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CORRECTION

Src-Like Adaptor Protein (SLAP) differentially regulates normal and oncogenic c-Kit signaling

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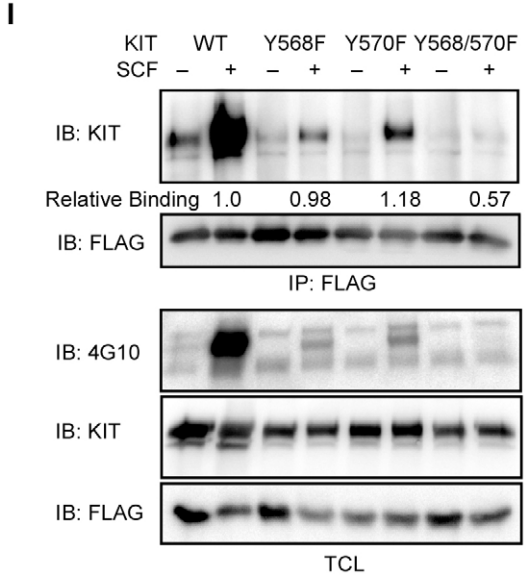
There was an error published in *J. Cell Sci.* **127**, 653–662.

The second panel of Fig. 1I was supposed to show immunoblotting of immunoprecipitated FLAG-tagged SLAP but, unfortunately, showed immunoblotting of whole-cell lysate (identical to the blot in the fifth panel of the figure). This has now been corrected so that the third panel shows immunoblotting of immunoprecipitated FLAG-tagged SLAP as indicated.



The authors apologise for this mistake.

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RESEARCH ARTICLE

Src-like-adaptor protein (SLAP) differentially regulates normal and oncogenic c-Kit signaling

Julhash U. Kazi[1], Shruti Agarwal[1], Jianmin Sun[1], Enrico Bracco[2] and Lars Rönnstrand[1,*]

ABSTRACT

The Src-like-adaptor protein (SLAP) is an adaptor protein sharing considerable structural homology with Src. SLAP is expressed in a variety of cells and regulates receptor tyrosine kinase signaling by direct association. In this report, we show that SLAP associates with both wild-type and oncogenic c-Kit (c-Kit-D816V). The association involves the SLAP SH2 domain and receptor phosphotyrosine residues different from those mediating Src interaction. Association of SLAP triggers c-Kit ubiquitylation which, in turn, is followed by receptor degradation. Although SLAP depletion potentiates c-Kit downstream signaling by stabilizing the receptor, it remains non-functional in c-Kit-D816V signaling. Ligand-stimulated c-Kit or c-Kit-D816V did not alter membrane localization of SLAP. Interestingly oncogenic c-Kit-D816V, but not wild-type c-Kit, phosphorylates SLAP on residues Y120, Y258 and Y273. Physical interaction between c-Kit-D816V and SLAP is mandatory for the phosphorylation to take place. Although tyrosine-phosphorylated SLAP does not affect c-Kit-D816V signaling, mutation of these tyrosine sites to phenylalanine can restore SLAP activity. Taken together the data demonstrate that SLAP negatively regulates wild-type c-Kit signaling, but not its oncogenic counterpart, indicating a possible mechanism by which the oncogenic c-Kit bypasses the normal cellular negative feedback control.

KEY WORDS: Kit, c-Kit-D816V, D816V, Receptor tyrosine kinase, Signal transduction, SLA, Ubiquitylation

INTRODUCTION

The Src-like adaptor protein (SLAP) is an adaptor protein containing both Src homology 2 (SH2) and SH3 domains. SLAP displays considerable structural homology with Src but lacks its kinase domain. Similar to Src, SLAP is myristoylated, thus it localizes to the cell membrane (Manes et al., 2000). Furthermore, the presence of an SH2 domain facilitates association of SLAP with many receptors through phosphotyrosine residues and thereby impairs Src-mediated signaling by competing with Src itself.

SLAP is primarily implicated in negative regulation of receptor signaling by facilitating the recruitment of E3 ubiquitin ligases. Because SLAP is capable of association with E3 ubiquitin ligase Cbl through its C-terminal tail in a phosphorylation-independent manner, it can form multi-proteins complex (Tang et al., 1999). The ability to self-dimerize also allows SLAP to recruit many proteins at the same time (Tang et al., 1999).

Despite its role in negative regulation of receptor signaling, SLAP is also capable of potentiating signals from certain receptors through unknown mechanism. SLAP transduces mitogenic signals from Syk to NAFT-AP1 and depletion of SLAP reduces FLT3 ligand-induced Akt and Erk1/2 phosphorylation (Kazi and Rönnstrand, 2012; Tang et al., 1999) suggesting that SLAP plays differential roles depending on its kinase partner.

Type III receptor tyrosine kinase (RTK) c-Kit (also known as Kit) is of importance in a variety of physiological conditions and is frequently mutated in many cancers (Kazi et al., 2008; Lennartsson and Rönnstrand, 2012). Whereas wild-type c-Kit receptor requires ligand stem cell factor (SCF) to trigger its dimerization and activation, the oncogenic c-Kit mutants display ligand-independent activation. One of the very first manifestations of an active c-Kit receptor is auto-phosphorylation on multiple tyrosine residues. These phosphotyrosine residues facilitate docking of multiple SH2-domain-containing proteins which, in turn, further propagate or counteract receptor signaling by distinct mechanisms (Masson and Rönnstrand, 2009). For example, association of Cbl through Y568 directs c-Kit to lysosomal degradation (Masson et al., 2006), whereas association of Src through the same tyrosine residue potentiates downstream signaling (Lennartsson et al., 1999). Many receptor-interacting proteins tightly regulate receptor stability, phosphorylation and signaling by linking kinases, ubiquitin ligases and other signaling molecules (Kazi and Rönnstrand, 2013a; Kazi and Rönnstrand, 2013b; Kazi et al., 2012; Kazi et al., 2013b; Lin et al., 2012).

Here, we show that SLAP associates with both normal and oncogenic mutants of c-Kit and that these associations have differential impacts on receptor downstream signaling.

RESULTS

SLAP associates with SCF-stimulated wild-type c-Kit as well as with oncogenic c-Kit-D816V

There is increasing evidence that SLAP regulates receptor signaling. We and other groups recently showed that SLAP associates with a variety of receptors including EphA (Semerdjieva et al., 2013) and FLT3 (Kazi and Rönnstrand, 2012) in a phosphorylation-dependent manner. FLT3 is a type III RTK that is found to be frequently mutated in hematopoietic malignancies (Kabir et al., 2013). In our previous report we also showed that SLAP is highly expressed in hematopoietic cells. Since c-Kit belongs to the same RTK family as FLT3 and is highly expressed in hematopoietic malignancies, we tested whether SLAP correspondingly associates with c-Kit. We co-transfected FLAG-tagged SLAP with wild-type c-Kit or an oncogenic version with an aspartic acid to valine mutation (c-Kit-D816V). We were only able to pull down c-Kit with an anti-FLAG antibody from cells that were stimulated with SCF prior to lysis (Fig. 1A, lane 3). The

