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Direct binding of Cbl to Y568 and Y936 of the stem cell factor receptor/c-Kit is required for ligand-induced ubiquitination, internalization and degradation

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Running title: Cbl-mediated ubiquitination, internalization and degradation of c-Kit

ABSTRACT

The ubiquitin E3 ligase Cbl has been shown to negatively regulate tyrosine kinase receptors, including the stem cell factor receptor/c-Kit. Impaired recruitment of Cbl to c-Kit results in a deregulated positive signaling that eventually can contribute to carcinogenesis. Here we present data showing that Cbl is activated by the Src family kinases and recruited to c-Kit in order to trigger receptor ubiquitination. We demonstrate that phosphorylated Y568 and Y936 in c-Kit are involved in direct binding and activation of Cbl and that binding of the TKB domain of Cbl to c-Kit is specified by the presence of an isoleucine or leucine residue in position +3 to the phosphorylated tyrosine residue on c-Kit. Apart from the direct association between Cbl and c-Kit, we show that phosphorylation of Cbl by Src family kinase members is required for activation of Cbl to occur. Moreover, we demonstrate that Cbl mediates monoubiquitination of c-Kit and that the receptor is subsequently targeted for lysosomal degradation. Taken together, our findings reveal novel insights into the mechanisms by which Cbl negatively regulates c-Kit mediated signaling.

Keywords: c-Kit, Cbl, ubiquitin ligase, stem cell factor, Src family kinases, degradation

INTRODUCTION

C-Kit belongs to the family of type III RTKs¹ and plays a major role in survival, expansion and differentiation of hematopoietic progenitor cells, as well as stem cells involved in reproduction and pigmentation (for review, see [1]). Moreover, it is essential for the function of mast cells. These c-Kit dependent processes are triggered by the binding of SCF to c-Kit which results in dimerization of the receptor and subsequent activation of its intrinsic tyrosine kinase activity. This leads to autophosphorylation of the receptors on specific tyrosine residues which then serve as docking sites for signaling molecules containing either a SH2 or PTB domains, respectively (for review, see [2]). Proteins reported to interact with ligand-stimulated c-Kit include PI3 kinase, PLC- γ 1, STATs, Src family kinases (SFK) and a number of adaptor proteins such as Grb2, Grb7, Grb10, Crk and APS. Their activation and recruitment normally constitute the first steps in the signal relay cascade from activated c-Kit to intracellular downstream targets.

Under normal circumstances, the activation of c-Kit is tightly regulated and its inappropriate activation is associated with development of a number of malignancies, including acute myeloid leukemia, small cell lung cancer, gastrointestinal stromal tumors and testicular cancer (for review, see [3]). Negative regulation of RTKs occurs through the action of protein tyrosine phosphatases as well as through proteolytic degradation.

Recent studies have shown that a number of RTKs undergo ubiquitination targeting them for degradation. Ubiquitination serves as a general key regulatory mechanism of signaling in normal cells and is mediated by ubiquitin ligases that attach single or multiple ubiquitin moieties to their targets resulting in mono- or polyubiquitinated proteins, respectively. Members of the Cbl family of ubiquitin ligases, namely Cbl, Cbl-b and Cbl-c, play a major role in the ligand-dependent ubiquitination of many RTKs [4]. They all possess a highly conserved N-terminal domain consisting of a TKB domain and a RING finger domain which are both necessary for ubiquitin ligase activity. The TKB domain is required for Cbl recruitment to tyrosine phosphorylated proteins [5]. The RING finger domain associates with the E2 ubiquitin-conjugating enzyme, UbcH7 [6] leading to the transfer of ubiquitin molecules to lysine residues on the target protein. The carboxyterminal half of Cbl contains a proline-rich region and potential phosphorylation sites which in turn can allow interaction of Cbl with

proteins possessing SH3 or SH2 domains, respectively, and confer adaptor functions to Cbl proteins (reviewed in [7]).

Examples of RTKs known to be targeted and negatively regulated by Cbl family members include the receptors for EGF, CSF-1, PDGF, VEGF and hepatocyte growth factor (c-Met) [8-12]. Upon mono- or polyubiquitination the proteins are destined for degradation via the lysosome or the proteasomes, respectively [13], although in some cases receptors are recycled to the plasma membrane. Dysfunction or impaired recruitment of Cbl to RTKs result in deregulated positive signaling that eventually can lead and contribute to carcinogenesis [14]

Several reports claim that internalization and degradation of c-Kit is mediated by Cbl in a Src-dependent manner. The current opinion suggests that upon SCF-stimulation Src family kinases are recruited to autophosphorylated Y568 of c-Kit and hereby activated. Active Src kinases then phosphorylate Cbl which in turn tags the receptor for proteasomal degradation [15]. The interaction of Cbl and c-Kit during these processes has been studied by some groups and so far it is assumed to be primarily mediated indirectly by the adaptor proteins APS or Grb2 [16-18].

In this paper, we present data suggesting that Cbl not only needs to be activated by Src but also to be recruited to c-Kit upon SCF stimulation in order to elicit receptor ubiquitination. We show that the N-terminal TKB domain of Cbl can directly interact with c-Kit at phosphorylated Y568 and Y936. The binding specificity of Cbl for these tyrosine residues is determined by I571 and L939 of c-Kit, respectively. The specific Cbl loss-of-binding mutant of c-Kit, namely c-Kit I571A/L939A, accordingly failed to bind Cbl while phosphorylation of Cbl appeared intact. Consequently, ubiquitination and degradation of I571A/L939A-c-Kit was retarded despite intact ligand-stimulated kinase activities of c-Kit and Src. Wild-type c-Kit was further found to be primarily monoubiquitinated and targeted to the lysosome for degradation. Thus, this report adds a novel perspective on the dynamics and regulation of c-Kit signaling with a sequential recruitment of signal transduction molecules such as Src and Cbl to Y568 of c-Kit.

EXPERIMENTAL PROCEDURES

Antibodies, ligands and inhibitors

Human recombinant SCF and murine recombinant IL-3 were obtained from ProSpec-Tany Technogene (Rehovot, Israel). The rabbit antiserum Kit-C1, recognizing the C-terminal domain of c-Kit, was affinity purified as described [19]. The antiphosphotyrosine antibody 4G10 was from Upstate Biotechnology (Charlottesville, VA), and anti-GST and anti-Cbl (c-15) antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). The 9E10 antibody recognizing Myc-tag was from Roche Diagnostics, anti-Ubiquitin P4D1 from Covance Research Products (Denver, PA), anti-ubiquitin FK1 from Biomol (Plymouth Meeting, PA). The Src antibody Ab-1 was from Oncogene Sciences. Redivue γ -[³²P]ATP was purchased from Amersham Biosciences. The Src inhibitor SU6656 was from SUGEN/Calbiochem (San Diego, CA). PE-conjugated monoclonal YB5.B8 against human c-Kit was from BD Pharmingen. Clasto-lactacystin (Biomol) or chloroquine (Sigma, St. Louis, MI) were used for proteasomal or lysosomal inhibition, respectively. GST fusion proteins of N- and C-terminal parts of Cbl have previously been described [20].

Cell culture

PAE cells were grown in Ham's F-12 medium with 10% FBS, 100 units/mL penicillin and 100 μ g/mL streptomycin. COS-1 cells were grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μ g/mL streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO₂. Cells were starved in 0.1 % FBS overnight and stimulated with 100ng/mL SCF for the indicated periods of time unless stated otherwise.

