# Src-like adaptor protein (Slap) is a negative regulator of mitogenesis

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**The Src-like adaptor protein (Slap) is a recently identified adaptor protein containing Src homology 3 (SH3) and SH2 domains. Slap is found in a wide range of cell types and was shown to interact with the Eck receptor tyrosine kinase in a yeast two-hybrid interaction screen [1]. Here, we found that Slap is expressed in NIH3T3 cells and could associate with the activated platelet-derived growth factor (PDGF) receptor. Using mutated versions of the PDGF receptor and phosphopeptide competition experiments, we determined that Slap has the highest affinity for the Src-binding site of the PDGF receptor. Our inability to produce cell lines that stably expressed Slap suggested that Slap inhibited cell growth. We further investigated this issue by transiently expressing Slap by microinjection. Overexpression of Slap by this method inhibited DNA synthesis induced by PDGF and serum, whereas overexpression of the adaptor proteins Grb2 and Shc did not. Finally, microinjection of a Slap antibody into NIH3T3 cells that had been stimulated with suboptimal doses of growth factors potentiated the effects of the growth factors. These data suggest that, unlike other adaptor proteins, Slap is a negative regulator of signalling initiated by growth factors.**

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Received: **13 February 1998**
Revised: **22 June 1998**
Accepted: **20 July 1998**

Published: **17 August 1998**

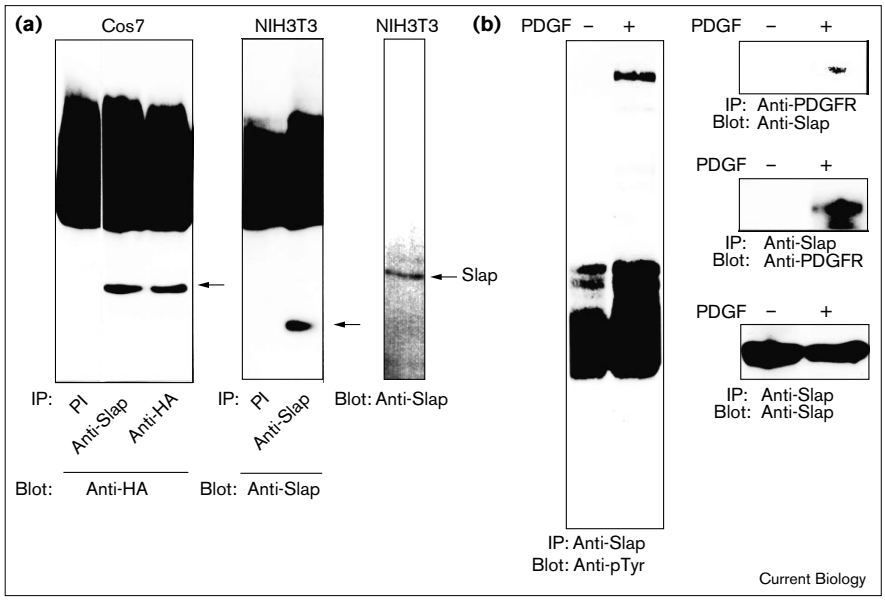
**Current Biology** 1998, 8:975–978
http://biomednet.com/elecref/0960982200800975



## Results and discussion

We first asked whether Slap is expressed in fibroblasts and whether it associates with the activated PDGF receptor. A polyclonal antibody raised against the carboxyl terminus of Slap recognised Slap in Cos7 cells that had been transfected with an expression vector encoding a haemagglutinin (HA) epitope-tagged version of Slap (Figure 1a). In addition, this antibody specifically immunoprecipitated a protein of the same size (34 kDa) from lysates of NIH3T3 fibroblasts, and the antibody also only recognised a single

