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**The Src-like adaptor protein regulates PDGF-induced actin dorsal ruffles
in a c-Cbl-dependent manner**

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Running head: SLAP as a negative regulator of F-actin assembly

Key words: SLAP, Src, PDGF, Cbl, DNA synthesis, F-actin assembly

ABSTRACT

The Src-like adaptor protein (SLAP) belongs to the sub-family of adapter proteins that negatively regulate cellular signalling initiated by tyrosine kinases. SLAP has a unique, myristylated N-terminus, followed by SH3 and SH2 domains with high homology to Src family tyrosine kinases (SFK), and a unique carboxyl-terminal tail, which is important for c-Cbl binding. We have previously shown that SLAP negatively regulates PDGF-induced mitogenesis in fibroblasts and we now report that it regulates F-actin assembly for dorsal ruffles formation. c-Cbl mediated SLAP inhibition towards actin remodelling. Moreover, SLAP enhanced PDGF-induced c-Cbl phosphorylation by SFK. In contrast, SLAP mitogenic inhibition was not mediated by c-Cbl, but it rather involved a competitive mechanism with SFK for PDGF receptor (PDGFR) association and mitogenic signalling. Accordingly, phosphorylation of the Src mitogenic substrates Stat3 and Shc were reduced by SLAP. We thus concluded that SLAP regulates PDGFR signalling by two independent mechanisms: a competitive mechanism for PDGF-induced Src mitogenic signalling and a non-competitive mechanism for dorsal ruffles formation mediated by c-Cbl.

SLAP is ubiquitously expressed at the mRNA level with a strong expression in spleen and lung (Pandey et al., 1995). Gene knock out in mice pointed to a crucial role for SLAP in thymocytes development (Sosinowski et al., 2001). The adapter and E3 ubiquitin ligase protein c-Cbl (Schmidt & Dikic, 2005) has been reported to bind to the SLAP C-terminus for negative regulation of T cell antigen receptor signalling including ubiquitination and down-regulation of the CD3 complex (Myers et al., 2006). We have previously reported that SLAP also negatively regulates mitogenesis induced by growth factors in fibroblasts including the PDGF (Roche et al., 1998a). Further experimental data suggested that it regulates mitogenesis by inhibition of a Src signalling pathway (Manes et al., 2000). Whether a SLAP-c-Cbl complex also regulates growth factor receptor signalling is however unknown.

In addition to mitogenesis, PDGF induces morphological changes leading to F-actin assembly for both lateral and dorsal/circular ruffles formation. While lateral ruffles mediate directional cell migration, dorsal ruffles were linked to cell invasion into the extracellular matrix (Suetsugu et al., 2003). This cytoskeletal rearrangement is regulated by cortical actin polymerisation through the activation of the Arp2/3 complex and a Rac/Wave1 specific pathway (Suetsugu et al., 2003). Ras and Rab5 pathways have been also documented (Lanzetti et al., 2004) and we have recently shown that this response implicates SFK. Additionally, our results suggest that in contrast to Src mitogenic function, SFK signal transduction promoting actin assembly does not require receptor association (Veracini et al., 2006). Rather, it involves the lipid second messenger sphingosine-1-phosphate that may activate SFK through a seven transmembrane receptor of the EDG family and an associated heterotrimeric Gi protein.

SLAP negatively regulates PDGF-induced dorsal ruffles.

Due to its homology with SFK, we investigated the role of SLAP on actin remodelling. We found that 65% of NIH 3T3 cells exhibited at least one dorsal ruffle after 5 minutes of PDGF stimulation. Interestingly, SLAP reduced this response by 70% (Figure 1b). A structure-function analysis implicated both the SLAP SH2 and C-terminus. The role of the endogenous protein was next investigated. Since SLAP is weakly expressed in NIH-3T3 cells, we used IMR90 human lung fibroblasts that express higher level of this adapter (not shown). PDGF induced dorsal ruffles in 30% of these cells and down-regulation of SLAP level enhanced this response by two (Figure 1c-e). This potentiating effect was specific to dorsal ruffles as lateral ruffles were not affected by SLAP depletion. We thus concluded that SLAP negatively regulates PDGF-induced dorsal ruffles.

c-Cbl mediates SLAP activity towards dorsal ruffles induction.

The mechanism by which SLAP regulates actin remodelling was next investigated. Since both SLAP and SFK require their SH2 domain in this cellular process (Veracini et al., 2006), we sought a SLAP competitive mechanism that prevents SFK signalling. However, Src co-expression had no rescuing effect, even when expressed at higher level, suggesting that SLAP does not act at the Src level (Figure 2d). Since c-Cbl has been implicated in F-actin assembly (Scaife et al., 2003; Swaminathan et al., 2007), we then addressed the role of this adapter on SLAP morphological activity. To this end, c-Cbl was depleted from cells with a shRNA that reduced 80% of protein level (Figure 2b). Interestingly, SLAP cytoskeleton inhibition was not observed anymore in c-Cbl deficient cells (Figure 2c). In contrast, PDGF-induced F-actin assembly still required SFK activity as shown by the inhibitory effect of SU6656, a pharmacological inhibitor of SFK (Figure 2c). This indicates that c-Cbl is required for this SLAP activity and that SFK use additional substrates for promoting dorsal ruffles. The role of c-Cbl was next confirmed by a dominant-negative approach. A c-Cbl mutant bearing

deletion at amino acid 480 ($\Delta 480$) has been shown to potentiate dorsal ruffles (Scaife et al., 2003), in agreement with a dominant negative activity towards c-Cbl-regulating F-actin assembly. Indeed, this mutant significantly restored ruffles in cells expressing SLAP (Figure 2d). $\Delta 480$ had lost its capacity to bind SH3-containing proteins and to be phosphorylated by SFK, implicating either molecular event for c-Cbl-mediated SLAP inhibitory effect. In contrast, c-Cbl mutants bearing inactive E3 ligase activity (3AHN) or tyrosine kinase domain (G306E) had a lower rescuing effect (Figure 2d). We concluded that this SLAP function is mediated by c-Cbl, implicating its C-terminus domain. Moreover, the absence of inhibitory effect observed with SLAP ΔC suggests that the interaction with c-Cbl may be necessary for in vivo activity (Figure 1b).

Cbl does not mediate SLAP mitogenic activity.