Transient and stable transfection

Transient transfection of COS-1 and PAE cells was conducted by employing Lipofectamine/Plus reagents (Invitrogen) according to the manufacturer's instructions. The mutants of c-Kit in pcDNA3 used in this paper as well as PAE cells stably expressing wild-type and mutant human c-Kit have previously been described [16, 21, 22]. In all experiments the GNNK+ isoform of c-Kit was used [23, 24].

Immunoprecipitation, pulldown and Western blotting

Experimental procedures were conducted as described in [24]. Immunodetection was performed by enhanced chemiluminescence using the Super Signal Dura from Pierce (Rockford, IL) and a CCD camera (LAS-3000; Fujifilm).

Affinity fishing of GST-fusion proteins with immobilized peptides

The following peptides were synthesized:

CEEINGNNY(p)VYIDPTQ (pY568), CEEINGNNY(p)AYIDPTQ (pY568, A569), CEEINGNYY(p)VAIDPTQ (pY568, A570), CEEINGNYY(p)VYADPTQ (pY568, A571), STNHIY(p)SNLANCS (pY936), STNHIY(p)ANLANCS (pY936,A937), STNHIY(p)SALANCS (pY936; A938), STNHIY(p)SNAANCS (pY936,A939), CSDSTNEY(p)MDMKPGV (pY721). Peptides were dissolved at 1mg/mL in 50 mM Tris buffer, 5 mM EDTA, pH 8, and immobilized on Affigel 10 beads (Bio-Rad, Hercules, CA; 1mg peptide/ml Affigel) according to manufacturers instructions. Fifty μ l of a 1:1 slurry was incubated with 1 μ g of purified GST-Cbl fusion protein and rolled end-over-end for 3 hrs in the cold with cell lysates. After thorough washing of the beads, bound protein was eluted by boiling SDS sample buffer, run out on a 10% SDS polyacrylamide gel, electrotransferred to Immobilon P and probed for GST. For competition experiments, the respective soluble protein (100 μ M) was added to the incubation mixture of fusion protein and immobilized peptides.

***In vitro* kinase assay**

Kinase activity of c-Src was assessed essentially as described in [22].

Internalization and degradation experiments

Internalization and degradation experiments using 125 I-SCF were performed as previously described [25].

Internalization of c-Kit was determined after stimulation with SCF (100ng/mL) for the indicated periods of time, using a PE-anti-human CD117 antibody (BD Biosciences Pharmingen) and analysis by flow cytometry using a FACSort instrument (BD Biosciences).

RESULTS

The N-terminal domain of Cbl interacts with c-Kit in a phospho-dependent manner

Several reports indicate that Cbl and tyrosine kinase receptors can interact both directly and indirectly through adaptor proteins such as Grb2, APS or the p85 subunit of PI3-kinase [26] [17, 27, 28]. To address the issue of whether the interaction between Cbl and c-Kit occurs in a direct or indirect manner, COS-1 cells were transfected with c-Kit and stimulated with 100ng/ml SCF for 5 minutes. Lysates were incubated with immobilized GST fusion proteins of either the N-terminal or the C-terminal half of Cbl, respectively. Pulled down proteins were separated on an 8% SDS-PAGE gel, followed by electrotransfer to Immobilon-P. The filter was first probed with a phosphotyrosine antibody (PY-99), followed by stripping and reprobing with Kit-C1 antibody. A phosphorylation-dependent interaction between the N-terminal region of Cbl and c-Kit could be detected, whereas the C-terminal region of Cbl did not interact with c-Kit (Fig. 1A). Efficient transfection and activation of c-Kit in COS-1 cells was confirmed by a parallel immunoprecipitation of c-Kit from the same lysates and probing sequentially for phosphotyrosine and c-Kit.

It has previously been suggested that the adaptor protein APS mediates an indirect interaction between Cbl and c-Kit [16]. In order to rule out the contribution of APS to the observed interaction between c-Kit and the N-terminal part of Cbl, we co-transfected COS-1 cells with Myc-tagged APS and c-Kit and performed an analogous pulldown experiment with GST-Cbl (N-term) as described above. Surprisingly, we did not detect an expected enhancement of Cbl/c-Kit interaction by co-transfection of APS (Fig. 1B) despite substantial amounts of exogenously expressed APS, while the interaction between Cbl and c-Kit appeared equally strong regardless of the presence or absence of APS. The interaction between APS and Cbl is to some extent SCF-independent, which can be explained by the overexpression of APS in this case. Similar experiments were performed using SH2B and Lnk, members of the same family of adaptor proteins as APS, where no binding of Cbl could be detected (data not shown). We observed that mutation of I571 and L939 in c-Kit (previously reported to be specific binding determinants for binding of APS to Y568 and Y936, respectively) abolished the observed interaction of c-Kit and Cbl-N. This finding suggests that Cbl interacts directly with c-Kit with the same binding determinants as APS (Fig. 1C). To verify this interaction *in*

in vivo with endogenous levels of Cbl, we performed co-immunoprecipitation between Cbl and c-Kit using cells transfected with either wild-type c-Kit or the I571A/L939A mutant (Fig. 1D).

The interaction between Cbl and c-Kit is direct and specifically determined by the +3 positions to tyrosines 568 and 936

To prove that neither any other molecule besides APS nor endogenous APS is mediating the interaction between Cbl and c-Kit at positions Y568 and Y936 of c-Kit, we incubated purified GST-Cbl (N-term) fusion protein with immobilized phosphopeptides containing pY568 and pY936. As positive controls for interaction with pY568 or pY936, GST-fusion proteins with the SH2 domain of Src or Grb2 were employed, respectively (data not shown). Bound proteins were eluted and immunoblotted for GST revealing that GST-Cbl (N-term) interacts with Y568 and Y936 in a phosphotyrosine-dependent manner (Fig. 2A). Furthermore, the observed interaction could be competed by an excess of soluble phosphopeptide. The competing effect was abolished when peptides with I571A or L939A mutations were used, further supporting the importance of isoleucine/leucine in position +3 to the tyrosine as an important determinant for binding of Cbl to c-Kit (Fig 2B). As a negative control for interaction with Cbl, a peptide based on the peptide sequence around pY721 in c-Kit was used.

Our data provide evidence that the N-terminal part of Cbl interacts with c-Kit at position pY568 and pY936 in a direct manner. As we furthermore determined I571 and L939 as specific determinants for this interaction, we chose the I571/L939A mutant of c-Kit as a specific Cbl loss-of-binding mutant of c-Kit for further phenotypic investigations.

The c-Kit double mutant I571A/L939A possesses intact intrinsic kinase activity and is able to activate Src upon ligand stimulation.