**Figure 1**




Slap associates with the PDGF receptor. **(a)** Characterisation of the anti-Slap antibody. Slap was immunoprecipitated (IP) from Cos7 cells expressing HA–Slap, or NIH3T3 cells, using preimmune serum (PI), anti-HA antibody, or anti-Slap antibody as indicated, and the immunoprecipitates were analysed by SDS–PAGE and western blotting. The blots were then probed with anti-HA or anti-Slap antibody as shown. The ability of the anti-Slap antibody to detect Slap in whole NIH3T3 cell lysates was also tested (right). Arrows mark the position of the Slap protein. **(b)** Association of Slap with the activated PDGF receptor. Slap and the PDGF receptor were immunoprecipitated using the antibodies indicated from quiescent NIH3T3 cells treated with or without PDGF. The immunoprecipitated proteins were subjected to SDS–PAGE and then visualised by western blotting using antibodies against Slap (anti-Slap), the PDGF receptor (anti-PDGFR) or phosphotyrosine residues (anti-pTyr).

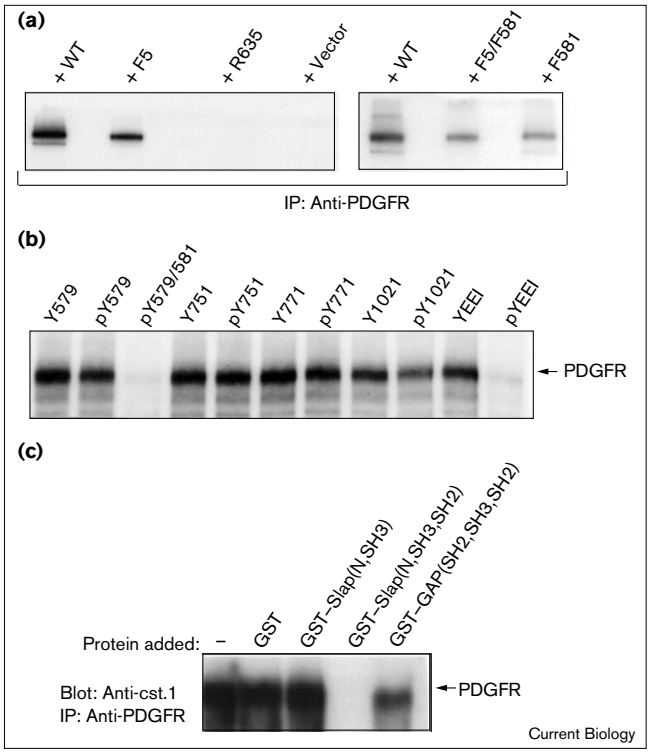
band of 34 kDa when whole cell lysates were probed, demonstrating its specificity for Slap (Figure 1a). In quiescent fibroblasts stimulated with PDGF, Slap was associated with the activated PDGF receptor, as revealed by probing both PDGF receptor and Slap immunoprecipitates with the appropriate antibodies (Figure 1b). As with the adaptor molecules, Grb2 and Grb10 [2,3], the association with the PDGF receptor did not, however, lead to tyrosine phosphorylation of Slap itself.

The predominant site on the PDGF receptor to which c-Src binds is tyrosine 579 (Y579), which is located in the juxtamembrane region; the phosphorylated Y581 residue is also involved in c-Src binding [4]. To determine whether these or other phosphotyrosine residues were critical for engaging Slap, HepG2 cells expressing various PDGF receptor mutants [5] were analysed (Figure 2a). Slap could still associate with a receptor in which five major tyrosine phosphorylation sites — not including Y579 and Y581 — had been mutated into phenylalanine residues (F5). Slap also associated with the Y581F mutant, but with apparently lower affinity than with the F5 mutant, suggesting that Y579 might be the binding site for Slap. This could not be tested directly, because of the reduced kinase activity of PDGF receptor Y579F mutants [4].