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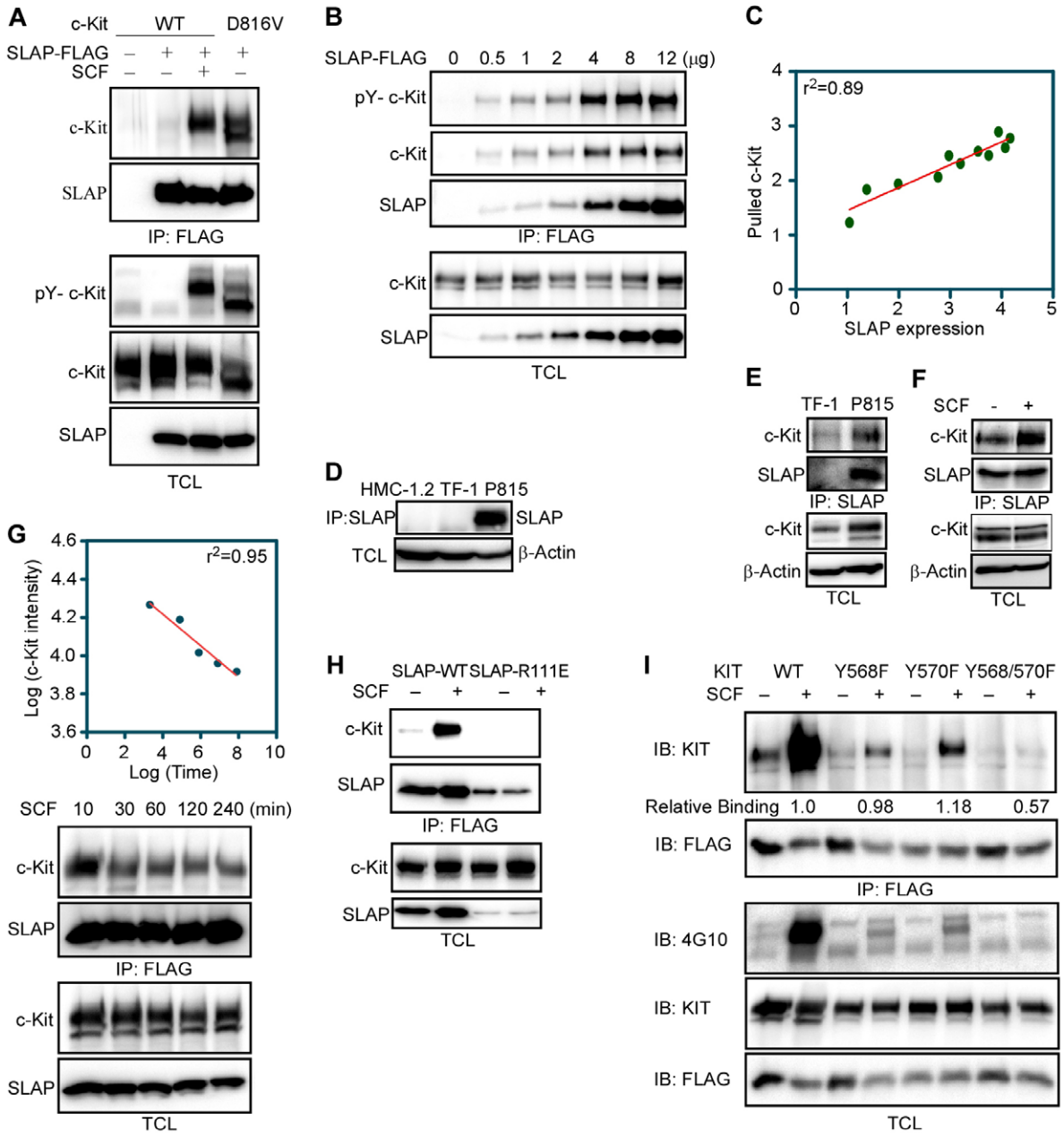

Fig. 1. SLAP associates with c-Kit-WT and c-Kit-D816V through the SLAP SH2 domain. (A) COS-1 cells were transfected with SLAP-FLAG and c-Kit-WT or c-Kit-D816V constructs. Cells were stimulated with SCF for 10 minutes where required and immediately lysed. Cell lysates were immunoprecipitated with an anti-FLAG antibody and then processed for western blot analysis. (B) COS-1 cells were transfected with c-Kit-WT and SLAP-FLAG and/or empty vector. Cells were stimulated with SCF for 10 minutes and processed for immunoprecipitation followed by western blotting. (C) COS-1 cells transfected with an equal amount of c-Kit-WT and increasing amount of SLAP-FLAG. Cells were stimulated with SCF for 10 minutes and cell lysates were subjected to FLAG immunoprecipitation. Western blots from two experiments with different concentrations of SLAP (relative SLAP expression, arbitrary units) and immunoprecipitated c-Kit (relative c-Kit amount that was pulled down by SLAP-FLAG, arbitrary units) were quantified and plotted using GraphPad Prism 5.0. (D) Cell lysates from HMC1.2, TF-1 and P815 cells were immunoprecipitated with an anti-SLAP antibody and then analyzed by western blotting. (E) Cell lysates from TF-1 and P815 cells were immunoprecipitated with an anti-SLAP antibody and then analyzed by western blotting. (F) Cell lysates from HL60 cells stimulated with SCF or not were immunoprecipitated with an anti-SLAP antibody and then analyzed by western blotting. (G) COS-1 cells were transfected with SLAP-FLAG and c-Kit-WT constructs. Cells were stimulated with SCF for different times followed by immunoprecipitation and western blotting analysis. The amount of c-Kit was quantified and the log value of the SCF stimulation time (min) was plotted against the log value of the c-Kit amount. (H) COS-1 cells were transfected with c-Kit-WT and FLAG-tagged SLAP-WT or SLAP-R111E constructs. Cells were stimulated with SCF or left unstimulated before lysis. Lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by western blotting. (I) COS-1 cells were transfected with SLAP-FLAG and c-Kit-WT or mutants. Cells were stimulated with SCF or left unstimulated before lysis. Lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by western blotting. Relative binding was calculated by normalization against total pY-c-Kit.

oncogenic c-Kit-D816V mutant is characterized by constitutive tyrosine kinase activity. In the case of the c-Kit-D816V mutant, a strong ligand-independent interaction between SLAP and c-Kit-D816V was detected (Fig. 1A, lane 4). These data suggest that SLAP associates with c-Kit only when c-Kit is tyrosine phosphorylated. The efficiency of c-Kit pull-down from cell lysates was independent of c-Kit

Journal of Cell Science (2014) 127, 653–662 doi:10.1242/jcs.140590

expression but was substantially affected by SLAP expression (Fig. 1B,C) indicating that association is dependent on availability of the SLAP protein. To explore endogenous binding, we checked SLAP expression in the human mastocytoma HMC-1.2, human erythroleukemia TF-1 and mouse mastocytoma P815 cell lines. The TF-1 cell line endogenously expresses wild-type c-Kit, whereas both HMC-1.2 and P815 cell lines express the oncogenic mutant, c-Kit-D816V. We observed that the P815 cell line expresses higher levels of SLAP than the other two cell lines (Fig. 1D). We then pulled-down SLAP from P815 cell lysate using an anti-SLAP antibody. TF-1 cell lysate was used as a negative control. We were able to detect c-Kit-SLAP complexes in P815 cells (Fig. 1E). We were also able to detect endogenous c-Kit and SLAP interaction in HL60 cells that expressed wild-type c-Kit, and the interaction was ligand dependent (Fig. 1F). In addition, c-Kit–SLAP interaction displayed a linear decrease over the time but complexes were detectable until 4 hours of SCF stimulation, suggesting that SLAP stably associates with c-Kit (Fig. 1G).