In order to exclude the possibility that the I571A/L939A double mutant of c-Kit blocks Cbl interaction because of its potential loss of intrinsic kinase activity, we examined ligand-stimulated autophosphorylation of the receptor. COS-1 cells transfected with either wild-type c-Kit or double mutant c-Kit I571A/L939A were stimulated with SCF and lysates were immunoprecipitated with a c-Kit antibody, Kit-C1. The samples were analyzed by Western blotting probing with a phosphotyrosine antibody (4G10) and reprobbed with Kit-C1 for confirmation of equal amount of protein. The kinase activity of the mutant receptor proved to

be intact (Fig.3A). Phosphorylated tyrosine residues 568 and 936 of c-Kit are known to bind c-Src and Grb2, respectively, and both Src and Grb2 have been reported to be involved in Cbl-mediated regulation of RTK signaling either via phosphorylation of Cbl [29] or indirect recruitment of Cbl to the receptors [30], respectively. Thus, to exclude that the observed Cbl phenotype of the I571A/L939A double mutant might be a consequence of impaired Src activation, we performed an *in vitro* Src kinase assay. For this purpose, Src was immunoprecipitated from lysates of cells expressing wild-type c-Kit, the I571/L939A mutant of c-Kit or the Y568F/Y570F mutant, known to be deficient in Src activation [22], and therefore serving as a negative control, respectively. Immunocomplexes were incubated with γ -[³²P]ATP and acid-denatured enolase as an exogenous substrate for Src. Reactions were terminated after 15 minutes, proteins were separated on an SDS polyacrylamide gel and electroblotted to PVDF membranes. Phosphorylated enolase was detected using a PhosphorImager and densitometrically analyzed. The membranes were reprobbed with a Src antibody to ensure equal levels of Src (data not shown). In parallel the same samples were analyzed using Western blotting and probed for anti-phosphotyrosine as well as c-Kit to verify equal amount of expressed protein and activation (data not shown). We could demonstrate that the wild-type c-Kit and the I571A/L939A double mutant show an increased Src kinase activity upon ligand stimulation whereas the Y568/570F mutant displayed a Src-negative phenotype as expected (Fig. 3B). Intact interaction of Grb2 with the I571A/L939A double mutant of c-Kit has previously been demonstrated by Wollberg et al [16].

These data demonstrate that the I571A/L939A double mutant of c-Kit is impaired in its ligand-induced binding of Cbl to the c-Kit receptor without abolishing kinase activities of either c-Kit or Src, respectively, nor does it inhibit binding of Grb2 to c-Kit.

Phosphorylation and recruitment of Cbl to c-Kit are separable events that both are necessary for receptor ubiquitination

So far we have shown that the I571A/L939A mutant of c-Kit is unable to bind Cbl, but is still capable of activating Src. Next we were interested to see whether Cbl needs to be recruited to c-Kit in order to be phosphorylated and activated by Src family kinases. To address this question COS-1 cells were transfected with wild-type c-Kit as well as the double mutants Y568/570F and I571A/L939A, respectively, stimulated with SCF and lysed. Cbl was

immunoprecipitated from the lysates and subjected to immunoblotting with anti-phosphotyrosine antibodies. Equal Cbl protein levels were confirmed by reprobing the membrane with an anti-Cbl antibody. Hereby we were able to reveal that phosphorylation of Cbl is deficient in the Y568/570F mutant, but unaffected in the Cbl-I571A/L939A mutant, further confirming that Src kinase activity is intact downstream of I571A/L939A c-Kit (Fig. 4). From this we conclude that Src-dependent phosphorylation of Cbl occurs independently from Cbl interaction with c-Kit and that both events can be temporally and spatially separated. Next, we aimed to examine the ubiquitination phenotype of I571A/L939A c-Kit. The question whether the receptor gets polyubiquitinated or monoubiquitinated following ligand stimulation has not been completely resolved yet in the case of c-Kit. It has been claimed that c-Kit is polyubiquitinated following ligand-stimulation and degraded in the proteasomes [15, 31] whereas others state that RTKs in general become monoubiquitinated through the action of Cbl, which targets them for degradation in the lysosomes [32, 33]. To escape the problem of using an antibody potentially raised against an inappropriate antigen, we chose an anti-ubiquitin antibody recognizing both mono- and polyubiquitin moieties (P4D1) for a first experiment. For this, c-Kit was immunoprecipitated from PAE cells that were transiently transfected with the wild-type form, the I571/L939A Cbl binding mutant or the Y568/570F Src binding mutant of c-Kit. Immunocomplexes were run on a gel, electrotransferred and probed for ubiquitin. Upon ligand stimulation for 10 minutes, wild-type c-Kit was markedly ubiquitinated while none of the c-Kit double mutants displayed receptor ubiquitination despite comparable expression and activation levels of each receptor isoform (Fig. 5A). This finding is in accordance with our hypothesis that ubiquitination of c-Kit requires both activation of Cbl by Src and recruitment of phosphorylated Cbl to c-Kit. Thus, binding of Cbl to I571/L939 in c-Kit appears to be a specific occurrence secondary to its phosphorylation.

C-Kit is selectively monoubiquitinated by Cbl

To address the issue of mono- vs. polyubiquitination of c-Kit, a similar experiment to fig 5A was conducted. This time, membranes containing immunoprecipitated c-Kit proteins were first probed with an antibody specifically reacting with poly-ubiquitin moieties (FK1), stripped and reprobed with the anti-mono/poly-ubiquitin antibody. As a positive control for a known poly-ubiquitinated protein, we included immunoprecipitated activated Src [34]. It was consistently

observed that only wild-type c-Kit with intact Cbl and Src binding displayed ubiquitination. Furthermore, the ubiquitination signal on c-Kit only became apparent when employing the antibody generated against both mono- and poly-ubiquitin, whereas the specific poly-ubiquitin antibody did not react with wild-type c-Kit (Fig. 5B). Thus it appears that c-Kit is primarily mono-ubiquitinated upon ligand stimulation in a process that requires Src-dependent phosphorylation and receptor association of Cbl.

Internalization and degradation of c-Kit is dependent on activation of Src and intact Cbl binding sites

Since it has been demonstrated that ligand-induced monoubiquitination regulates internalization and degradation of several RTKs we wanted to investigate the effect of inhibition of monoubiquitination on c-Kit internalization and degradation. PAE cells expressing either Y568/570F or I571/L939A mutant forms of c-Kit, which we have shown to inhibit Src-dependent phosphorylation of Cbl or Cbl association, respectively, displayed impaired ligand internalization and degradation when compared to wild-type c-Kit (Fig. 6A). In addition, inhibition of Src kinase activity by 2 μ M SU6656 interfered with degradation of WILD-TYPE-receptor, which is in agreement with previous reports [24]. We also analyzed receptor internalization in Ba/F3 cells expressing either wild-type, Y568F/570F or I571A/L939A mutant c-Kit by flow cytometry using an antibody against the extracellular part of c-Kit. Ba/F3 cells were used since they grow in suspension culture and thus do not need to be treated with trypsin in order to do the flow cytometry analysis, which could potentially interfere with receptor detection. In both the Y568F/Y570F and the I571A/L939A mutant c-Kit, internalization of the receptor was decreased compared to wild-type receptor expressing cells (Fig. 6B).

Internalized c-Kit is degraded via the lysosomal pathway

It has recently been shown that the EGF receptor is sorted to lysosomes upon EGF stimulation in a Cbl dependent manner [35]. Moreover, lysosomal degradation has been suggested to be the general degradation pathway following mono-ubiquitination of proteins (reviewed by [36]). To test whether c-Kit is degraded in the lysosomes, we examined receptor degradation indirectly by assessing degradation of bound ¹²⁵I-labeled ligand in the presence or absence of the specific

proteasomal or lysosomal inhibitors, lactacystin or chloroquine, respectively. Measurement of ligand degradation showed that inhibition of lysosomal action by 20 μ M chloroquine completely abolished receptor degradation whereas inhibition of the proteasome by 100 μ M lactacystin only retarded receptor degradation slightly (Fig. 6C). Therefore we propose that degradation of c-Kit mainly occurs via mono-ubiquitination and subsequent targeting to the lysosomes.