We next addressed whether c-Cbl plays a similar role in SLAP mitogenic inhibition. First, cells with reduced c-Cbl gave a significant higher PDGF response, in agreement with a negative function for this adapter on mitogenic signalling (Broome et al., 1999; Miyake et al., 1999). Nevertheless, SLAP retroviral expression still inhibited this response (Figure 2e), suggesting that c-Cbl does not mediate its mitogenic activity. Similarly, SFK activities were necessary for mitogenesis (Figure 2e) in agreement with the requirement of additional Src substrates in this signalling process (Bromann et al., 2004). We next confirmed this data with a dominant-negative approach. G306E exhibits dominant negative activity towards c-Cbl mitogenic function (Broome et al., 1999; Miyake et al., 1999), therefore this mutant was co-expressed with SLAP for c-Cbl inhibition. However, it did not overcome the block induced by SLAP (Figure 2f), confirming the c-Cbl-independent nature of this SLAP signalling. It should be mentioned that $\Delta 480$ gave a partial rescuing effect, while inhibiting mitogenesis *per se* (Figure 2f). It thus may affect SLAP activity by a mechanism independent of c-Cbl.

SLAP mitogenic inhibition involves a SFK competitive mechanism.

We next investigated the mechanism by which SLAP inhibits the PDGF mitogenic response. Our previous studies indicated that SLAP interferes with Src signalling including the association of Src-SH2 with the PDGFR at phospho-Tyr579 (Manes et al., 2000; Roche et al., 1998b). SFK activation induces a Rac/Myc signalling cascade necessary for DNA synthesis induction that can be suppressed by a p53-dependent activity (Bromann et al., 2004). PDGF mitogenic response was inhibited by a moderate expression of SLAP (Figures 3a and 3b, right panel). Moreover, this inhibition was by-passed when co-expressing constitutive active elements of the Src pathway, ie Myc and active V12Rac, and the dominant-negative p53H273 (Figure 3a). More importantly, Src also significantly rescued this mitogenic blockade (Figure 3b) suggesting that SLAP acts, at least in part, at the Src level. We attributed our inability to previously rescue SLAP inhibition (Manes et al., 2000) to a higher expression of this adapter (Figure 3b, right panel).

The impact of SLAP on Src mitogenic signaling was next confirmed at the molecular level. SLAP exhibited a strong anti-proliferative effect in mouse fibroblasts (Roche et al., 1998a). Nevertheless, we could generate NIH-3T3 cells stably expressing SLAP when transduced by retroviral infection (Figure 3c). PDGF-induced SFK association with the receptor and kinase activation were both reduced by SLAP (Figure 3c). Accordingly, phosphorylation of Src mitogenic substrates Stat3 and Shc (Bromann et al., 2004) were inhibited (Figure 3d). Specificity was shown by the inability of SLAP to affect PDGFR activation (Figure 3e). Moreover, this adapter had a low impact on Ras-induced MAPKs activation, indicating that it does not inhibit all signalling pathways (Figure 3e).

SLAP associates with c-Cbl and affects Rac activation.

SLAP associated with the activated PDGFR in agreement with a reduction of SFK-PDGFR complex formation and it was tyrosine phosphorylated upon PDGF stimulation (Figure 4a). Moreover, SLAP associated with a tyrosine phosphorylated protein of 120 kDa that was further identified as c-Cbl (Figure 4a). SLAP-c-Cbl complex formation was however independent of growth factor stimulation. Nevertheless, we found that SLAP potentiated PDGF-induced c-Cbl tyrosine phosphorylation that was regulated by SFK (Figure 4b). Since this phosphorylation has been implicated in c-Cbl activities (Schmidt & Dikic, 2005), we suggest that SLAP promotes c-Cbl functions for negative regulation of F-actin assembly. C-Cbl also inhibits Rac activity, required for dorsal ruffle formation (Scaife et al., 2003; Swaminathan et al., 2007). Accordingly, PDGF-induced Rac activation was also affected by SLAP. This suggests that dorsal ruffle inhibition by SLAP implicates a c-Cbl/Rac pathway.

Here we show that in addition to mitogenesis, SLAP negatively regulates PDGF-induced dorsal ruffles and that this activity is mediated by c-Cbl. We propose that SLAP promotes Src-induced c-Cbl phosphorylation at the C-terminus for inhibition of Rac activation. Given the proposed function of dorsal ruffles formation in cell invasion (Suetsugu et al., 2003), we suggest that SLAP may play a role in this biological process induced by growth factors. Nonetheless, additional cytoskeletal function for SLAP-c-Cbl may be expected (Teckchandani et al., 2005). c-Cbl has been also implicated in PDGFR β degradation for down-regulation of mitogenesis (Broome et al., 1999; Miyake et al., 1999). However, our data suggest that in contrast to F-actin assembly, c-Cbl does not mediate SLAP mitogenic inhibition. Therefore, c-Cbl may use distinct mechanisms for PDGFR association and ubiquitination (Reddi et al., 2007). We thus propose that SLAP dictates c-Cbl specificity towards PDGFR signalling. Finally, this report suggests the existence of two distinct pools of SLAP for regulation of SFK signalling. SFK regulates mitogenesis by direct association of the

receptor in cholesterol-enriched microdomains while SFK-promoting dorsal ruffles is localized at the actin cytoskeleton (Veracini et al., 2006). Therefore, we suggest that SLAP negatively regulates mitogenesis by directly interfering with SFK-PDGFR complex formation in caveolae while a SLAP-c-Cbl complex regulates actin remodelling by targeting SFK-induced Rac activation. Since SFK play crucial roles in neoplastic transformation (Ishizawar & Parsons, 2004), we anticipate negative functions for SLAP in human cancer.

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LEGENDS TO FIGURES

Figure 1. SLAP negatively regulates PDGF-induced dorsal ruffles.

(a). SLAP constructs. (\\\\), myristylation; *, point mutations in the SH3 (W58A) or SH2 (R111L) domain (SH3* and SH2*); ΔC, C-terminal truncation. (b) SLAP inhibits PDGF-induced dorsal ruffles. Left panel: statistical analysis of dorsal ruffles formation in PDGF-stimulated cells expressing indicated SLAP constructs. Right panel: level of SLAP mutants. (c) SLAP mRNA and protein levels in IMR90 cells infected with retroviruses expressing shRNA specific to luciferase (mock) or SLAP sequence (GACCTGGTGAACCACTATT) and assessed by Q-PCR (forward CCGGAGGGACTGGATAGC and reverse ACAGCCAGCCATGGTAAAC primers) and Western-blotting (sc-1215, Santa Cruz) from a whole cell-lysate. Tubulin level is also shown. (d) and (e) SLAP depletion potentiates PDGF-induced dorsal ruffles. An example (d) and statistical analysis (e) of ruffles formation in cells stimulated or not with PDGF that were transduced with indicated shRNA. Cells grown on coverslips were transfected or not with indicated construct, serum-starved and stimulated with PDGF-BB (Abcys) (20 ng/ml) for 5 min. Cells were fixed and preceded for actin staining using rodhamin-phalloïdin and ectopic protein expression. Ruffles formation (%) = [number of ruffles-positive transfected cells] / [number of transfected cells] X 100. The mean ± s.d. from 3-5 independent experiments is shown. SLAP constructs (Manes et al., 2000) were sub-cloned into pBABE. Cell culture, transfection, infection, immunofluorescence analysis, Q-PCR and biochemistry have been described in (Veracini et al., 2006). White arrows indicate cells with dorsal ruffles. *** P<0.001; ** P<0.01 using a student's *t*-test.