SLAP associates with c-Kit through its SH2 domain at a site different from the Src-binding site

SLAP associates only with the activated form of c-Kit suggesting that the association is mediated through phosphotyrosine residues of c-Kit and the SLAP SH2 domain. To test this hypothesis, we generated the SLAP-R111E mutant in which the positively charged crucial arginine residue in the phosphotyrosine binding pocket of the SH2 domain was replaced with a negatively charged glutamic acid. Thereby, this mutant has a negatively charged phosphotyrosine binding pocket that will repulse phosphotyrosine residues. We co-expressed c-Kit-WT and SLAP-WT-FLAG or SLAP-R111E-FLAG in COS-1 cells. Although wild-type SLAP efficiently pulled down c-Kit, the interaction was completely absent when using the SLAP-SH2-domain-mutated form SLAP-R111E (Fig. 1H), confirming that SLAP associates with c-Kit through its SH2 domain. Because SLAP shares considerable structural similarity with Src, we also checked whether SLAP associates with c-Kit through the same tyrosine phosphorylation sites as Src. For this purpose we used the c-Kit-Y568F, c-Kit-Y570F and c-Kit-Y567F/Y570F mutants that fail to associate with Src. The results indicated that this mutant of c-Kit is unable to block the association with SLAP, although the binding was diminished because of the lower total tyrosine phosphorylation of these mutants (Fig. 1I). We therefore propose that SLAP associates with c-Kit through its SH2 domain and that the binding site is different from the one mediating binding to the structurally related protein Src.

SLAP depletion increases c-Kit tyrosine phosphorylation

The association of adaptor proteins with the RTK proteins affects receptor auto-phosphorylation (Lin et al., 2012). We transfected COS-1 cells with increasing amounts of SLAP plasmid while keeping the amount of c-Kit plasmid constant. Cells were serum-starved overnight and stimulated with SCF for a fixed period of time before lysis. We observed a linear decrease of c-Kit tyrosine phosphorylation as well as c-Kit expression with increasing SLAP expression but the net c-Kit tyrosine phosphorylation remained unchanged (Fig. 2A). Because overexpression of exogenous proteins can affect cellular behavior, we intended to check the effect of SLAP on c-Kit phosphorylation using endogenous proteins. Ba/F3 cells express a reasonable level of SLAP but lack c-Kit expression (Kazi and Rönnstrand, 2012). Thus, we used Ba/F3 cells stably transfected with c-Kit (Ba/F3-c-Kit cells) (Kazi et al., 2013b), and further stably transfected with

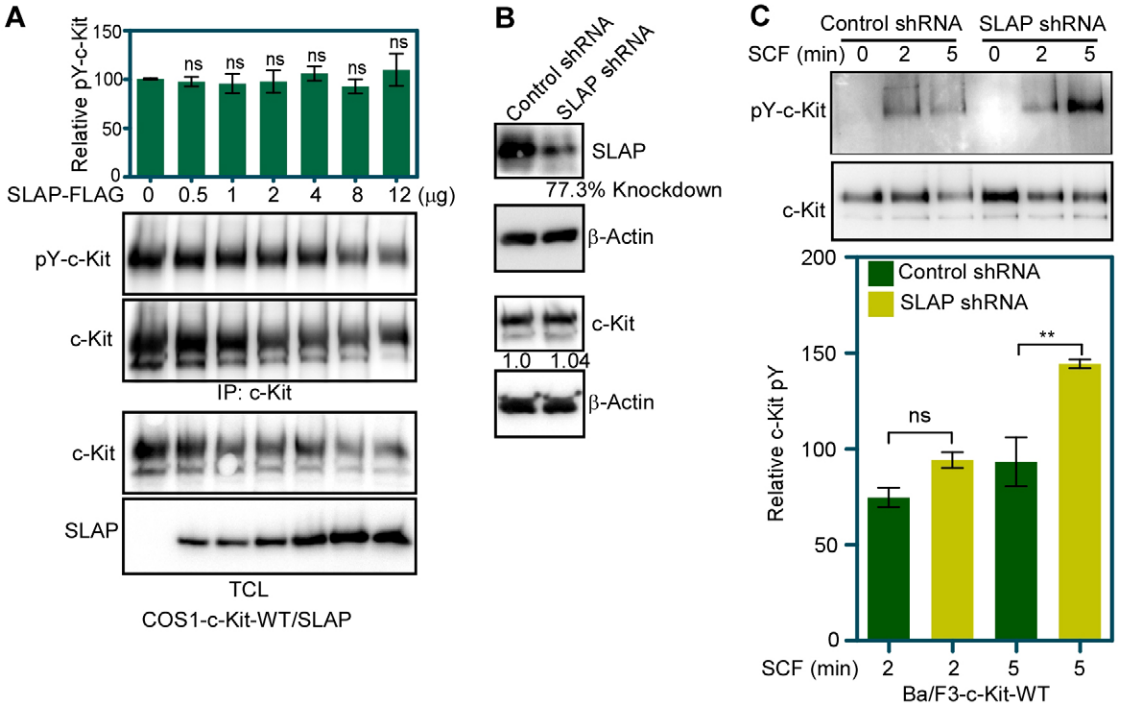

Fig. 2. SLAP depletion increases c-Kit phosphorylation. (A) COS-1 cells were transfected with c-Kit-WT and different amount of SLAP-FLAG and/or empty control vector. Cells were stimulated for 5 minutes before lysis and subjected to immunoprecipitation and western blotting analysis. (B) Ba/F3 cells stably transfected with SLAP shRNA or empty control vector were lysed and processed for immunoprecipitation with an anti-SLAP antibody followed by western blotting. (C) Ba/F3 c-Kit-WT-SLAP-shRNA and Ba/F3-c-Kit-WT-control-shRNA were stimulated with SCF for different times before lysis. Lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by western blotting. Blots from three experiments were quantified using Multi Gauge software. ns, not significant; $^{**}P<0.01$.

Journal of Cell Science (2014) 127, 653–662 doi:10.1242/jcs.140590

control shRNA vector (Ba/F3-c-Kit-control cells) or SLAP shRNA vector (Ba/F3-c-Kit-SLAP-shRNA cells). We were able to achieve a 77.3% SLAP knockdown while keeping the c-Kit expression unchanged (Fig. 2B). Ba/F3-c-Kit-control and Ba/F3-c-Kit-SLAP-shRNA cells were stimulated with SCF for different periods of time and then subjected to immunoprecipitation with an anti-c-Kit antibody. Upon SLAP depletion c-Kit tyrosine phosphorylation was significantly ($P<0.01$) increased (Fig. 2C) suggesting that SLAP is required in order to maintain the normal level of receptor tyrosine phosphorylation.

SLAP depletion decreases c-Kit ubiquitylation and degradation

SLAP association with FLT3 increases receptor ubiquitylation by recruiting the Cbl E3 ubiquitin ligase to the receptor (Dragone et al., 2006; Kazi and Rönnstrand, 2012). We used Ba/F3-c-Kit-control and Ba/F3-c-Kit-SLAP-shRNA cells to evaluate the effect of SLAP on c-Kit ubiquitylation. Cells were serum-starved and treated with MG132 and chloroquine diphosphate prior to SCF stimulation. We observed that depletion of SLAP significantly ($P<0.05$) decreased c-Kit ubiquitylation in response to SCF (Fig. 3A), which is in line with a previous finding that SLAP potentiates ubiquitylation of RTKs (Dragone et al., 2006; Kazi and Rönnstrand, 2012). Because SLAP expression resulted in increased ubiquitylation of c-Kit, we then tested whether increased ubiquitylation leads to increased degradation of the receptor. We observed that although SLAP expression significantly ($P<0.001$) increased c-Kit degradation in COS-1 cells (Fig. 3B), SLAP depletion significantly ($P<0.05$) stabilized c-Kit in Ba/F3 cells (Fig. 3C).