DISCUSSION

The TKB domain of Cbl mediates binding to target proteins containing phosphorylated tyrosine residues and is indispensable for Cbl to induce ubiquitination of target RTKs [5]. Cbl can either associate to the activated RTK directly or through adaptor proteins. The adaptor protein Grb2 is able to associate with Cbl through proline-rich regions in Cbl interacting with the SH3 domain of Grb2 [27]. It was recently suggested that direct versus indirect association of Cbl to the EGF receptor has distinct function [35]. This further suggests that the sites of ubiquitination might differ depending on a direct or indirect interaction with Cbl which in turn might differentially regulate association with downstream signal transduction molecules. Here we present evidence that Cbl can interact with c-Kit in a direct manner at position Y568 and Y936, and that this interaction is dependent on a leucine or isoleucine residue in position +3. Since Y568 of c-Kit is the binding site for several signal transduction molecules besides Cbl, including Src family kinases, SHP-2 and the adaptor protein APS, we made the I571A mutant of c-Kit to specifically knock out binding of Cbl without affecting Src kinase activity. This also enabled us to study the role of Src kinase activity versus Cbl association to Y568 for the activation of Cbl following c-Kit activation. Hereby we could reveal that Cbl phosphorylation by Src and Cbl recruitment to c-Kit are separate events that are both pivotal for Cbl-mediated ubiquitination of c-Kit. This also includes a sequential binding of Src and Cbl to pY568 of c-Kit (see scheme Fig. 7).

A number of tyrosine kinases and RTKs have been shown to bind to Cbl through the TKB of Cbl, including the epidermal growth factor (EGF) receptor [28], vascular endothelial growth factor (VEGF) receptor (Flt-1) [37], macrophage colony-stimulating factor-1 (CSF-1) receptor [38], Met receptor [39], the p75 neurotrophin receptor [40], the PDGF α -receptor [11],

Zap-70 [41], Syk [42], Src [29] and Fyn [43]. Studies on the consensus sequence for binding of the Cbl TKB using degenerate peptide libraries, suggested a consensus of NXpY(S/T)XXP [41]. Many Cbl binding proteins fulfill this consensus sequence requirements, but there are exceptions (Table 1).

The Met receptor has been described to associate to Cbl via an atypical sequence motif [39]. In that case the required sequence was found to be DpYR. Also the amino acid sequence surrounding Y568 and Y936 in c-Kit does not strictly meet the requirements the “traditional” consensus sequence for binding of Cbl to phosphotyrosine residues. Y936 has a serine residue in position +1, but otherwise it shows little similarity to the typical Cbl association sites. The two Cbl recruitment sites in c-Kit both have isoleucine/leucine residues in position +3. This is also present in the Cbl association site of the p75 neurotrophin receptor. However, there are no striking similarities. This might point to requirements for Cbl binding that are rather determined by specific tertiary structures than by primary amino acid sequences. One should also keep in mind that the data presented on the binding specificity of Cbl to c-Kit arises from the use of synthetic phosphopeptides derived from the c-Kit sequence and might not fully represent the role of c-Kit in a physiological context.

Ubiquitination of protein has for long time been thought exclusively to occur through polyubiquitination and to target proteins for degradation in the proteasomes. However, in the case of RTKs, it was recently shown that at least some of them undergo multiple monoubiquitination, which targets them for endocytosis and degradation in the lysosomes [32, 33]. Several studies have suggested that c-Kit is polyubiquitinated upon ligand stimulation [15, 31], but the methods to detect ubiquitination in those studies were not able to distinguish between polyubiquitination versus monoubiquitination. In this study we have used two types of ubiquitin antibodies; one that is able to recognize both monoubiquitin as well as polyubiquitin (P4D1), and one recognizing only polyubiquitin (FK1). Thus, we could show that ubiquitination of c-Kit following ligand stimulation occurs exclusively as monoubiquitination.

Other studies have presented evidence that mono- and polyubiquitination serve distinct purposes. In the case of the receptors for PDGF and EGF several studies have linked monoubiquitination to regulation of receptor internalization and endosomal routing of receptors for lysosomal degradation [32, 33]. By use of a proteasomal inhibitor, lactacystin, and a lysosomal inhibitor, chloroquine, we showed that degradation of c-Kit is to a large extent

dependent on the lysosomal pathway and only to a minor extent on the proteasomal pathway. It has been shown that proteasome inhibitors block lysosomal degradation of ligand, which remains bound to their receptors within the endocytic pathway [44].

Monoubiquitination and degradation of RTKs through the lysosomal pathways are important pathways for regulating receptor activity. It is striking that in many cases transforming mutants of RTKs and other tyrosine kinases carry mutations that render them unable to bind to the Cbl family of ubiquitin E3 ligases (reviewed by [14]). Thus, escape of lysosomal degradation will allow the receptor to signal for a prolonged period of time and contribute to the transformed phenotype.

In this paper we describe a direct interaction between Cbl and c-Kit. Based on our data we propose a model in which Cbl is activated through Src mediated phosphorylation and then associates with the receptor. The physical association of Cbl with c-Kit is necessary for c-Kit to become ubiquitinated (Fig. 7). The monoubiquitinated receptor is then internalized, sorted to the lysosomes and degraded. In other systems, such as the EGF receptor, direct association versus indirect association via Grb2 has been shown to have distinct functions [35]. One obvious possibility is that direct versus indirect association with Cbl leads to ubiquitination at distinct lysine residues with distinct functional consequences. Future studies aiming at identifying the sites of ubiquitination in wild-type c-Kit, versus the I571A/L939A mutant c-Kit (that is deficient in direct association), versus the Y703F/Y936F mutant that is defective in Grb2 association [45], will shed light on these issues.

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FIGURE LEGENDS

Figure 1: Interaction of the N-terminal half of Cbl with c-Kit at position Y568 and Y936.

A: COS-1 cells were transiently transfected with wild-type c-Kit expression plasmids and stimulated with SCF as indicated. After lysis, cell lysates were subjected to either a immunoprecipitation with c-Kit antibody or a pulldown with GST fusion proteins of either the N-terminal or C-terminal half of Cbl. Immunocomplexes were separated on an 8% SDS gel, blotted and sequentially probed for c-Kit (*upper panel*) or anti-phosphotyrosine (*lower panel*).

B: COS-1 cells were co-transfected with expression plasmid for c-Kit and Myc-tagged APS and stimulated with SCF as indicated. After lysis, cell lysates were subjected to immunoprecipitation with anti-c-Kit as well to a pulldown with GST-Cbl (N-term) fusion protein. Complexes were analyzed by Western blotting and the membranes were sequentially probed for c-Kit, phosphorylated c-Kit and APS. C: COS-1 cells were transfected with the c-Kit mutant I571A/L939A and samples were treated as described above. D: Cbl was immunoprecipitated from COS-1 cells transfected with either wild-type c-Kit or the I571A/L939A mutant and samples were analyzed by Western blotting.