Figure 2. c-Cbl mediates SLAP activity towards actin remodelling but not mitogenesis.

(a) c-Cbl constructs. 4H, four-helix bundle; EF, EF hand; *, point mutations in tyrosine kinase-binding (TKB) or ring finger (RF) domains; PRR, proline-rich repeat; LZ, leucine zipper; G306E (a gift from Dr Gross), tyrosine kinase-binding domain mutant, C3AHN (a gift from Prof. Band), multiple point mutations in ring finger domain; Δ480 (a gift from Prof Langdon), truncation at amino acid 480. (b) c-Cbl level in NIH-3T3 cells infected with retroviruses expressing scramble (mock) or shRNA specific to the Cbl sequence (ACACTTTCCGGATTACTA) and assessed by Western-blotting of immunoprecipitated c-Cbl (sc-170, Santa Cruz). The level of tubulin from a whole cell-lysate is shown. (c) SLAP does not inhibit dorsal ruffles in c-Cbl depleted cells. (d) SLAP inhibition towards dorsal ruffles induction is overcome in cells with inactive c-Cbl. (e) SLAP still inhibits mitogenesis in c-Cbl depleted cells. (f) SLAP still inhibits mitogenesis in cells co-expressing c-Cbl inactive mutants. Indicated quiescent cells that were transfected or not with indicated constructs, treated or not with SU6656 (2 μM) (Calbiochem) and stimulated with PDGF-BB for 5 min (ruffles formation) or 18 h in the presence of bromo-deoxyuridine (BrdU) (0.1 mM, Sigma) (BrdU incorporation). Cells were fixed and processed for immunostaining as described in (Veracini et al., 2006). BrdU incorporation (%) = [number of BrdU-positive transfected cells] / [number of transfected cells] X 100. The mean ± s.d. from 3-5 independent experiments is shown.

Figure 3. SLAP inhibits SFK mitogenic signalling.

(a) SLAP inhibits PDGF-induced Src mitogenic signalling (b) at the Src level. BrdU incorporation assays in quiescent NIH-3T3 cells transfected with indicated constructs and stimulated or not with PDGF-BB. Src, Myc and RacV12 constructs have been described in (Boureaux et al., 2005) and p53H273 was from Dr Hibner. Right panel: SLAP level in HEK

293 cells transfected with indicated constructs. (c) PDGF-induced SFK activation and (d) phosphorylation of Src mitogenic substrates were reduced in NIH-3T3 cells stably expressing SLAP. (e) SLAP does not affect all PDGFR signalling pathways. Cell-lysates were made from quiescent cells that were infected as indicated and stimulated for 5 min with indicated concentrations of PDGF-BB. The level of immunoprecipitated SLAP [α SLAP1, (Manes et al., 2000)], immunoprecipitated SFK with associated tyrosine phosphorylated proteins and PDGFR are shown (left). An example (left) and statistical analysis (right) (mean \pm s.d., n = 3) of in vitro SFK activities (relative to activities from quiescent mock-infected cells) is shown and was assessed by the capacity to purified SFK to phosphorylate denatured enolase (32 P-enolase) as in (Veracini et al., 2006). (d) Levels of immunoprecipitated Stat3 and Shc and their phosphorylation on Tyr705 and Tyr239 and 240 respectively are shown from indicated cell-lysates. (e) (top) Kinase activity (32 P-PDGFR) and tyrosine phosphorylation content of immunoprecipitated PDGFR is shown from indicated cell-lysates. The levels of phosphorylated MAPKs (p-MAPKs) and MAPKs are also shown. Used antibodies are described in (Veracini et al., 2006).

Figure 4. SLAP associates with c-Cbl and inhibits Rac activation.

(a) SLAP associates with PDGFR and c-Cbl. (b) SLAP potentiates PDGF-induced c-Cbl phosphorylation in a SFK-dependent manner. Cell-lysates were made from quiescent cells that were infected with as indicated and stimulated for 5 min with indicated concentrations of PDGF-BB. Left: levels of immunoprecipitated SLAP with associated tyrosine phosphorylated proteins, PDGFR and c-Cbl are shown. Right: the level of immunoprecipitated c-Cbl and its tyrosine phosphorylation content is shown from indicated cells stimulated with PDGF-BB and treated or not with SU6656 (2 μ M). (c) SLAP affects PDGF-induced Rac activation. An example (left) and statistical analysis (right) (mean \pm s.e., n = 3) of Rac1-GTP level (% of

maximum) in cells stimulated with PDGF (10ng/ml) is shown and was assessed as in (Boureaux et al., 2005). **(d)** A model for SLAP-interfering with PDGF-induced SFK signalling leading to DNA synthesis and dorsal ruffles. Cholesterol-enriched microdomains are shown in grey; S1P: sphingosine 1 phosphate, endothelial differentiation gene (EDG) receptors. Gi: heterotrimeric protein of the Gi sub-family.