SLAP depletion increases SCF-induced Akt and Erk1/2 phosphorylation

SCF stimulation activates PI3K and MAPK pathways resulting in phosphorylation of Akt, Erk1/2 and p38 (Lennartsson and Rönnstrand, 2012; Masson and Rönnstrand, 2009). To assess the role of SLAP in c-Kit downstream signaling, we stimulated Ba/F3-c-Kit-control shRNA and Ba/F3-c-Kit-SLAP-shRNA cells with SCF after 4 hours starvation. SCF stimulation significantly ($P<0.05$) increased Akt phosphorylation at 5 minutes (Fig. 4A). Erk1/2 phosphorylation was decreased at 2 minutes but increased at 5 minutes (Fig. 4B). Phosphorylation of p38 remained mostly unchanged (Fig. 4C). Furthermore, SCF stimulation for an extended time period also had a similar effect on Akt (Fig. 4D) and Erk1/2 (Fig. 4E) phosphorylation. Thus, we conclude that SLAP negatively regulates SCF-induced c-Kit downstream signaling in a selective manner.

SLAP controls Akt and Erk1/2 phosphorylation through SHC and Gab2

Stimulation with SCF induces Akt and Erk1/2 phosphorylation through several pathways. To understand which pathways are affected by SLAP, we investigated the phosphorylation status of SHC, SHP2 and Gab2. SHC phosphorylation was significantly ($P<0.05$) decreased after 2 minutes of SCF stimulation whereas it was increased after 5 minutes of SCF stimulation in SLAP-depleted cells (Fig. 5A). SLAP depletion did not alter SHP2 phosphorylation (Fig. 5B) but enhanced Gab2 phosphorylation after 5 minutes of SCF stimulation (Fig. 5C).

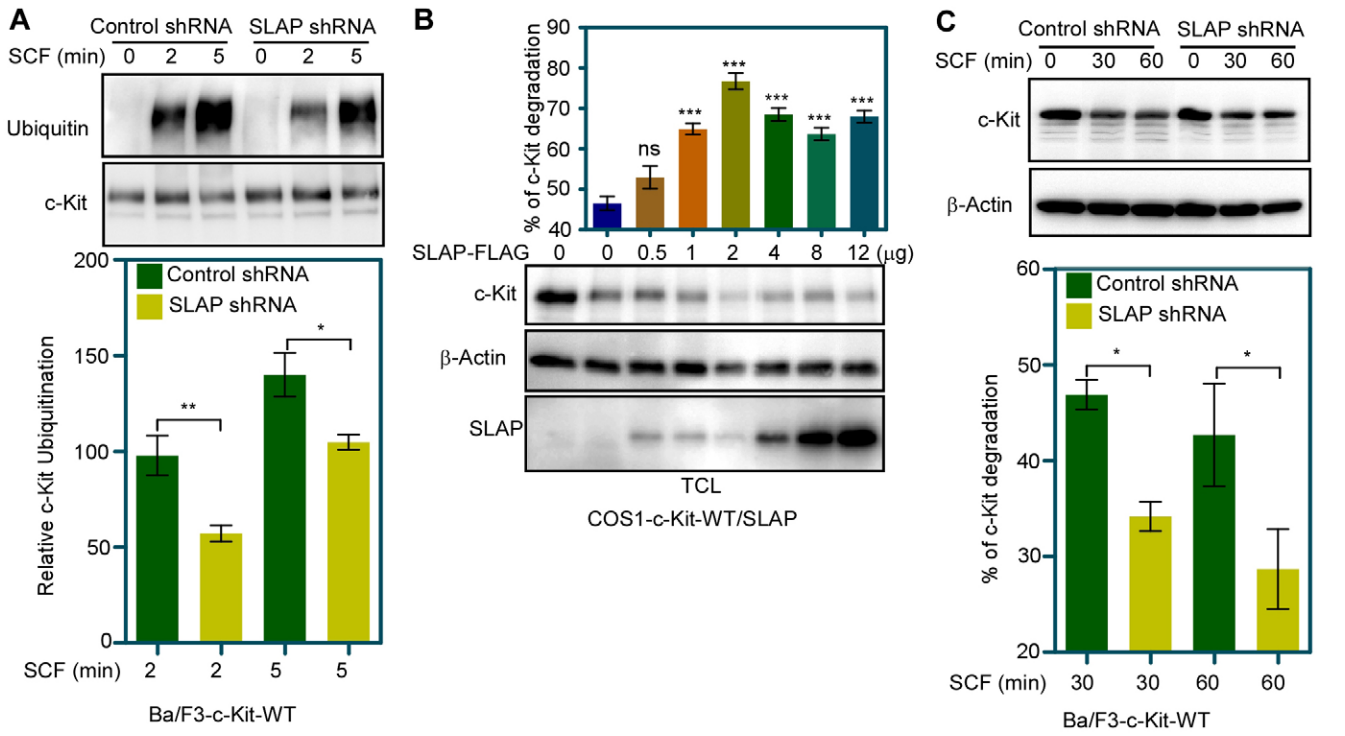

Fig. 3. SLAP depletion decreases c-Kit ubiquitylation and degradation. (A) Ba/F3-c-Kit-WT-SLAP-shRNA and Ba/F3-c-Kit-WT-control-shRNA cells were stimulated with SCF for different times before lysis. Lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by western blotting. Relative levels of ubiquitylation were then determined. (B) COS-1 cells were transfected with c-Kit-WT and different amount of SLAP or empty control vector. Cells were incubated with cycloheximide for 30 minutes before 30 minutes of SCF stimulation. Then cells were lysed and lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by western blotting. The percentage of c-Kit degradation was then determined. (C) Ba/F3-c-Kit-WT-SLAP-shRNA and Ba/F3-c-Kit-WT-control-shRNA cells were incubated with cycloheximide for 30 minutes before 30 and 60 minutes of SCF stimulation. Cells were then lysed and lysates were processed for immunoprecipitation with an anti-c-Kit antibody followed by western blotting. Blots from three experiments were quantified using Multi Gauge software. ns, not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Journal of Cell Science (2014) 127, 653–662 doi:10.1242/jcs.140590