Instead, a phosphopeptide competition approach was used [6]. A glutathione-S-transferase (GST) fusion protein containing the amino terminus and the SH3 and SH2 domains of Slap — GST–Slap(N,SH3,SH2) — bound to the PDGF receptor *in vitro*. Addition of a peptide corresponding to the Src-binding site on the PDGF receptor, containing phosphorylated Y579 and Y581 residues, inhibited this binding (Figure 2b). Peptides corresponding to binding sites for phosphatidylinositol (PI) 3-kinase, ras GTPase-activating protein (rasGAP) and phospholipase Cγ did not compete. A peptide containing phosphorylated YEEI (pYEEI, the general consensus binding sequence for c-Src [7]) also abolished the binding of Slap to the PDGF receptor. These data suggest that the Slap SH2 domain has a similar binding specificity to the Src SH2 domain. To test this, Src was immunoprecipitated from a cell lysate of Sf9 insect cells infected with a Src-producing baculovirus, and incubated with baculovirus-produced PDGF receptor protein, in the presence or the absence of purified GST fusion proteins, as indicated in Figure 2c. GST–Slap competed efficiently with Src for PDGF receptor binding. GST alone, a Slap construct deleted in the SH2 domain — GST–Slap(N,SH3) — and a GST construct containing the SH2 and SH3 domains of rasGAP — GST–GAP(SH2,SH3,SH2) — did not compete for binding, however. Furthermore, in other experiments, we determined that the SH2 domains of Slap and the Src family kinase Fyn bound to the PDGF receptor *in vitro* with similar affinities (data not shown). Collectively, these data suggest that Slap uses the same binding site(s) on the PDGF receptor as c-Src, although we cannot rule out that it might also associate with other phosphorylated tyrosine residues that were not tested here.

To investigate the effect of Slap overexpression, NIH3T3 cells were transfected with an expression vector encoding