Figure 1a, b

a



b

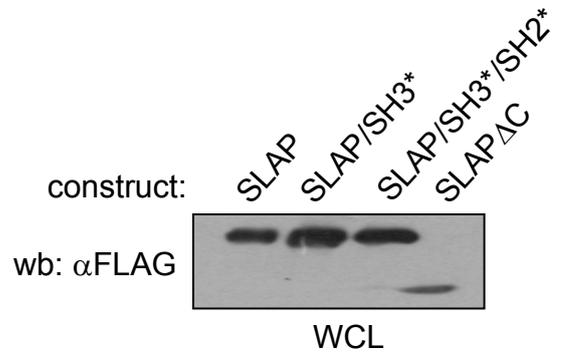
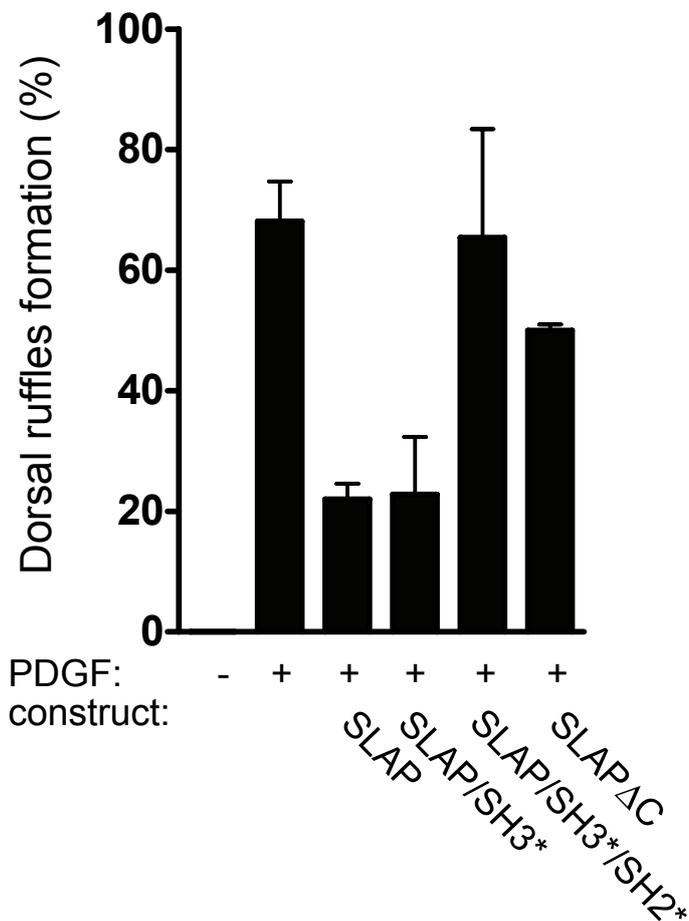


Figure 1c-e

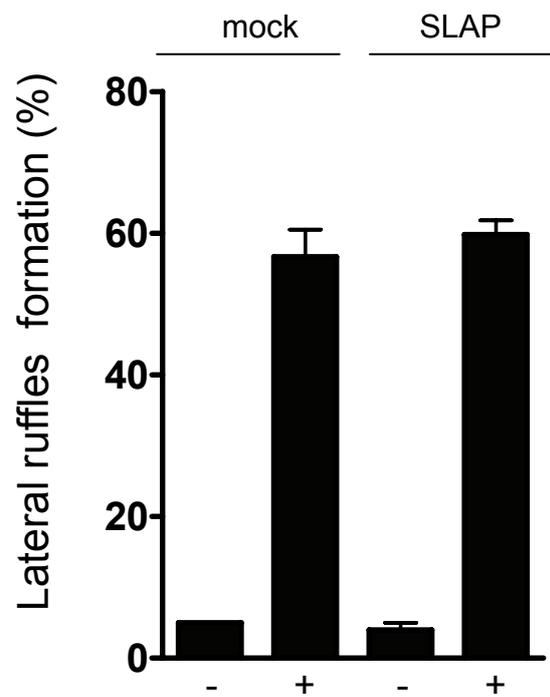
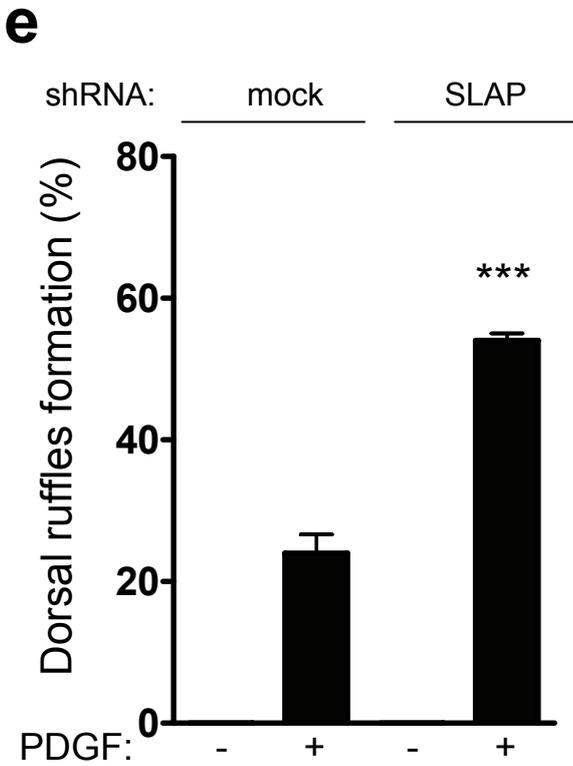
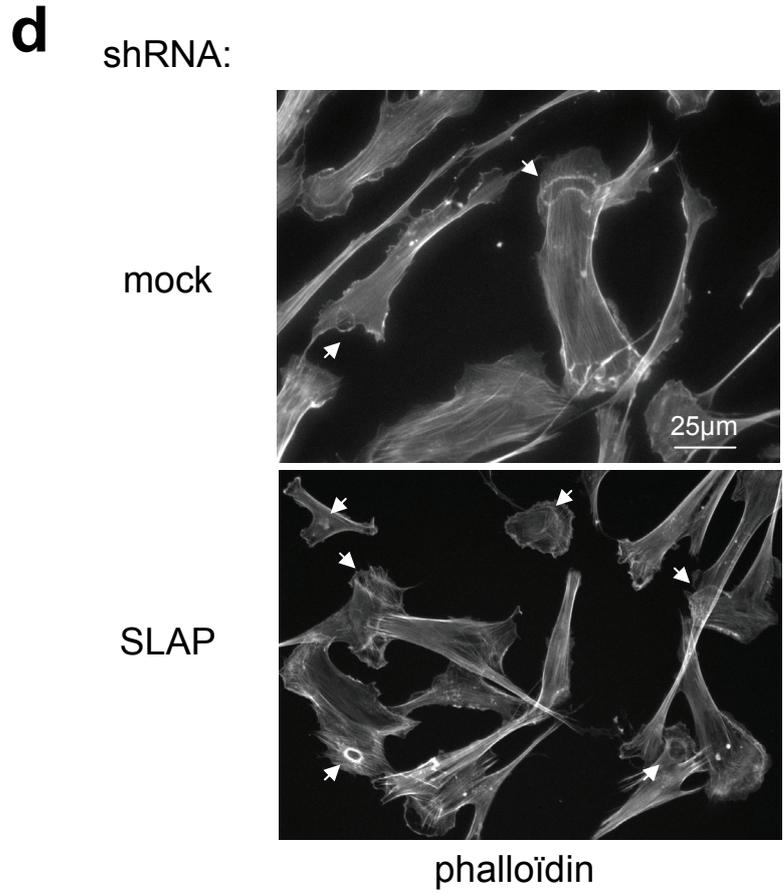
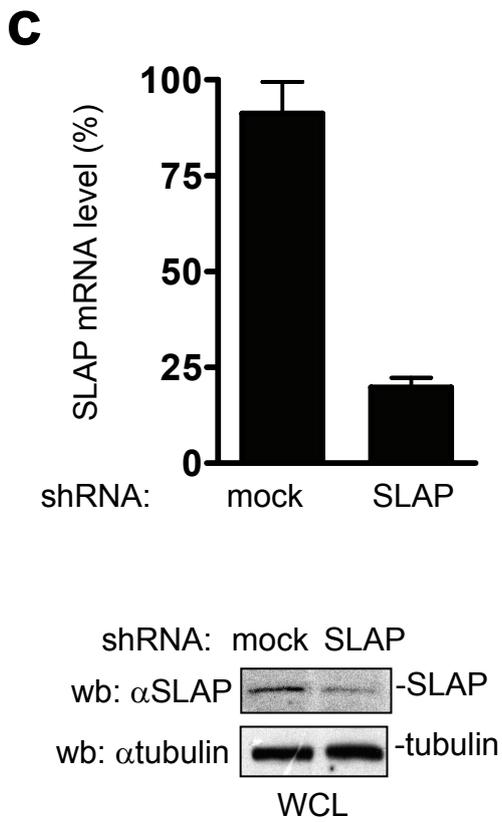