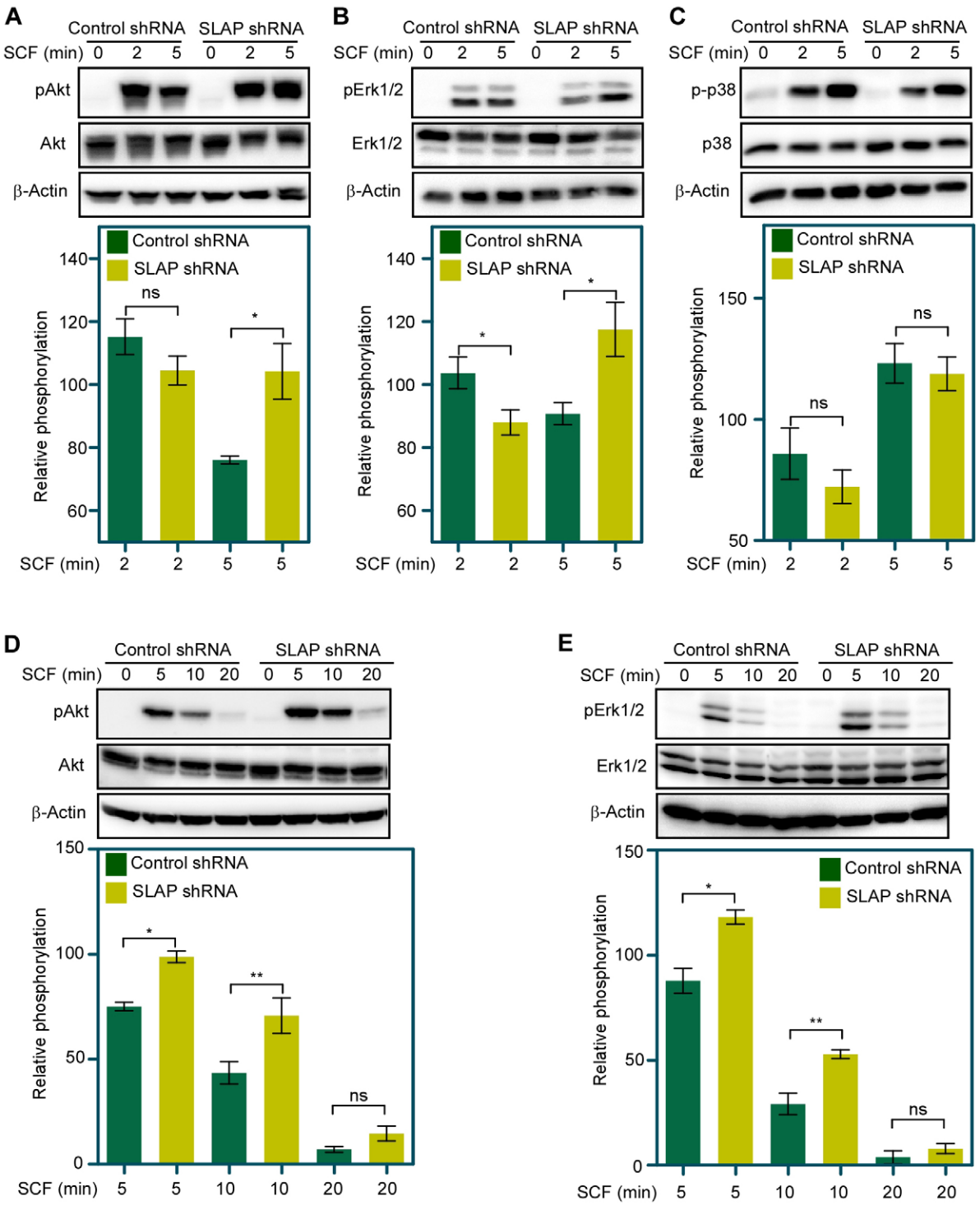

Fig. 4. SLAP depletion accelerates Akt and Erk1/2 phosphorylation. (A–E) Ba/F3-c-Kit-WT-SLAP-shRNA and Ba/F3-c-Kit-WT-control-shRNA cells were serum starved for 4 hours before SCF stimulation for 0, 2 or 5 minutes (A–C) or 0, 5, 10 or 20 minutes (D,E). Cells were then lysed and lysates were subjected for western blotting analysis using (A,D) anti-phospho-Akt, anti-Akt and anti-β-actin, (B,E) anti-phospho-Erk1/2, anti-Erk and anti-β-actin, and (C) anti-phospho-p38, anti-p38 and anti-β-actin. Signal intensities were quantified using Multi Gauge software from three experiments. ns, not significant; *$P<0.05$.

SLAP does not block c-Kit-D816V-induced Akt and Erk phosphorylation

c-Kit and its oncogenic mutant c-Kit-D816V have different effects in cellular signaling (Lennartsson et al., 2003; Sun et al., 2009). Because SLAP associates with c-Kit-D816V, we hypothesized that SLAP might negatively regulate c-Kit-D816V signaling in a similar fashion to wild-type c-Kit. We transfected wild-type SLAP or empty vector into the HMC-1.2 cells that harbor the oncogenic mutant of c-Kit. Surprisingly, SLAP expression did not block SCF-induced Akt and Erk1/2 phosphorylation (Fig. 6A). Similar results were obtained from SLAP-depleted Ba/F3-c-Kit-D816V cells (Fig. 6B). Furthermore,

Journal of Cell Science (2014) 127, 653–662 doi:10.1242/jcs.140590

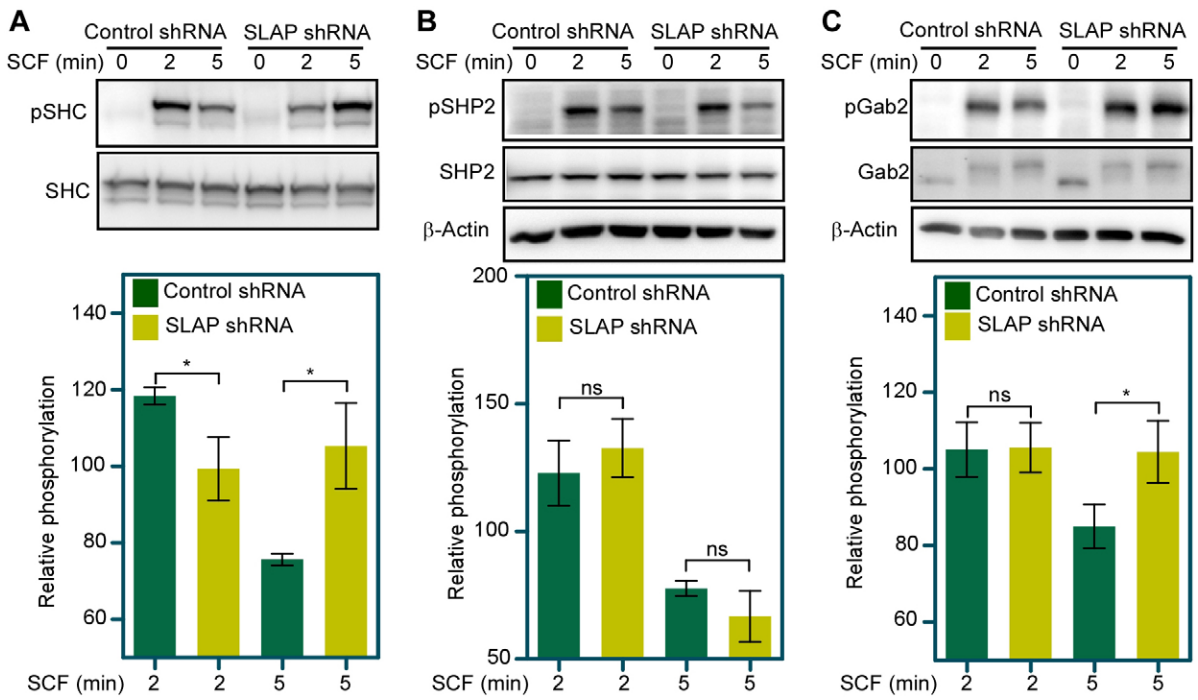

Fig. 5. SLAP depletion accelerates SHC and Gab2 phosphorylation. Ba/F3-c-Kit-WT-SLAP-shRNA and Ba/F3-c-Kit-WT-control-shRNA cells were serum starved for 4 hours before SCF stimulation followed by lysis. (A) Cell lysates were immunoprecipitated with an anti-SHC antibody and then analyzed by western blotting. (B) Cell lysates were subjected to western blotting analysis using anti-phospho-SHP2, anti-SHP2 and anti-β-actin. (C) Cell lysates were subjected to western blotting analysis using anti-phospho-Gab2, anti-Gab2 and anti-β-actin. Signal intensities were quantified using Multi Gauge software from three experiments. ns, not significant; $*P<0.05$.

although SLAP depletion accelerated SCF-induced cell proliferation, it did not affect c-Kit-D816V-induced cell proliferation (Fig. 6C). Therefore, we suggest that SLAP negatively regulates wild-type c-Kit signaling but not oncogenic c-Kit-D816V signaling.

SLAP shows similar subcellular localization patterns with c-Kit-WT and c-Kit-D816V

Because SLAP has different impacts on c-Kit signaling, we checked whether this is due to the differential sub-cellular localization of SLAP. We transfected COS-1 cells with SLAP-FLAG and c-Kit-WT or c-Kit-D816V. Cells were serum-starved overnight and the c-Kit-WT-transfected cells were stimulated with SCF before paraformaldehyde fixation. Cells were then stained with Alexa-Fluor-647-conjugated anti-FLAG (DYKDDDDK) and PE-conjugated c-Kit antibodies. Subcellular localization was visualized with a laser scanning confocal microscope. As expected SLAP was found to be localized near the inner surface of the cell membrane in both unstimulated (Fig. 7A) and SCF-stimulated cells (Fig. 7B) as well as in c-Kit-D816V-expressing cells (Fig. 7C). In addition, SCF stimulation and expression of c-Kit-D816V significantly ($P<0.05$) increased colocalization of SLAP with c-Kit, but colocalization of SLAP and c-Kit-WT or SLAP and c-Kit-D816V were not affected (Fig. 7D).

