the erythropoietin, these tyrosine residues are not followed by either leucine or isoleucine residues in position +3.

c-Cbl is an adapter protein involved in negative regulation of growth-factor signalling [27]. It was later shown that c-Cbl is an E3 ubiquitin ligase, conjugating ubiquitin moieties to growth-factor receptors, targeting them for degradation in the lysosomes [12,28]. Thus, loss of the Cbl association site in APS would likely lead to decreased degradation of the receptor. Alternatively, loss of association of APS to a growth-factor receptor would likely lead to decreased recruitment of c-Cbl and reduced degradation of the receptor. The viral form of Kit, v-Kit, has two deletions that are of importance for its transforming ability [29]: a deletion in the juxtamembrane region of Tyr-568 and Val569, and a deletion of 50 amino acids in the C-terminus of c-Kit. The

juxtamembrane deletion leads to a loss of an isoleucine residue in position +3, and thus a loss of APS binding. The 50-amino-acid deletion in the C-terminus leads to loss of Tyr-936. In other words, APS is no longer able to physically associate with v-Kit. It is likely that the decreased recruitment of c-Cbl to the receptor that these deletions will cause, will increase the time span within which Kit will signal and potentially drive the proliferation of a tumour.

Future studies will aim at elucidating the functional role of APS in v-Kit induced transformation.