Figure 2 a-d

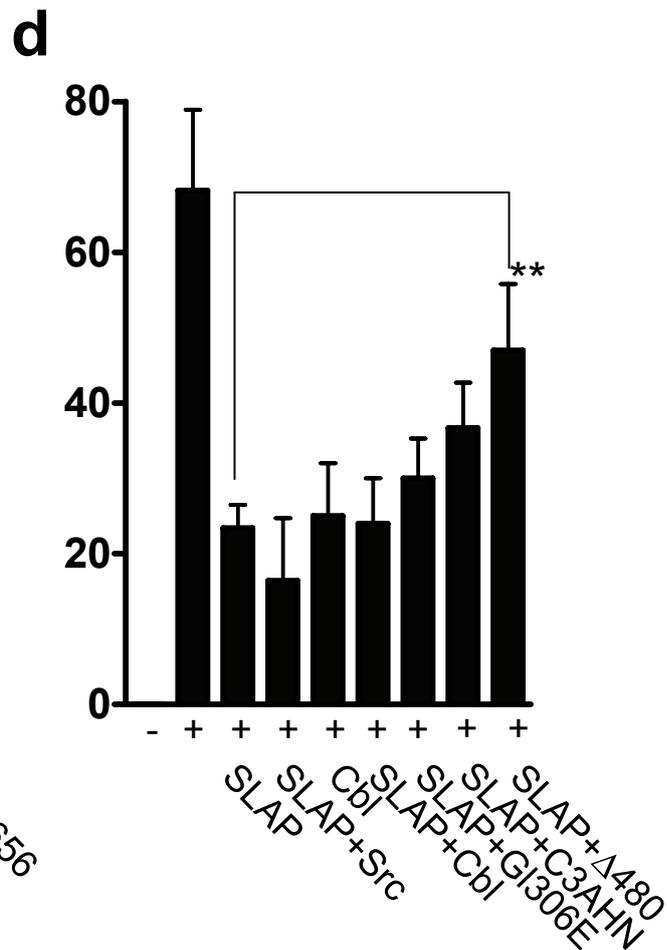
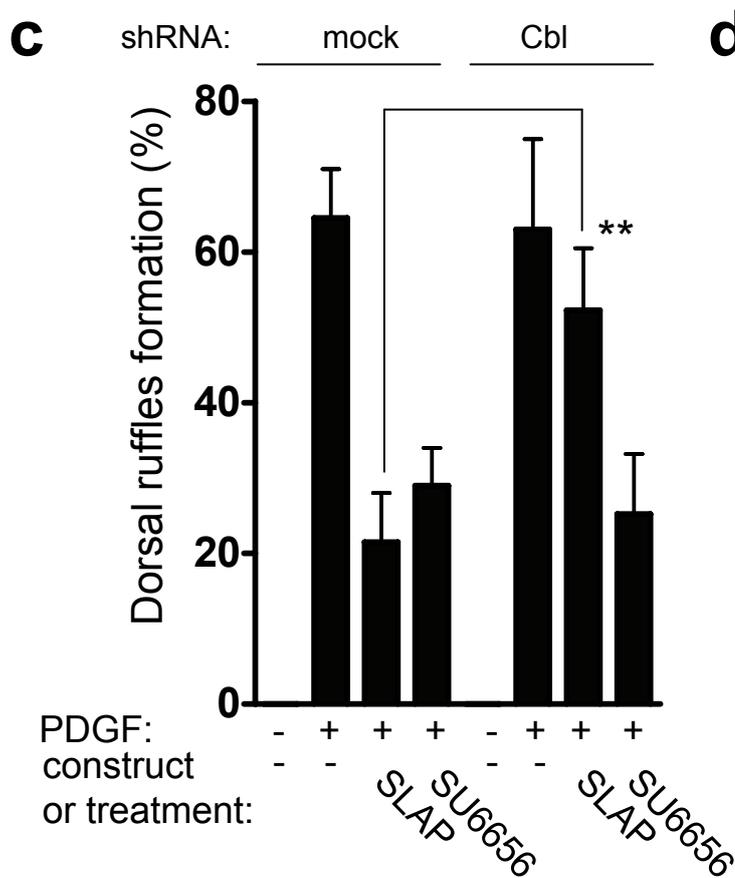
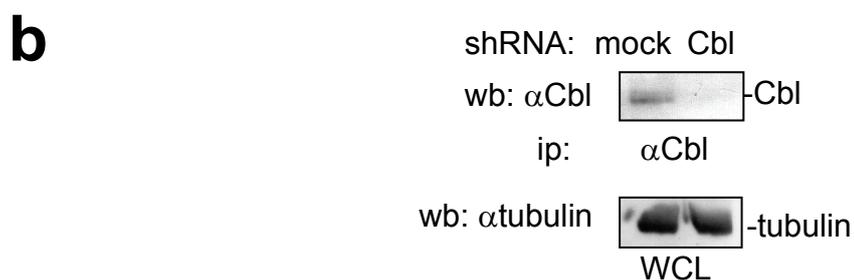
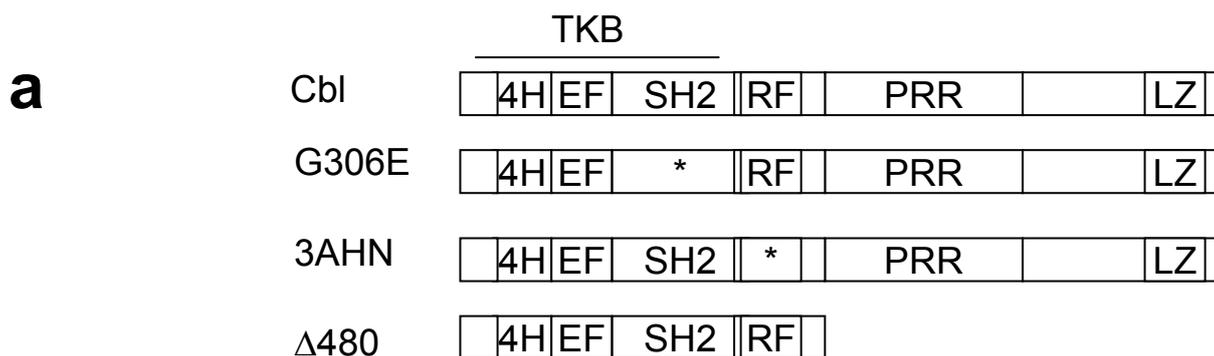
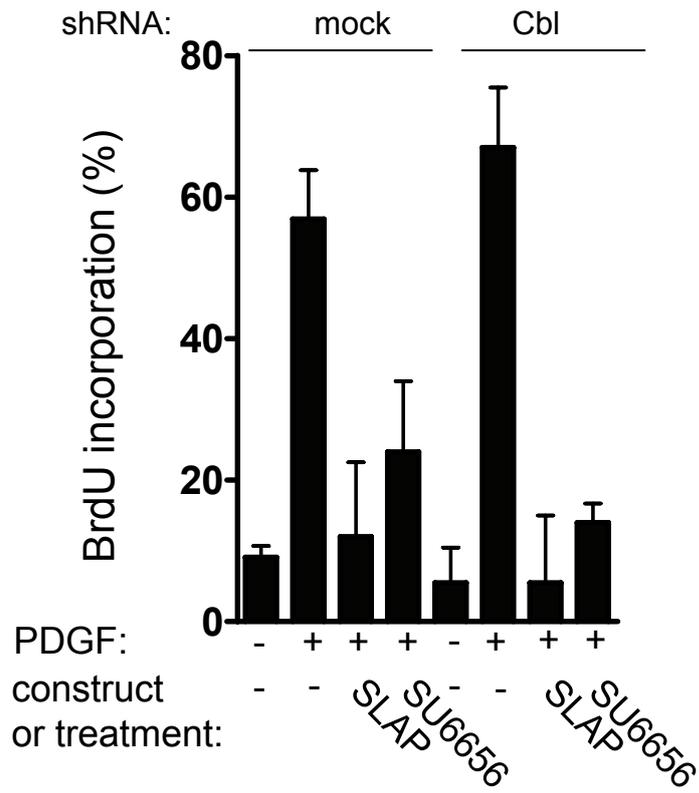