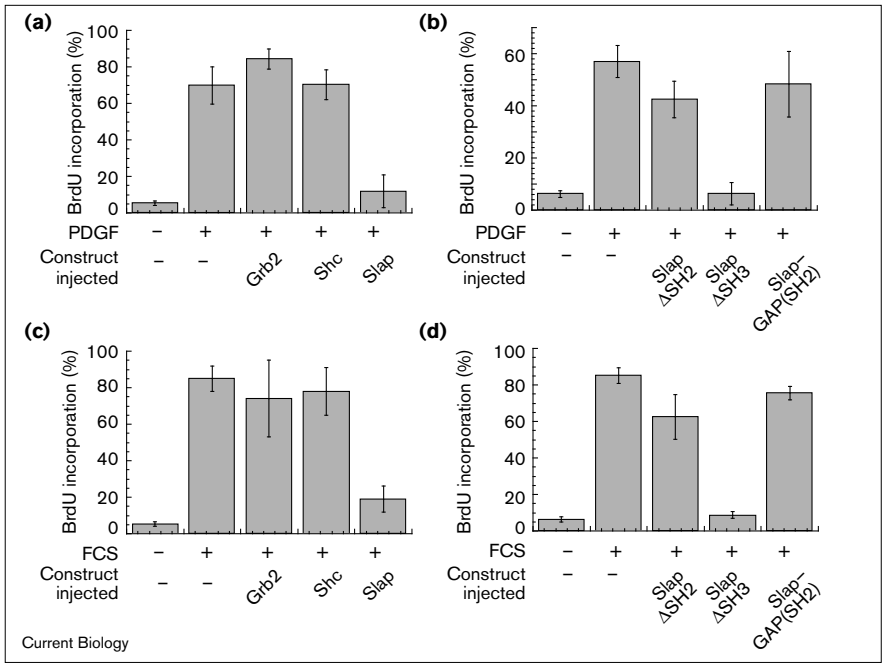
**Figure 2**




Slap and Src share a binding site on the PDGF receptor. **(a)** Slap association with mutant PDGF receptors *in vitro*. HepG2 cells expressing an empty expression vector (vector) or various PDGF receptor mutants were metabolically labelled with $^{35}$S-methionine/cysteine for 6 h, treated with PDGF (100 ng/ml) for 5 min and then lysed. After incubation with GST–Slap(N,SH3,SH2), the PDGF receptor was immunoprecipitated using anti-PDGF receptor (anti-PDGFR) antibody, subjected to SDS–PAGE and visualised by autoradiography. The various PDGF receptors tested were: wild type (WT); the R635 kinase-deficient receptor mutant (R635); the F5 mutant, which has five major tyrosine phosphorylation sites mutated to phenylalanine; the F581 mutant, which carries the Y581 to phenylalanine mutation; and the F5/581 mutant, which corresponds to the F5 mutant with the additional Y581 to phenylalanine mutation. **(b)** Inhibition of binding of Slap to the PDGF receptor by phosphotyrosine-containing peptides. Immobilised GST–Slap(N,SH3,SH2) was incubated with different peptides containing the indicated tyrosine residues (phosphorylated and unphosphorylated) before a lysate of insect cells expressing the human PDGF receptor was added. After washing, the presence of the bound receptor was detected by an *in vitro* kinase assay [6]. **(c)** Slap competes with Src for binding to the PDGF receptor *in vitro*. Src was immunoprecipitated from baculovirus-infected Sf9 cells, and incubated *in vitro* with baculovirus-produced PDGF receptor in the presence and absence of purified GST fusion proteins as indicated. The association of the PDGF receptor with Src was monitored by immunoprecipitating Src with the anti-cst.1 antibody (which is raised against the seven carboxy-terminal amino acids of Src) and then immunoblotting with an anti-PDGF receptor antibody .

**Figure 3**

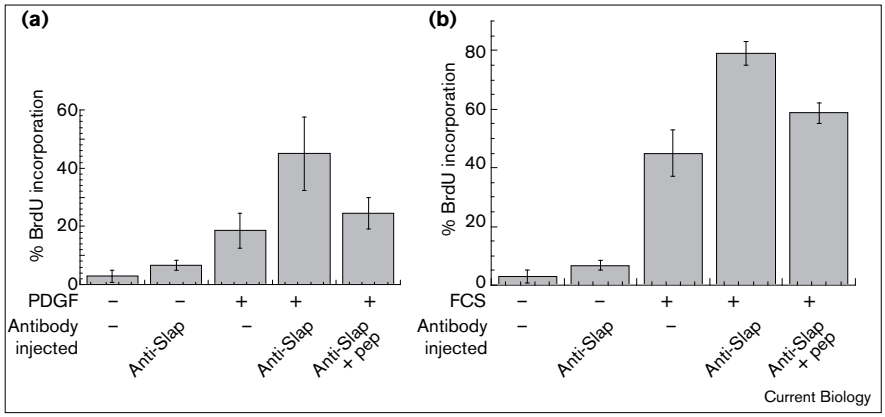
Slap and SlapΔSH3 inhibit the mitogenic response induced by both PDGF and serum, whereas Shc, Grb2, SlapΔSH2 and Slap–GAP(SH2) have no effect. Quiescent NIH3T3 cells were microinjected with the indicated constructs, stimulated with **(a,b)** PDGF or **(c,d)** foetal calf serum (FCS), in the presence of bromodeoxyuridine (BrdU) for 18 h, labelled with an anti-BrdU antibody and processed for immunofluorescence. Statistical analysis of cells that incorporated BrdU as a marker of DNA synthesis is represented on the graph. Values shown are ± SD.




FLAG-epitope-tagged Slap (Slap–FLAG), and subsequently selected for G418 resistance. Surprisingly, no Slap-expressing colonies were obtained, although several colonies (495 per μg DNA) were obtained with the empty vector. Colonies were obtained when the adaptor proteins Grb2 (484 per μg DNA) and Shc (450 per μg DNA) were transfected. These observations suggest that, in contrast to other adaptor proteins, overexpression of Slap was either toxic or growth inhibitory for NIH3T3 fibroblasts. Transient expression by microinjection was used to investigate this further [8,9]. Data from several experiments, when pooled and analysed, indicated that overexpression of Slap inhibited more than 90% of cells from entering S phase in response to PDGF, when compared with control cells (Figure 3). Microinjection of Shc and Grb2 did not inhibit DNA synthesis. (Figure 3a). Slap inhibition required an intact SH2 domain, but not the SH3 domain (Figure 3b). This effect was specific for the SH2 domain of Slap because a chimeric molecule in which the Slap SH2 domain was replaced by the amino-terminal SH2 domain of Gap — Slap–GAP(SH2) — was also not inhibitory (Figure 3b). Similar results were obtained when GST fusions of Slap were microinjected into fibroblasts (data not shown). Overexpression of Slap with an intact SH2 domain also inhibited the mitogenic response of cells to serum (Figure 3c,d).