Figure 2. Cbl binds directly to phosphorylated Y568 and Y936 and the specificity is determined by positions Y+3

A: Phosphopeptides pY568 (*left panel*) and pY936 (*right panel*) (sequences in Materials and Methods) immobilized to Affigel 10 beads were incubated with the indicated purified GST fusion proteins in the presence of either un-phosphorylated or phosphorylated peptides in solution as indicated. Samples were analyzed by SDS-PAGE and Western blotting, and membranes were probed with an anti-GST antibody. B: Pulldown experiment performed as described above with competition by soluble peptides (100 μ M) as indicated.

Figure 3: Intrinsic kinase activity and ability to phosphorylate Src of the c-Kit double mutant I571A/L939A.

A: COS-1 cells were transfected with wild-type c-Kit (*lane 1 and 2*) as well as the I571A/L939A (*lane 3 and 4*) double mutant and stimulated with SCF for the indicated periods of time and concentrations. Lysates were immunoprecipitated with a c-Kit antibody and analyzed by Western blotting. The membrane was probed for tyrosine phosphorylated residues (4G10) and reprobed for c-Kit as loading control. B: Densitometric analysis of a Src *in vitro* kinase assay. Src was immunoprecipitated from an aliquot of cell

lysate prepared as described above, followed by incubation with 50 μ M [γ - 32 P]ATP in kinase buffer (described in detail in Materials and Methods). Samples were separated on an 8% SDS-PAGE gel and blotted to Immobilon. The filter was incubated with 1 M KOH for 1 hr at 55°C to hydrolyze non-specific phosphoserine present in the proteins, exposed to the Phosphorimager and densitometrically analyzed. Src activity upon stimulation in the respective mutant (grey bars) is expressed as fold activity in unstimulated cells (white bars). Mean values and standard deviations of three independent experiments are shown.

Figure 4: The effect of the wild-type c-Kit vs the two c-Kit double mutants Y568/570F and I571A/L939A on Cbl phosphorylation. Transiently transfected COS-1 cells expressing wild-type c-Kit or one of the two double mutants Y568/570F or I571A/L939A, respectively, were stimulated with SCF for the indicated periods of time, immunoprecipitated with a Cbl antibody and subjected to Western blotting. The membrane was probed for tyrosine phosphorylation and Cbl, respectively.

Figure 5: Monoubiquitination of c-Kit upon phosphorylation of Y568 and Y936 and recruitment of Cbl. A: PAE cells expressing wild-type, Y568F/Y570F or I571A/L939A c-Kit were stimulated with SCF for the indicated time points, followed by lysis and immunoprecipitation with c-Kit antibody. Samples were subjected to a Western blot analysis for ubiquitin (P4D1) and c-Kit. B: An identical experiment was performed, with the exception that the membrane was probed with a selective poly-ubiquitin antibody (FK1) as well as the P4D1 ubiquitin antibody recognizing both poly- and mono-ubiquitin. Immunoprecipitated Src was included as a positive control for the poly-ubiquitin antibody.

Figure 6: Analysis of degradation of internalized SCF in cells expressing wild-type c-Kit vs. I571A/L939A mutant c-Kit and their intracellular processing. A: PAE cells expressing wild-type (\blacklozenge), Y568/570F (\blacktriangle) or I571A/L939A (\bullet) c-Kit were incubated with 125 I-SCF for 60 min on ice, followed by incubation at 37°C for the indicated periods of time. Cells were washed with an acidic buffer and surface released radioactivity was determined. Loss of surface bound radioactivity was taken as a measure of internalization. The medium was collected and subjected to precipitation with 10% trichloroacetic acid (TCA). TCA-soluble radioactivity was taken as a measure of degradation of 125 I-SCF. As a negative control wild-type c-Kit-expressing PAE cells were treated with 2 μ M SU6656 (\blacksquare) to inhibit Src activity before examining internalization and SCF degradation. B: Flow cytometry analysis of

internalization. Ba/F3 cells transfected with either wild-type (◆), Y568F/Y570F (▲) or I571A/L939A (●) mutant c-Kit were incubated with 100ng/mL of SCF for the indicated periods of time, followed by flow cytometry analysis using an antibody against the extracellular part of c-Kit. The following color coding of the histograms was used: grey: negative control; black; 0 min. ; blue: 2 min.; red: 5 min.; green: 15 min.; purple: 30 min. C: PAE cells expressing wild-type c-Kit were preincubated either in the absence of inhibitor (◆) or presence of either 100μM lactacystin (■) or 20μM chloroquine (▲) for 4 hrs, followed by the assessment of binding, internalization and degradation of ¹²⁵I-SCF as described above. Mean values and standard deviations of three independent experiments are shown.

Figure 7: Proposed model for the sequential Src- and Cbl-dependent steps during the ubiquitination of c-Kit. Activation of Src family kinases, initiated by Src binding to pY568 of ligand stimulated c-Kit, leads to phosphorylation of Cbl. Phosphorylated Cbl is then recruited to pY568 and pY936 of c-Kit which in turn results in monoubiquitination of the receptor and its subsequent internalization and degradation in the lysosomes.

Table 1. Sequence comparison of Cbl binding sites in various proteins. The upper part of the table shows the sequences of Cbl binding sites that conform with the previously described as traditional consensus sequences for the binding of Cbl to phosphorylated tyrosine residues. The lower part of the table depicts the recently reported sequences of Cbl binding sites that do not match the previously proposed consensus (APS, Met and c-Kit).