Oncogenic c-Kit-D816V but not SCF stimulated wild-type c-Kit induces SLAP tyrosine phosphorylation

To understand the mechanism of the differential regulation of normal and oncogenic c-Kit signaling, we checked whether SLAP was differentially modified by wild-type and oncogenic c-Kit. We observed that only c-Kit-D816V but not wild-type c-Kit was able to phosphorylate SLAP on tyrosine residues (Fig. 8A). Thus, we suggest that SLAP-mediated differential regulation of c-Kit downstream signaling might be due to the differential post-translational modification of SLAP by oncogenic c-Kit.

Oncogenic c-Kit-D816V phosphorylates SLAP on residues Y120, Y258 and Y273

SLAP tyrosine phosphorylation sites have not been well studied. The PhosphoSitePlus database (www.phosphosite.org) describes three different predicted phosphorylation sites of SLAP in three different species (Fig. 8B). To explore SLAP tyrosine phosphorylation sites in living cells, we generated Y to F mutants of the corresponding human SLAP tyrosine residues and observed that mutation in any of those three sites reduced the total tyrosine phosphorylation of SLAP, and that Y120 and Y258 sites are the major tyrosine phosphorylation sites in SLAP phosphorylated by c-Kit-D816V (Fig. 8C). Then we generated a SLAP-Y120F-Y258F-Y273F (SLAP-YYYFFF) mutant to check whether SLAP has any other minor tyrosine phosphorylation sites other than those predicted. The triple mutant completely abolished c-Kit-D816V-mediated tyrosine phosphorylation (Fig. 8D) of SLAP, indicating the presence of only three tyrosine phosphorylation sites (pY120, pY258 and pY273) in SLAP.

Association of SLAP with c-Kit-D816V is required for tyrosine phosphorylation of SLAP

We then checked whether association of SLAP with c-Kit-D816V is required for c-Kit-D816V-mediated tyrosine phosphorylation of SLAP. We transfected cells with wild-type SLAP and non-functional SH2 domain mutant SLAP-R111E together with c-Kit-D816V. We observed that while c-Kit-D816V strongly phosphorylates wild-type SLAP, SLAP-R111E remains mostly non-tyrosine-phosphorylated (Fig. 8E) indicating that association of SLAP with c-Kit-D816V is required for tyrosine phosphorylation of SLAP.

Journal of Cell Science (2014) 127, 653–662 doi:10.1242/jcs.140590

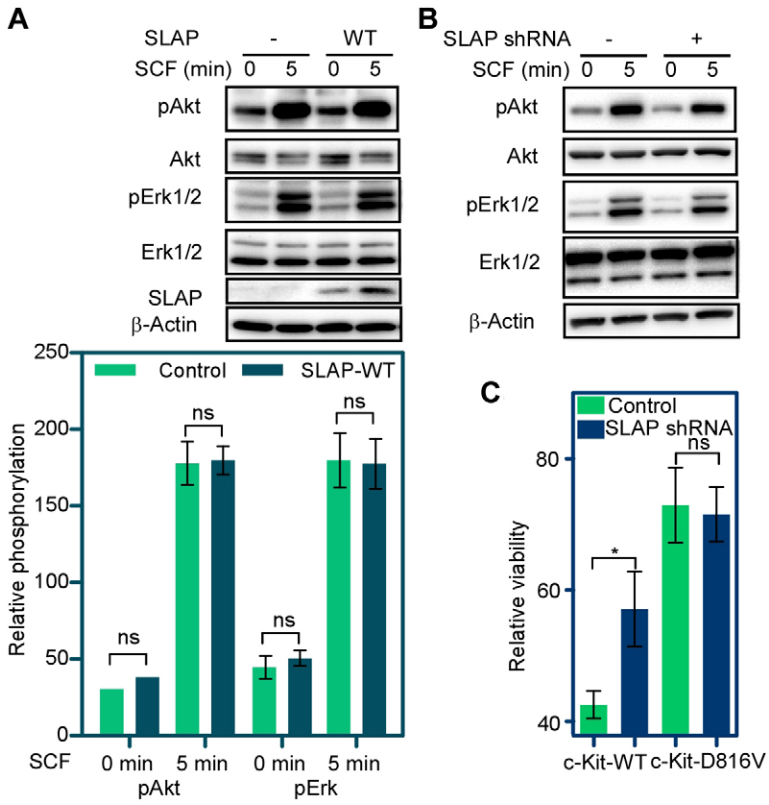

Fig. 6. SLAP expression does not alter c-Kit-D816V-mediated Akt or Erk1/2 phosphorylation. (A) HMC-1.2 cells transfected with SLAP-WT or empty control vector were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were processed for western blotting analysis using anti-phospho-Akt, anti-Akt, anti-phospho-Erk1/2, anti-Erk, anti-SLAP and anti-β-actin. (B) Ba/F3-c-Kit-D816V-SLAP-shRNA and Ba/F3-c-Kit-D816V-control-shRNA cells were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were subjected for western blotting analysis using anti-phospho-Akt, anti-Akt, anti-phospho-Erk1/2, anti-Erk and anti-β-actin. Signal intensities were quantified using Multi Gauge software from three experiments. ns, not significant; *$P<0.05$.

Mutation of the SLAP tyrosine phosphorylation sites rescues its activity

Since we observed that c-Kit and its oncogenic counterpart c-Kit-D816V differentially mediate SLAP tyrosine phosphorylation, and SLAP differentially regulates wild-type and mutant c-Kit signaling, we suggest that tyrosine phosphorylation of SLAP inactivates SLAP. To verify this hypothesis we transfected wild-type SLAP, SLAP-YYYFFF and empty vector into the HMC-1.2 cell line. We observed that SLAP-YYYFFF significantly ($P<0.05$) decreased SCF-induced Akt and Erk1/2 phosphorylation (Fig. 9A,B) suggesting that tyrosine phosphorylation of SLAP disrupts SLAP activity. Single tyrosine mutants (SLAP-Y120F or -Y258F or -Y273F) also exhibited similar reduction in phosphorylation (Fig. 9C). These results are further supported by the observation that expression of SLAP-YYYFFF but not SLAP-WT significantly ($P<0.05$) decreased c-Kit-D816V-mediated cell growth (Fig. 9D).