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REFERENCES

- Lev, S., Blechman, J. M., Givol, D. and Yarden, Y. (1994) Steel factor and c-kit protooncogene: genetic lessons in signal transduction. *Crit. Rev. Oncog.* **5**, 141–168
- Galli, S. J., Zsebo, K. M. and Geissler, E. N. (1994) The kit ligand, stem cell factor. *Adv. Immunol.* **55**, 1–96
- Linnekin, D., DeBerry, C. S. and Mou, S. (1997) Lyn associates with the juxtamembrane region of c-Kit and is activated by stem cell factor in hematopoietic cell lines and normal progenitor cells. *J. Biol. Chem.* **272**, 27450–27455
- Lennartsson, J., Blume-Jensen, P., Hermanson, M., Pontén, E., Carlberg, M. and Rönnstrand, L. (1999) Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. *Oncogene* **18**, 5546–5553
- Serve, H., Hsu, Y. C. and Besmer, P. (1994) Tyrosine residue 719 of the c-kit receptor is essential for binding of the P85 subunit of phosphatidylinositol (PI) 3-kinase and for c-kit-associated PI 3-kinase activity in COS-1 cells. *J. Biol. Chem.* **269**, 6026–6030
- Thömmes, K., Lennartsson, J., Carlberg, M. and Rönnstrand, L. (1999) Identification of Tyr-703 and Tyr-936 as the primary association sites for Grb2 and Grb7 in the c-Kit/stem cell factor receptor. *Biochem. J.* **341**, 211–216
- Yokouchi, M., Suzuki, R., Masuhara, M., Komiya, S., Inoue, A. and Yoshimura, A. (1997) Cloning and characterization of APS, an adaptor molecule containing PH and SH2 domains that is tyrosine phosphorylated upon B-cell receptor stimulation. *Oncogene* **15**, 7–15
- Yokouchi, M., Wakioka, T., Sakamoto, H., Yasukawa, H., Ohtsuka, S., Sasaki, A., Ohtsubo, M., Valius, M., Inoue, A., Komiya, S. and Yoshimura, A. (1999) APS, an adaptor protein containing PH and SH2 domains, is associated with the PDGF receptor and c-Cbl and inhibits PDGF-induced mitogenesis. *Oncogene* **18**, 759–767
- Ahmed, Z., Smith, B. J., Kotani, K., Wilden, P. and Pillay, T. S. (1999) APS, an adapter protein with a PH and SH2 domain, is a substrate for the insulin receptor kinase. *Biochem. J.* **341**, 665–668
- Qian, X., Riccio, A., Zhang, Y. and Ginty, D. D. (1998) Identification and characterization of novel substrates of Trk receptors in developing neurons. *Neuron* **21**, 1017–1029
- Wakioka, T., Sasaki, A., Mitsui, K., Yokouchi, M., Inoue, A., Komiya, S. and Yoshimura, A. (1999) APS, an adaptor protein containing Pleckstrin homology (PH) and Src homology-2 (SH2) domains inhibits the JAK-STAT pathway in collaboration with c-Cbl. *Leukemia* **13**, 760–767
- Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T. and Liu, Y. C. (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science (Washington, D.C.)* **286**, 309–312
- Liu, J., Kimura, A., Baumann, C. A. and Saltiel, A. R. (2002) APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. *Mol. Cell. Biol.* **22**, 3599–3609
- Ahmed, Z. and Pillay, T. S. (2001) Functional effects of APS and SH2-B on insulin receptor signalling. *Biochem. Soc. Trans.* **29**, 529–534
- Blume-Jensen, P., Siegbahn, A., Stabel, S., Heldin, C. H. and Rönnstrand, L. (1993) Increased Kit/SCF receptor induced mitogenicity but abolished cell motility after inhibition of protein kinase C. *EMBO J.* **12**, 4199–4209
- Arteaga, C. L., Johnson, M. D., Todderud, G., Coffey, R. J., Carpenter, G. and Page, D. L. (1991) Elevated content of the tyrosine kinase substrate phospholipase C γ 1 in primary human breast carcinomas. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10435–10439
- Andrews, D. M., Kitchin, J. and Seale, P. W. (1991) Solid-phase synthesis of a range of O-phosphorylated peptides by post-assembly phosphitylation and oxidation. *Int. J. Pept. Protein Res.* **38**, 469–475
- Mori, S., Rönnstrand, L., Yokote, K., Engström, A., Courtneidge, S. A., Claesson-Welsh, L. and Heldin, C. H. (1993) Identification of two juxtamembrane autophosphorylation sites in the PDGF beta-receptor; involvement in the interaction with Src family tyrosine kinases. *EMBO J.* **12**, 2257–2264
- Rönnstrand, L., Mori, S., Arvidsson, A. K., Eriksson, A., Wernstedt, C., Hellman, U., Claesson-Welsh, L. and Heldin, C. H. (1992) Identification of two C-terminal autophosphorylation sites in the PDGF beta-receptor: involvement in the interaction with phospholipase C-gamma. *EMBO J.* **11**, 3911–3919
- Rönnstrand, L., Arvidsson, A. K., Kallin, A., Rorsman, A., Hellman, U., Engström, U., Wernstedt, C. and Heldin, C. H. (1999) SHP-2 binds to Tyr763 and Tyr1009 in the PDGF beta-receptor and mediates PDGF-induced activation of the Ras/MAP kinase pathway and chemotaxis. *Oncogene* **18**, 3696–3702
- Kozlowski, M., Larose, L., Lee, F., Le, D. M., Rottapel, R. and Siminovich, K. A. (1998) SHP-1 binds and negatively modulates the c-Kit receptor by interaction with tyrosine 569 in the c-Kit juxtamembrane domain. *Mol. Cell. Biol.* **18**, 2089–2099
- Price, D. J., Rivnay, B., Fu, Y., Jiang, S., Avraham, S. and Avraham, H. (1997) Direct association of Csk homologous kinase (CHK) with the diphosphorylated site Tyr568/570 of the activated c-KIT in megakaryocytes. *J. Biol. Chem.* **272**, 5915–5920
- Takaki, S., Morita, H., Tezuka, Y. and Takatsu, K. (2002) Enhanced hematopoiesis by hematopoietic progenitor cells lacking intracellular adaptor protein, Lnk. *J. Exp. Med.* **195**, 151–160
- Ohtsuka, S., Takaki, S., Iseki, M., Miyoshi, K., Nakagata, N., Kataoka, Y., Yoshida, N., Takatsu, K. and Yoshimura, A. (2002) SH2-B is required for both male and female reproduction. *Mol. Cell. Biol.* **22**, 3066–3077
- Odai, H., Sasaki, K., Iwamatsu, A., Hanazono, Y., Tanaka, T., Mitani, K., Yazaki, Y. and Hirai, H. (1995) The proto-oncogene product c-Cbl becomes tyrosine phosphorylated by stimulation with GM-CSF or Epo and constitutively binds to the SH3 domain of Grb2/Ash in human hematopoietic cells. *J. Biol. Chem.* **270**, 10800–10805
- Qian, X. and Ginty, D. D. (2001) SH2-B and APS are multimeric adapters that augment TrkA signaling. *Mol. Cell Biol.* **21**, 1613–1620
- Miyake, S., Lupher, M. L., Jr, Druker, B. and Band, H. (1998) The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor α . *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7927–7932
- Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A. et al. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell* **4**, 1029–1040
- Herbst, R., Munemitsu, S. and Ullrich, A. (1995) Oncogenic activation of v-kit involves deletion of a putative tyrosine-substrate interaction site. *Oncogene* **10**, 369–377

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