Figure 2e, f

e



f

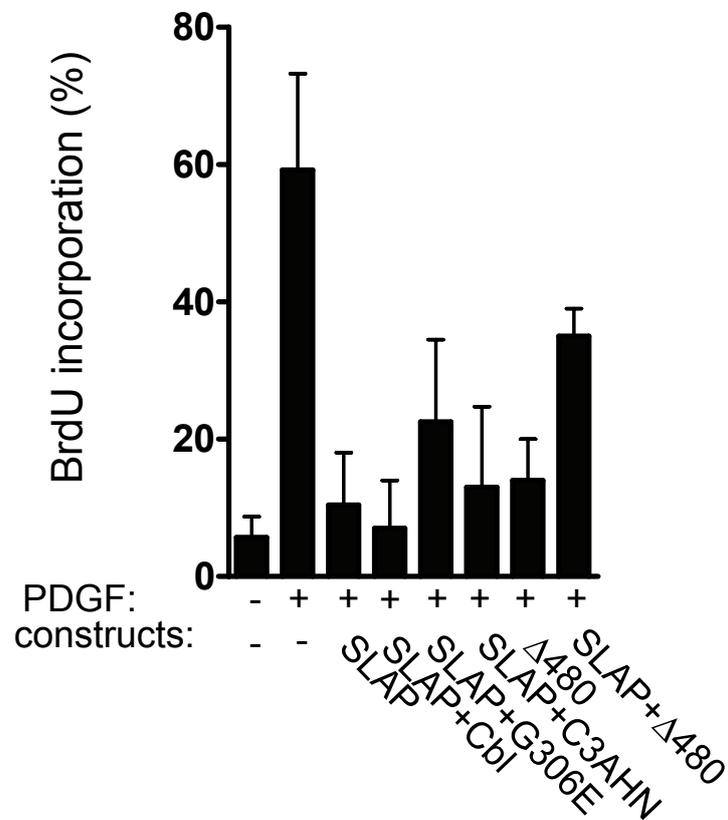
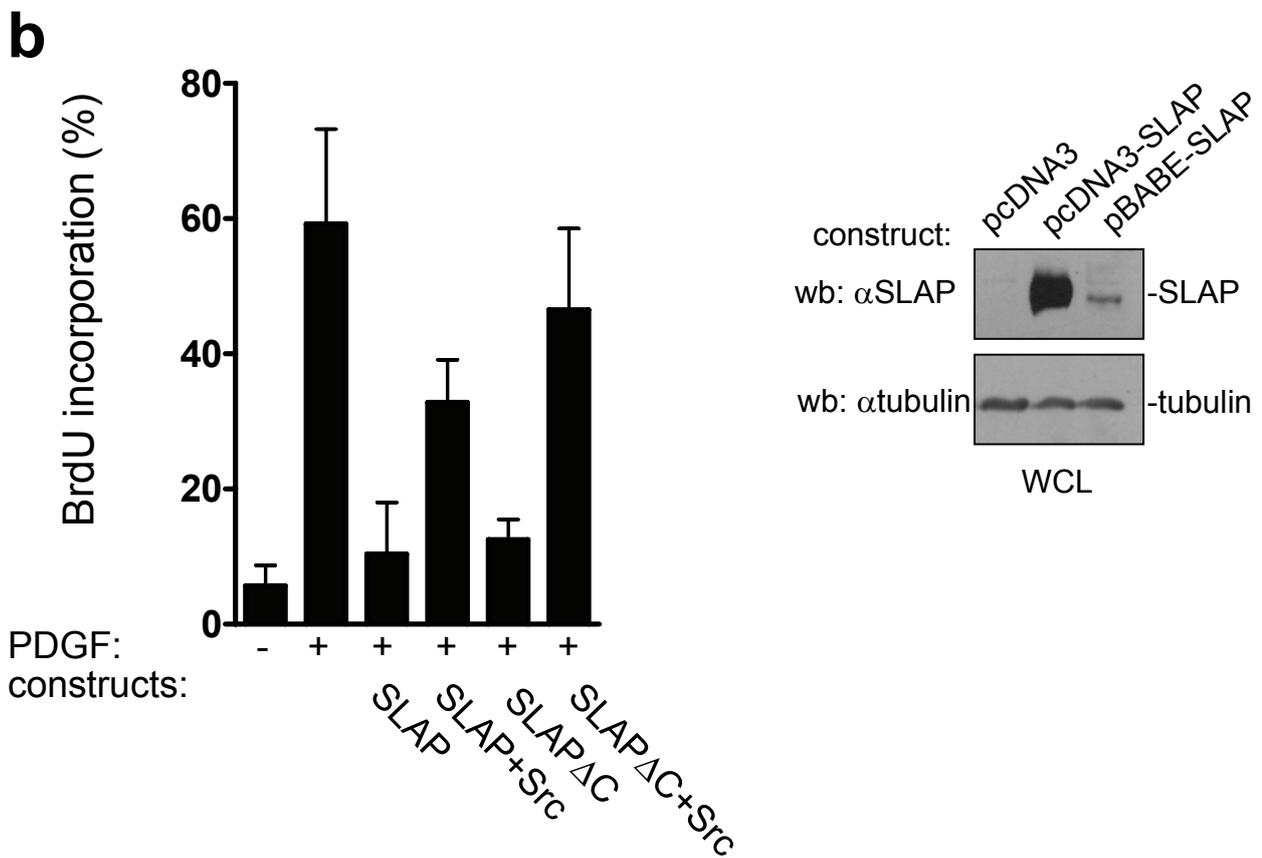