DISCUSSION

Adaptor proteins control RTK signaling either by recruiting ubiquitin ligases, phosphatases, kinases and other signaling proteins, or competing with kinases or phosphatases for association with receptors. Although the adaptor protein SLAP negatively regulates RTK signaling by influencing degradation and thereby reducing receptor turnover (Dragone et al., 2006; Semerdjieva et al., 2013; Sosinowski et al., 2000), a couple of reports suggest that SLAP propagates additional receptor signaling through, as yet, poorly understood mechanisms (Kazi and Rönnstrand, 2012; Tang et al., 1999). In this report we show

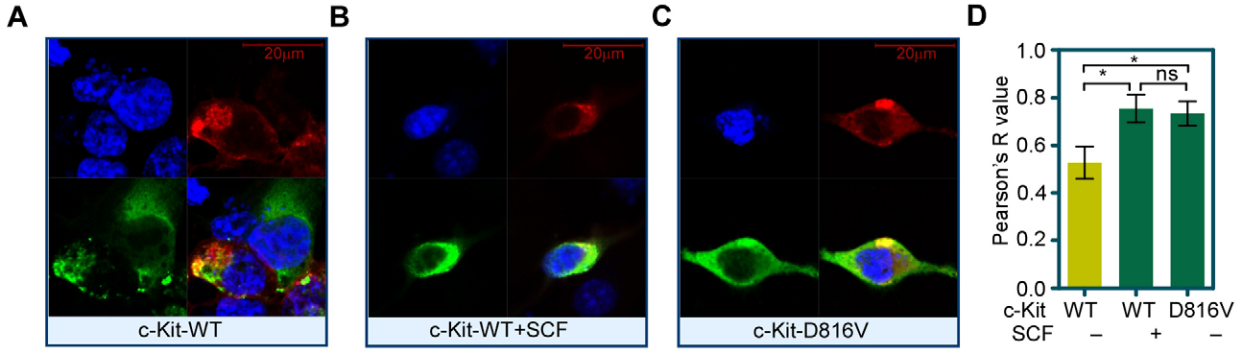

Fig. 7. SCF stimulation increases colocalization of SLAP with c-Kit. (A–C) COS-1 cells were co-transfected with SLAP-FLAG and c-Kit-WT or c-Kit-D816V plasmids. Cells were serum starved overnight before stimulation and then fixed, permeabilized and stained with fluorophore-conjugated anti-FLAG antibody, anti-c-Kit antibody and DAPI . Blue, DAPI; red, c-Kit; green, SLAP–FLAG. (D) Colocalization was quantified using CoLocalizer Pro 2.7.1. Pearson's *R*-value was used to measure colocalization of red and green colors from five cells. ns, not significant; *$P<0.05$.

Journal of Cell Science (2014) 127, 653–662 doi:10.1242/jcs.140590

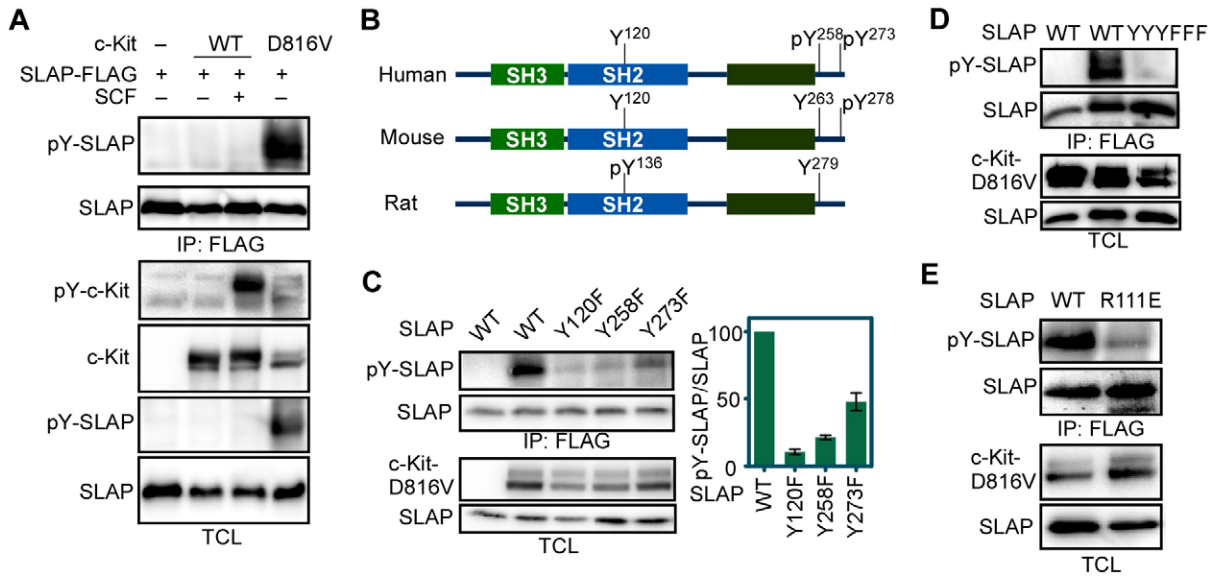

Fig. 8. c-Kit-D816V induces tyrosine phosphorylation of SLAP. (A) Cells were transfected with SLAP-FLAG and c-Kit-WT or c-Kit-D816V. Cells were serum starved overnight before SCF stimulation. Cells were then lysed and lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by western blotting. (B) Schematic showing the conserved tyrosine residues in human, mouse and rat. (C,D) Cells were transfected with FLAG-tagged SLAP mutants and c-Kit-D816V. Cells were serum starved overnight before lysis and lysates were immunoprecipitated with an anti-FLAG antibody and analyzed by western blotting. (E) Cells were transfected with c-Kit-D816V and FLAG-tagged SLAP-WT or SLAP-R111E mutant. Cells were serum starved overnight before lysis. Lysates were immunoprecipitated with an anti-FLAG antibody and then analyzed by western blotting. pY-SLAP, tyrosine phosphorylated SLAP.

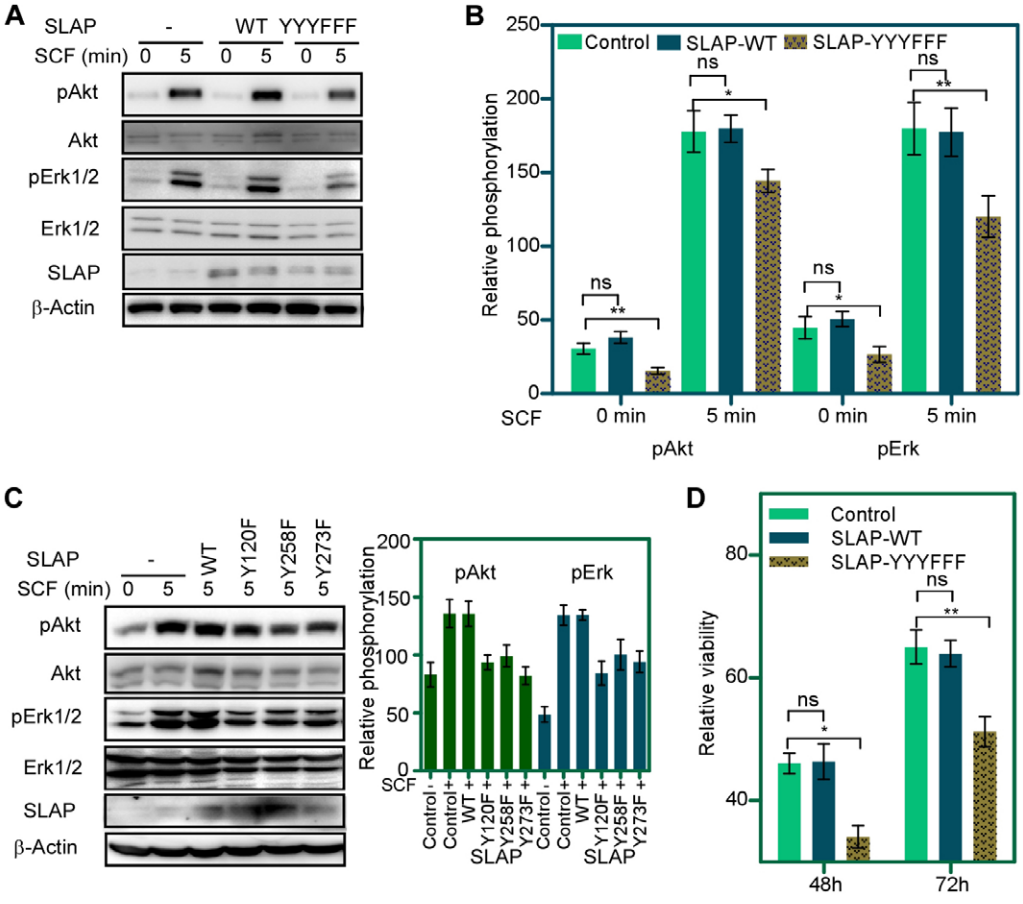

Fig. 9. SLAP mutant negatively regulates c-Kit-D816V-mediated Akt or Erk1/2 phosphorylation. (A) HMC-1.2 cells transfected with SLAP-WT, SLAP-YYYFFF or empty control vector were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were processed for western blot analysis. (B) Signal intensities from three experiments were quantified using Multi Gauge software. (C) HMC-1.2 cells transfected with SLAP-WT, SLAP-Y120F, SLAP-Y258F, SLAP-Y273F or empty control vector were serum starved for 4 hours before SCF stimulation. Cells were then lysed and lysates were processed for western blotting. Signal intensities from three experiments were quantified using Multi Gauge software. (D) HMC-1.2 cells transfected with SLAP-WT, SLAP-YYYFFF or empty control vector were seeded in 96-well plates and cultured for different time periods. Cell viability measured using PrestoBlue (Life Technologies) assays according to the manufacturer's protocol. ns, not significant; *$P<0.05$; **$P<0.01$.