Fig. 1A

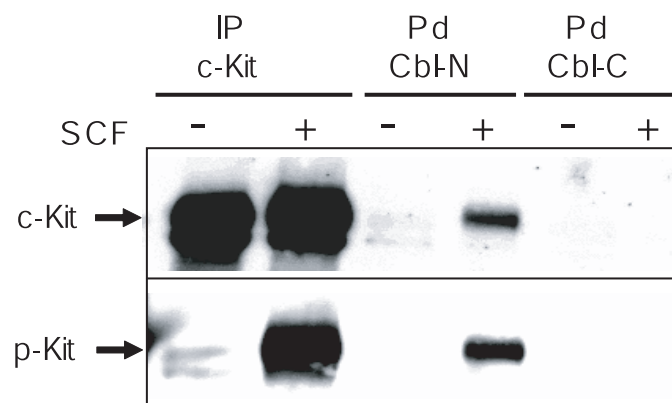


Fig 1B

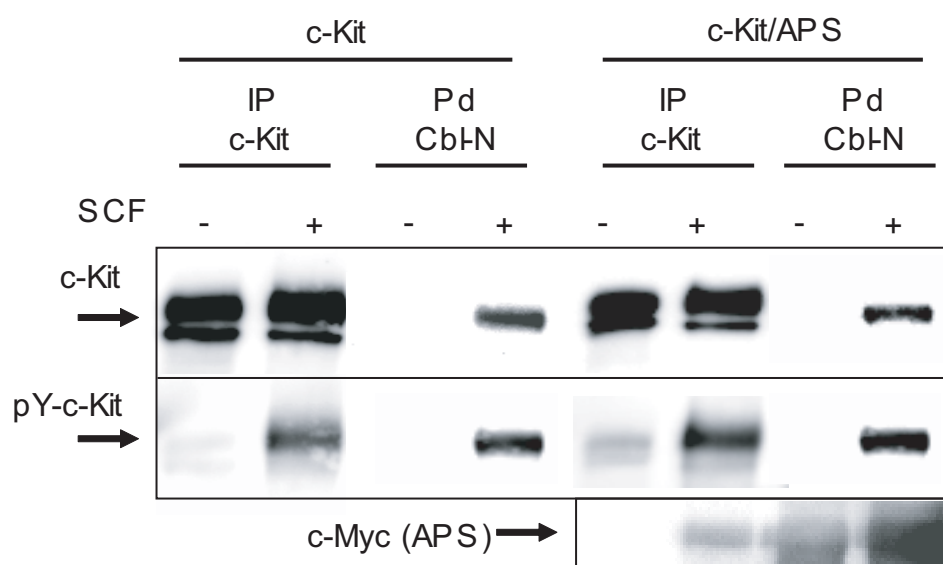


Fig. 1C

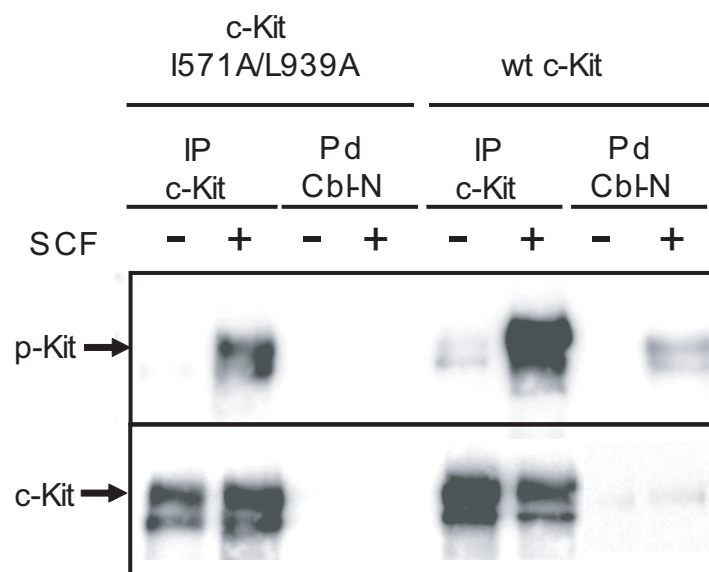


Fig. 1D

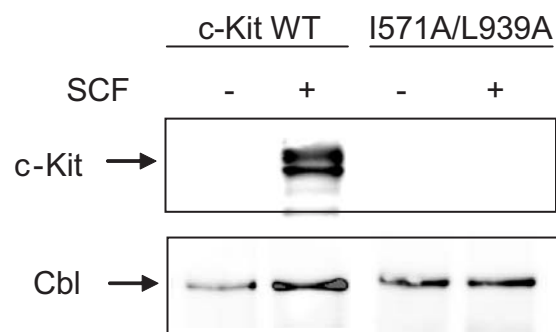


Fig. 2A

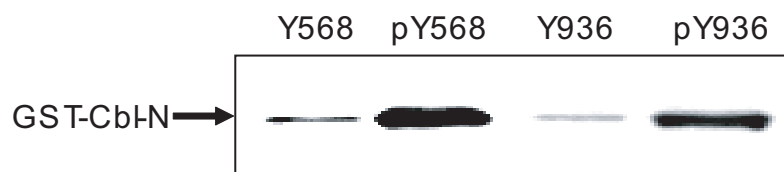
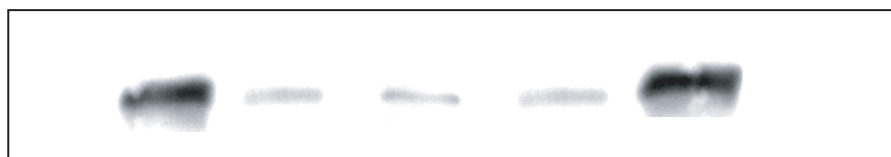


Fig. 2B

Immobilized Peptide	<u>Y568</u>	<u>pY568</u>				<u>pY721</u>	
Competition:	-	-	pY568	pY568 A569	pY568 A570	pY568 A571	-



Immobilized Peptide	<u>Y936</u>	<u>pY936</u>				<u>pY721</u>	
Competition:	-	-	pY936	pY936 A937	pY936 A938	pY936 A939	-