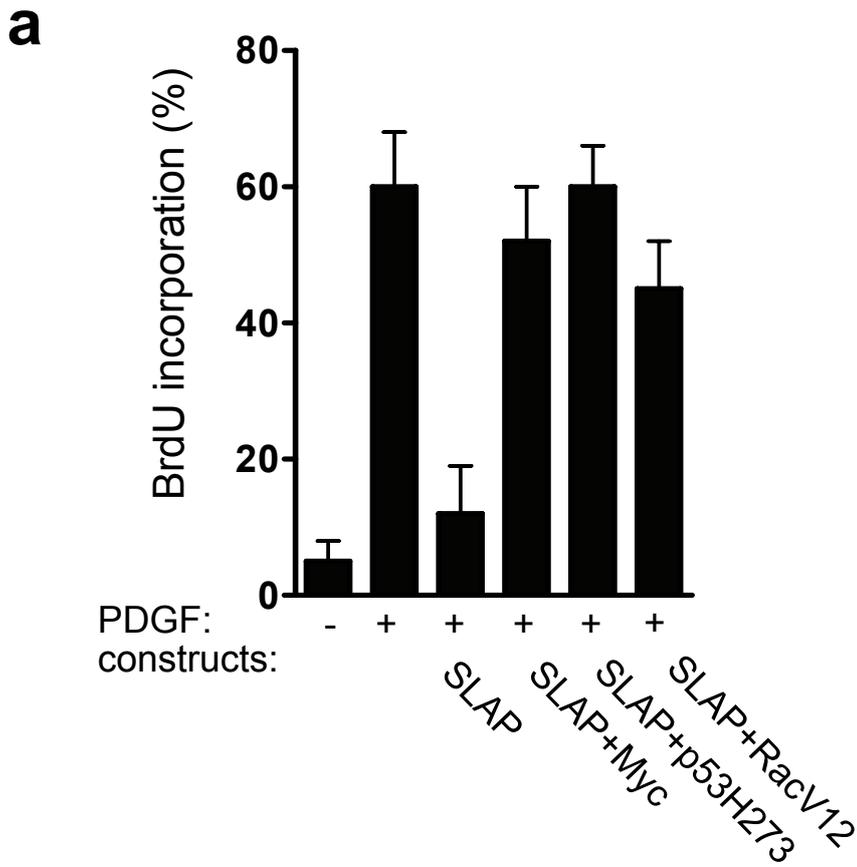
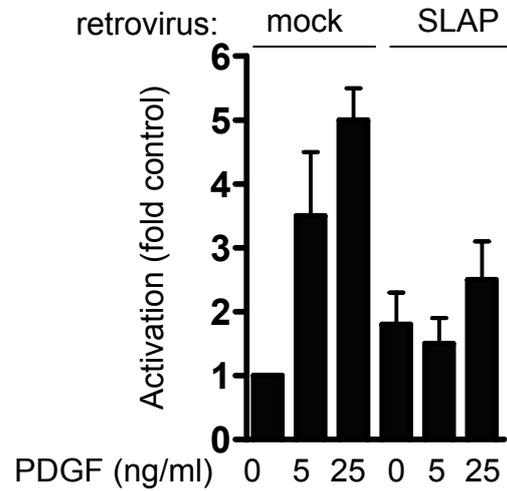
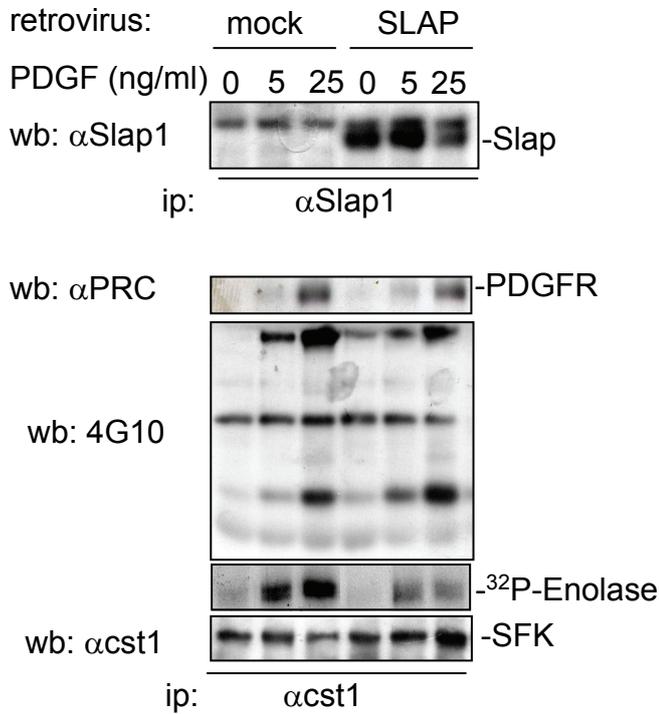