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that SLAP plays dual roles in RTK signaling, depending on its tyrosine phosphorylation status.

SLAP interacts with ligand stimulated c-Kit as well as with its oncogenic form c-Kit-D816V. This association is mediated through the SLAP SH2 domain and results in accelerated ubiquitylation followed by receptor degradation. These findings indicate that the c-Kit–SLAP interaction is dependent on c-Kit tyrosine phosphorylation and that SLAP recruits ubiquitin ligases which tag c-Kit for degradation. This is the most common mechanism through which adaptor proteins regulate receptor stability as well as downstream signaling (Dragone et al., 2006; Dragone et al., 2009). Although, SLAP has a significant ($P<0.05$) effect on c-Kit ubiquitylation, it is not the only factor that regulates c-Kit stability. Several studies suggest that c-Kit directly interacts with several ubiquitin ligases, such as Cbl (Masson et al., 2006) and SOCS6 (Bayle et al., 2004), or through indirect association of Cbl through Grb2 (Sun et al., 2007). Thus, SCF-induced c-Kit degradation is controlled through a number of different pathways and SLAP contributes to these pathways by accelerating ubiquitylation.

Because SLAP accelerates receptor ubiquitylation followed by degradation, it was expected that SLAP depletion would positively regulate c-Kit downstream signaling. The fact that SLAP depletion enhanced Akt, Erk1/2, SHC and Gab2 phosphorylation at 5 minutes partially supports the idea that this effect is mediated through alteration of receptor stability, but early decrease of Erk1/2 or SHC phosphorylation and equal phosphorylation of p38 or SHP2 cannot be explained by the same mechanism. Therefore, it is of interest to identify how SLAP selectively controls c-Kit signaling. Enhanced Akt, Erk1/2 phosphorylation also correlates with increased receptor activation that was observed at 5 minutes of SCF stimulation in SLAP-depleted cells. It is not clear why SLAP depletion increases c-Kit tyrosine phosphorylation. One possible explanation is that SLAP is regulating ubiquitylation and degradation of another kinase that is involved in c-Kit phosphorylation. Src family kinases (SFKs) promote c-Kit tyrosine phosphorylation and SLAP is known to associate with SFKs (Lennartsson et al., 2003; Sosinowski et al., 2000). Another explanation could be that SLAP competes with SFKs for the same binding site. However, we showed that SLAP does not associate with the known Src-binding sites in c-Kit, so it is possible that other SFKs associate with c-Kit through different tyrosine residues, which overlaps with SLAP-binding sites. The non-receptor protein tyrosine kinase Syk is another candidate (Tang et al., 1999). If there is a depletion of SLAP, maybe more of the candidate tyrosine kinase will bind to c-Kit and be activated, and this would then contribute to c-Kit phosphorylation as well as downstream signaling. We know from previous studies that other tyrosine kinases can associate with and phosphorylate c-Kit (Lennartsson et al., 2003).

The inability of SLAP to elicit a negative regulation of c-Kit-D816V-mediated signaling is probably due to the tyrosine phosphorylation of SLAP, since the effect was restored in the triple tyrosine mutant of SLAP. Because Cbl-N interacts with SLAP through the C-terminus of SLAP and SLAP also forms homodimers through its C-terminus (Tang et al., 1999), tyrosine phosphorylation of SLAP in the C-terminus might disrupt homodimerization as well as interaction with Cbl and thereby disable the block of c-Kit-D816V-mediated signaling.

In conclusion we propose that SLAP negatively regulates signal transduction events triggered by activated wild-type c-Kit. In contrast, the oncogenic c-Kit-D816V form bypasses the negative control of SLAP by phosphorylating SLAP on tyrosine residues. Taken together, we demonstrate that an adaptor protein, SLAP, is able to dramatically influence signaling downstream of c-Kit despite the fact that it lacks enzymatic activity by itself.

MATERIALS AND METHODS

Plasmids, antibodies and reagents

The pcDNA3-c-Kit-WT, pcDNA3-c-Kit-D816V, pMSCV-c-Kit-WT, pMSCV-c-Kit-D816V and pcDNA3-SLAP-WT plasmids were described elsewhere (Kazi and Rönnstrand, 2012; Kazi et al., 2013b). The pc-DNA3-c-Kit-Y568F, pcDNA3-c-Kit-Y570F, pcDNA3-c-Kit-Y568F/Y570F, pcDNA3-SLAP-Y120F, pcDNA3-SLAP-Y258F, pcDNA3-SLAP-Y273F, pcDNA3-SLAP-Y120F/Y258/Y273F and pcDNA3-SLAP-R111E plasmids were generated using the QuikChange site-directed mutagenesis kit (Stratagene). All reagents and antibodies used in this study were described previously (Kazi et al., 2013b).

Cell lines and culture conditions

COS-1 and P815 cells were cultured in DMEM supplemented with 10% FBS. Ba/F3-c-Kit-WT and Ba/F3-c-Kit-D816V cells (Kazi et al., 2013b) were cultured in RPMI 1640 medium supplemented with 10% FBS and 10 ng/ml recombinant murine IL-3, as recommended (Kazi et al., 2013a). HMC-1.2 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS. TF-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 10 ng/ml recombinant human IL-3.

Electroporation, transient transfection and western blotting

Electroporation, transient transfection and western blotting methods were described previously (Kazi and Rönnstrand, 2012; Kazi et al., 2012; Kazi et al., 2013b). For electroporation of HMC-1.2 cells, 4D-Nucleofector (Lonza) was used. Cells were washed with PBS before electroporation in serum-free solution (Lonza) using the DS104 program. Where statistical analysis was applied, a minimum three replicates were used to calculate mean value and standard error of the mean.

Confocal microscopy

Cells were fixed with 4% paraformaldehyde and then permeabilized and blocked with a mixture of 0.5% Triton X-100 and 5% goat serum. Cells were then stained with fluorophore-conjugated anti-c-KIT and anti-FLAG antibodies for 1 hour. Nuclei were stained with DAPI before mounting. Subcellular localization of c-Kit and SLAP–FLAG was visualized with a Carl Zeiss LSM 710 laser scanning microscope. CoLocalizer Pro 2.7.1 (www.colocalizer.com) was used to measure the co-localization of green and red signals.

Competing interests

The authors declare no competing interests.

Author contributions

J.U.K. conceived, designed and performed the experiments, analyzed the data and wrote the manuscript. S.A. contributed in the c-Kit–D816V signaling experiments in Ba/F3 cells. J.S. generated c-Kit mutants. E.B. conceived the experiments and wrote the manuscript. L.R. conceived and designed the experiments, discussed data and wrote the manuscript. All authors read and approved the final manuscript.

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