Fig. 3A

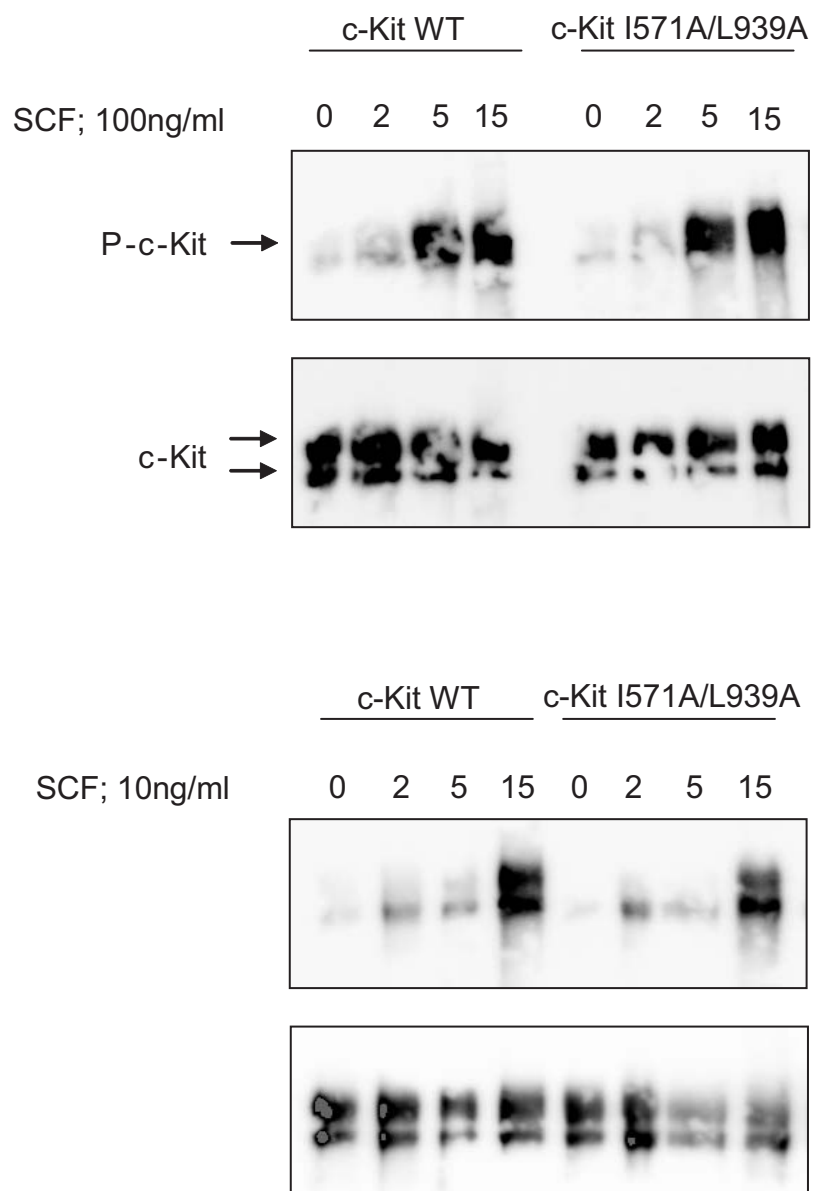


Fig. 3B

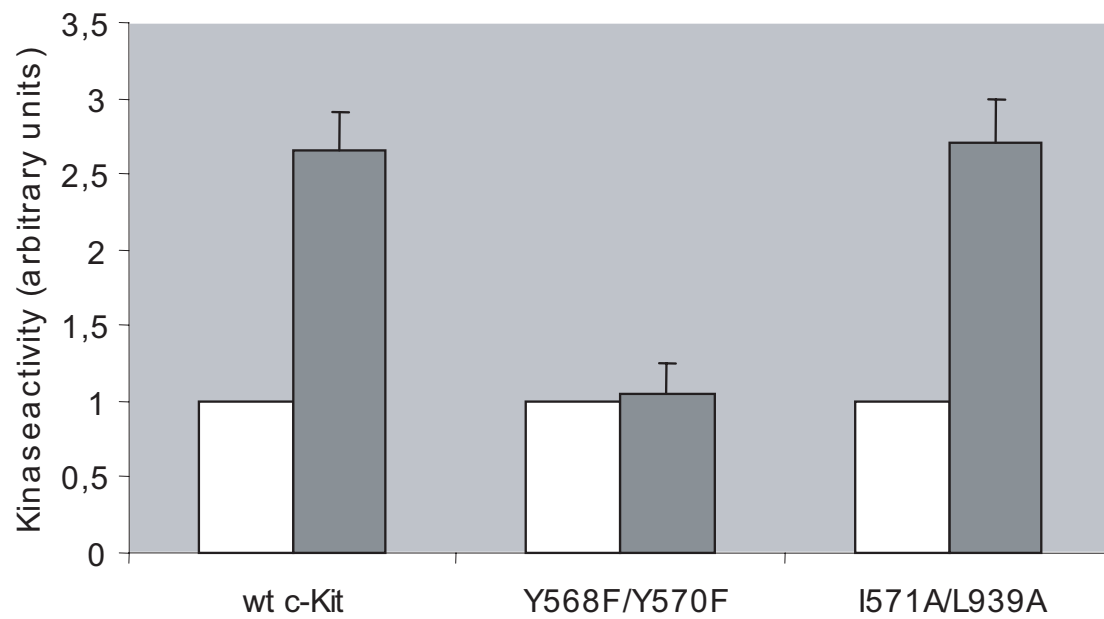


Fig. 4

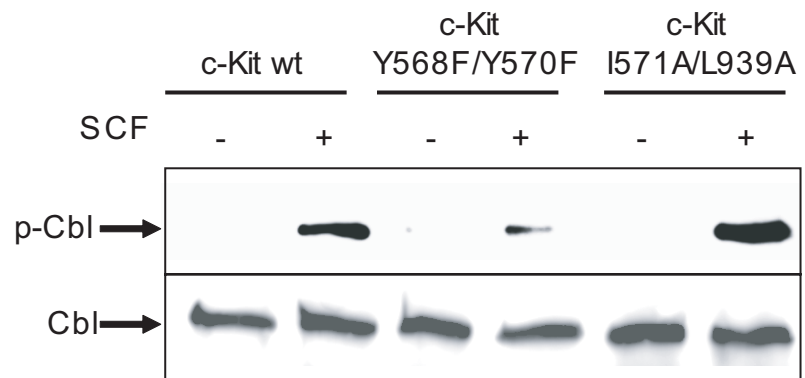


Fig. 5A

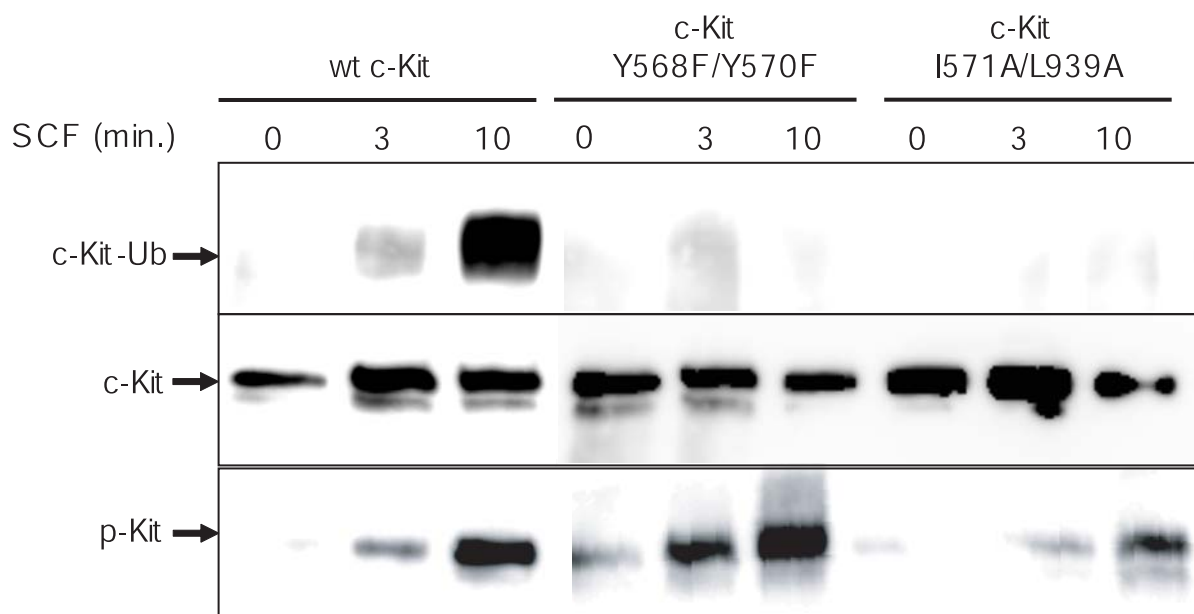