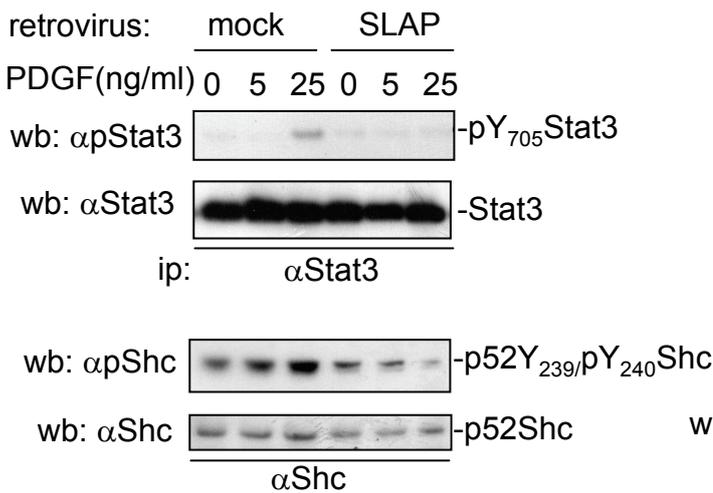
Figure 3a, b



c



d



e

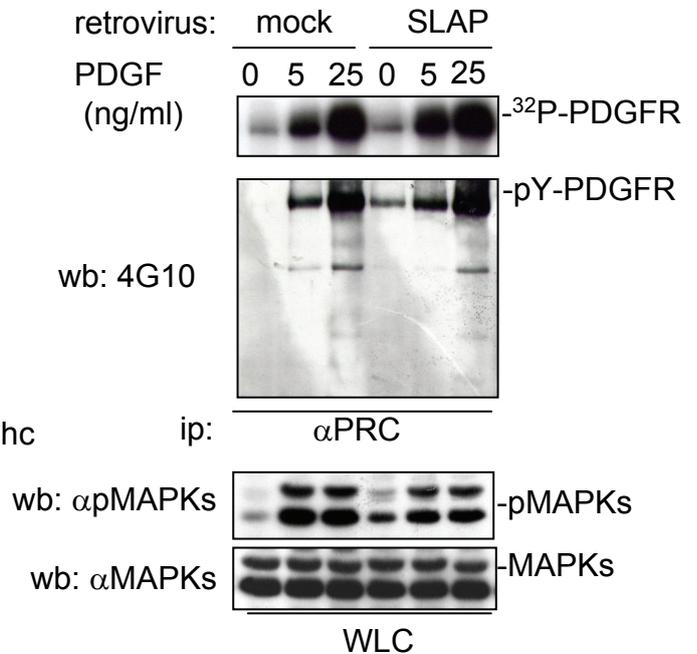
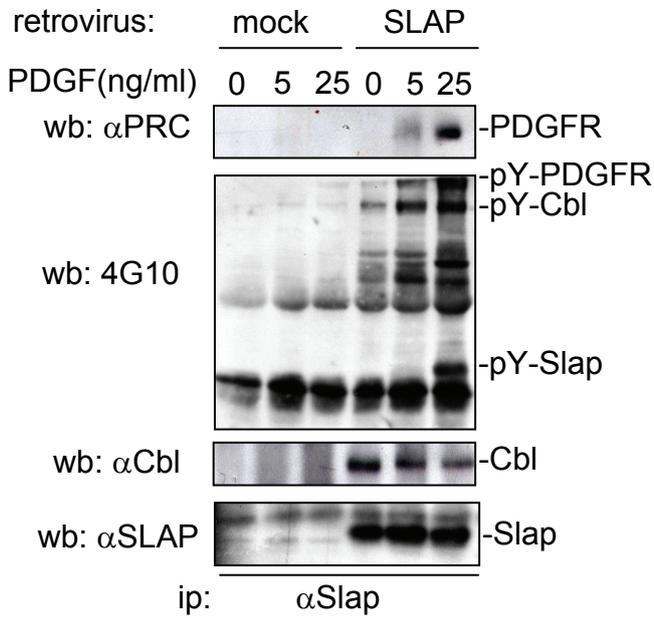
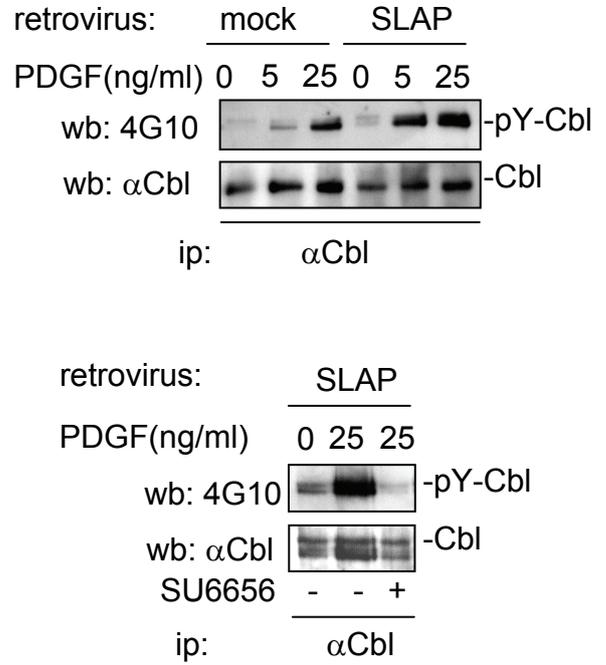


Figure 4a-c

a



b



c

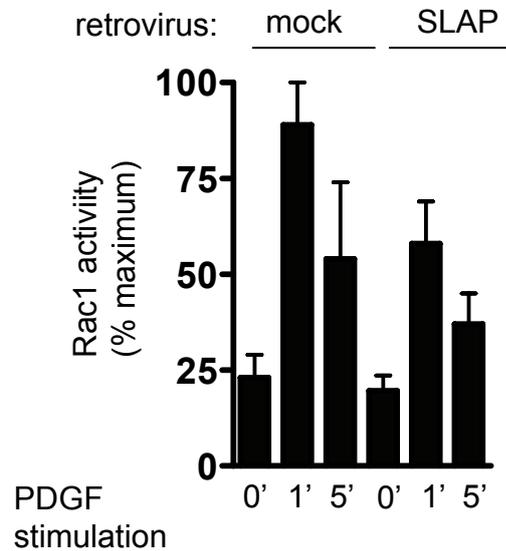
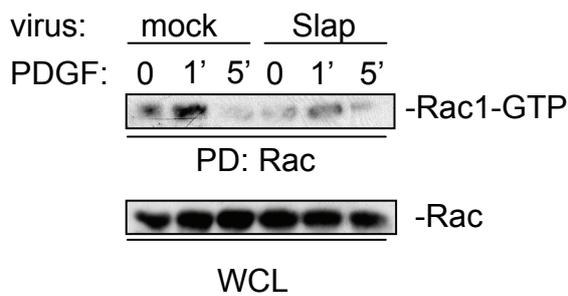


Figure 4d

d