**Figure 4**

Inhibition of Slap potentiates mitogenesis. Quiescent NIH3T3 cells were microinjected with an affinity-purified anti-Slap antiserum, or anti-Slap antiserum preincubated with the immunising peptide (pep), as indicated. Cells were then stimulated with a suboptimal concentration of **(a)** PDGF (5ng/ml) or **(b)** serum (2.5%) for 18 h, and DNA synthesis was measured as described in the legend to Figure 3. Values shown are ± SD.



If inhibition of mitogen-induced DNA synthesis is the physiological role of Slap, then removal of Slap should potentiate signalling. To test this, cells were microinjected with the Slap antibody, and then stimulated with a suboptimal dose of PDGF or serum. We observed that more microinjected cells entered S phase than control cells (Figure 4a,b). Preincubation of the antibody with the immunising peptide demonstrated that these potentiation effects were specific for Slap. We conclude that the endogenous level of Slap in NIH3T3 cells can influence whether the cells progress though the cell cycle in response to a particular concentration of growth factor.

It is not clear how Slap inhibits the cell cycle. Preliminary data indicate that Slap inhibition can be overcome by heterologous expression of Myc (S.R., unpublished observations) suggesting that Slap antagonises the Src pathway [10]. Negative regulation of receptor-activated signalling pathways may be a general theme in cell growth control. Other recent examples include FRNK (FAK-related non-kinase), the Cis family of proteins that regulate cytokine signalling, and the T-cell adaptor protein Cbl [11–16].

## Materials and methods

Slap and various mutants were derived by PCR, tagged with a FLAG epitope, and then cloned into pcDNA3 (Invitrogen). Slap(GAPSH2) contains the amino-terminal SH2 domain of GAP in place of the Slap SH2 domain. Expression constructs encoding Myc-tagged Grb2 and Shc were a gift from Ben Margolis (University of Michigan). Rabbit anti-Slap serum against the nine carboxy-terminal residues of the mouse Slap sequence was affinity-purified as described [9]. Fusion proteins were purified as described [17]. Other antibodies, immunoprecipitation techniques, kinase assays, and peptide and peptide competition methodology have been described previously [6,18]. For microinjection analysis, NIH3T3 cells were seeded, synchronised and microinjected as described [19]. Plasmids (100 ng/ml) were injected into the nucleus 4–6 h before cell stimulation. Affinity-purified antibody (1–2 mg/ml) was injected into the cytoplasm. Cells were stimulated for 18 h with PDGF (25 ng/ml unless otherwise indicated) or FCS (10% unless otherwise indicated), in medium containing BrdU (Sigma; 0.1 mM). BrdU incorporation was measured as described [9]. For transfection, NIH3T3 cells were seeded into 6-well dishes and transfected with 2 μg DNA per well using lipofectamine (Gibco BRL) according to manufacturer's instructions. The cells were trypsinised 24 h later and placed in selection medium containing 0.4 mg/ml G418. After 2–3 weeks, the number of colonies were visualised and counted by staining with crystal violet. For PDGF receptor association assays, cell lysates made from PDGF-treated or PDGF-untreated HepG2 cells expressing mutant PDGF receptors [5] were incubated with GST–Slap(N,SH3,SH2) or GST–Src(SH2) for 2 h at 4°C. Pelleted beads were then washed, boiled in 1% SDS to elute the bound material, diluted to a final concentration of 0.1% SDS, and immunoprecipitated using an antibody against the PDGF receptor. Association of the PDGF receptor with Slap in fibroblasts was assayed from lysates of quiescent fibroblasts that had been stimulated with PDGF (50 ng/ml) for 5 min or left unstimulated.

## Acknowledgements

S.A.C. thanks the members of her laboratory, particularly Martin Broome, for critical reading of the manuscript, and Martha Velarde for her cheerful help with the preparation of the figures. We thank Ian Jones for technical assistance and Claude Granier for peptide synthesis. V.M.D. is supported by National Institutes of Health grant number DK 54386. S.R. was supported by INSERM, EMBO, Association pour la Recherche contre le Cancer (ARC) and Fondation pour la Recherche Medicale (FRM).

## References

1. Pandey A, Duan H, Dixit VM: **Characterization of a novel Src-like adapter protein that associates with the Eck receptor tyrosine kinase.** *J Biol Chem* 1995, **270:**19201-19204.
2. Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, *et al.*: **The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling.** *Cell* 1992, **70:**431-442.
3. Pandey A, Duan H, Di Fiore PP, Dixit V: **The Ret receptor protein tyrosine kinase associates with the SH2-containing adapter Grb10.** *J Biol Chem* 1995, **270:**21461-21463.
4. Mori S, Rönnstrand L, Yokote K, Engström A, Courtneidge SA, Claesson-Welsh L, *et al.*: **Identification of two juxtamembrane autophosphorylation sites in the PDGF β-receptor. Involvement in the interaction with Src family tyrosine kinases.** *EMBO J* 1993, **12:**2257-2264.
5. Valius M, Kazlauskas A: **Phospholipase C-γ1 and phosphatydilinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal.** *Cell* 1993, **73:**321-334.
6. Alonso G, Koegl M, Mazurenko N Courtneidge SA: **Sequence requirements for binding of Src family tyrosine kinases to activated growth factor receptors.** *J Biol Chem* 1995, **270:**9840-9848.
7. Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, *et al.*: **SH2 domains recognize specific phosphopeptide sequences.** *Cell* 1993, **72:**767-778.
8. Twamley-Stein GM, Pepperkok R, Ansorge W Courtneidge SA: **The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH-3T3 cells.** *Proc Nat Acad Sci USA* 1993, **90:**7696-7700.
9. Roche S, Koegl, M, Courtneidge SA: **The phosphatidylinositol 3-kinase α is required for DNA synthesis induced by some, but not, all growth factors.** *Proc Natl Acad Sci USA* 1994, **91:**9185-9189.
10. Barone MV Courtneidge SA: **Myc but not Fos rescue of PDGF signaling block caused by kinase inactive Src.** *Nature* 1995, **378:**509-512.
11. Richardson A Parsons T: **A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125$^{FAK}$.** *Nature* 1996, **380:**538-540.
12. Yishimura T, Ohkubo T, Kiguchi T, Jenkins NA, Gilbert DJ, Copeland NG, *et al.*: **A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds tyrosine-phosphorylated interleukin 3 and erythropoietin receptors.** *EMBO J* 1995, **14:**2816-2826.
13. Endo TA, Mashura M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, *et al.*: **A new protein containing-SH2 domain that inhibits JAK kinases.** *Nature* 1997, **387:**921-924.
14. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, *et al.*: **Structure and function of a new STAT-induced STAT inhibitor.** *Nature* 1997, **387:**924-929.
15. Starr R, Willson TA, Viney EM, Murray LJL, Rayner JR, Jenkins BJ, *et al.*: **A family of cytokine-inducible inhibitors of signalling.** *Nature* 1997, **387:**917-921.
16. Ota Y, Samelson LE: **The product of the proto-oncogene c-cbl: a negative regulator of the Syk tyrosine kinase.** *Science* 1997, **276:**418-420.
17. Frangioni JV, Neel BG: **Solubilization and purification of enzymatically active gluthathione S-transferase (pGEX) fusion proteins.** *Anal Biochem* 1993, **210:**179-187.
18. Roche S, Dhan R, Waterfield MD, Courtneidge SA: **The catalytic subunit of phosphatidylinositol 3-kinase is a substrate for the platelet-derived growth factor receptor, but not for middle-T antigen-pp60c-src complexes.** *Biochem J* 1994, **301:**703-711.
19. Roche S, Koegl M, Barone VM, Roussel M, Courtneidge SA: **DNA synthesis induced by some, but not all, growth factors requires Src family protein tyrosine kinases.** *Mol Cell Biol* 1995, **15:**1102-1109.