Fig. 5B

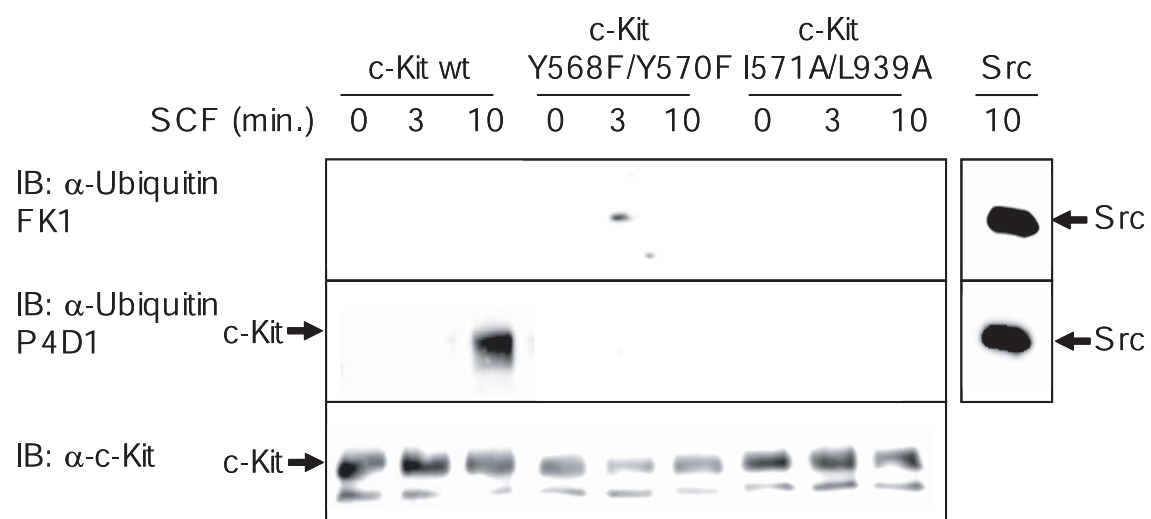


Fig. 6A

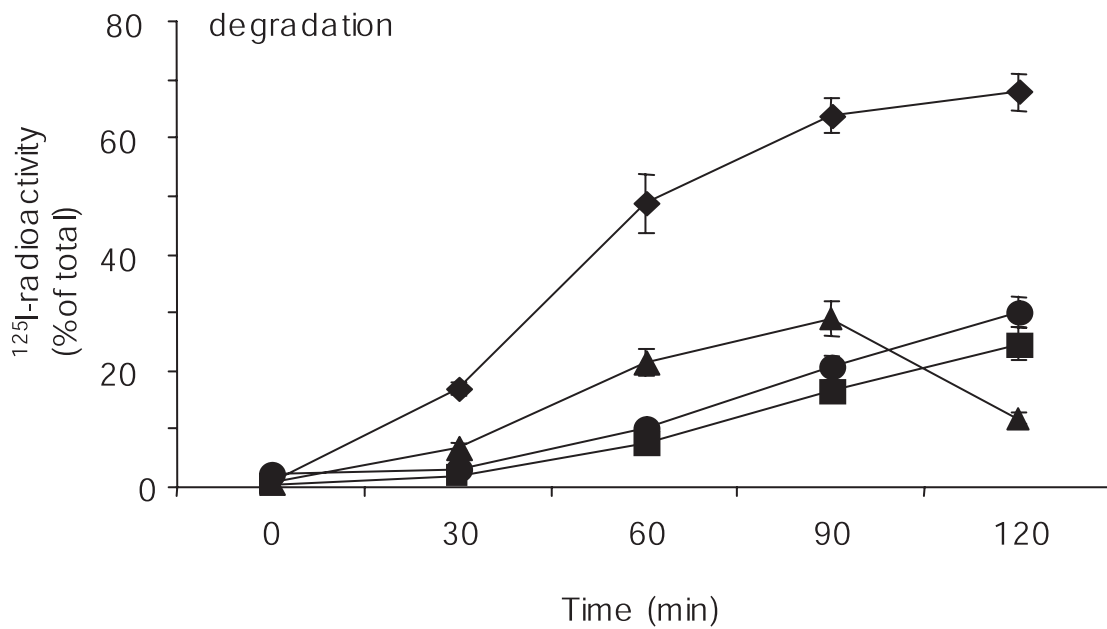
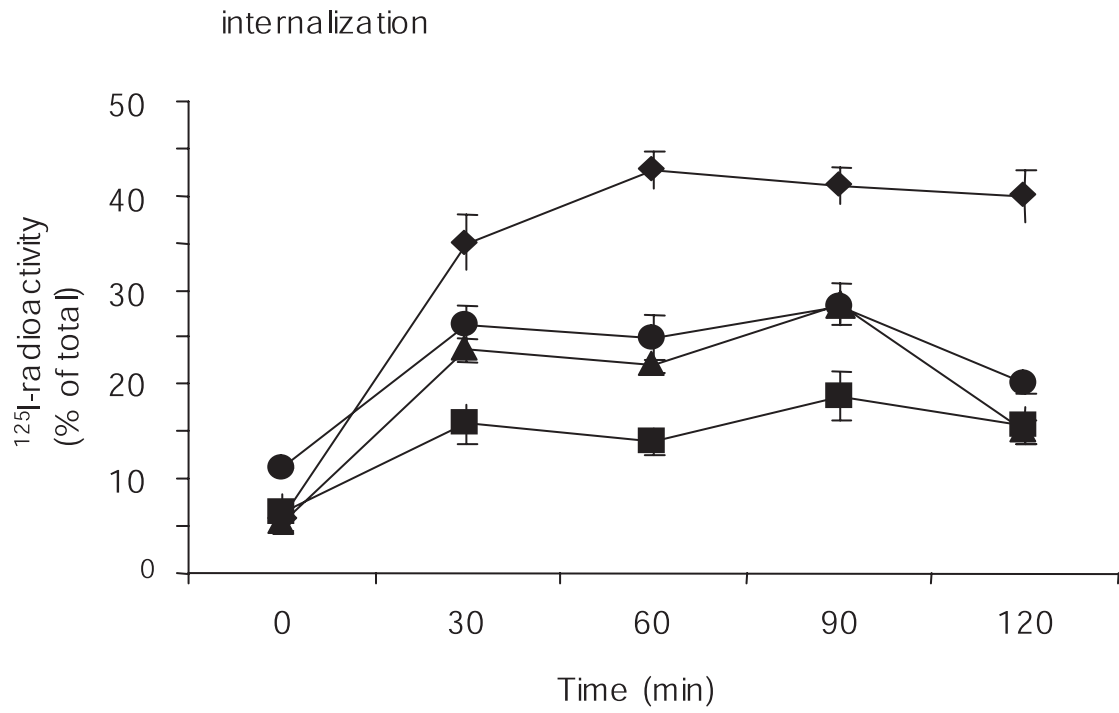


Fig. 6B

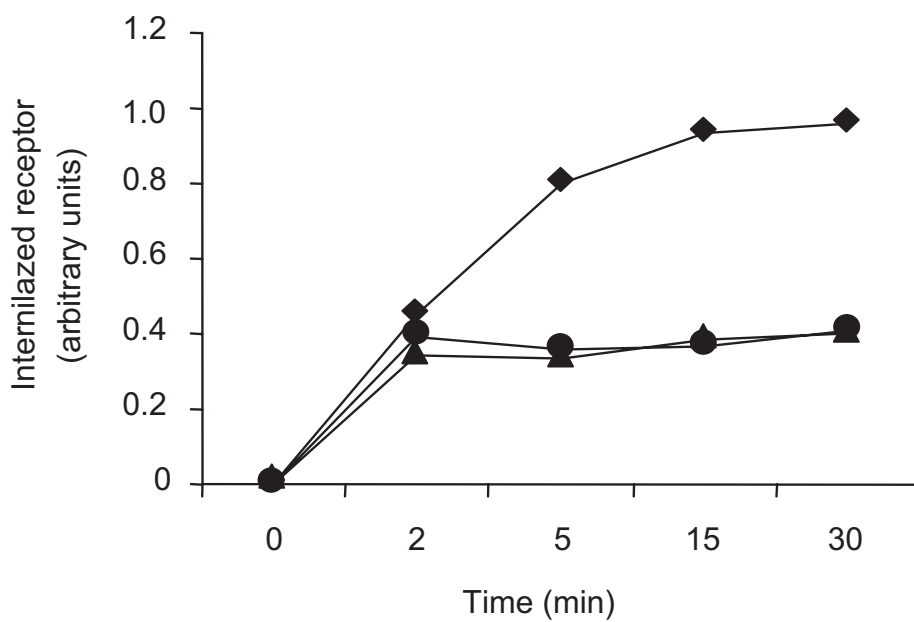
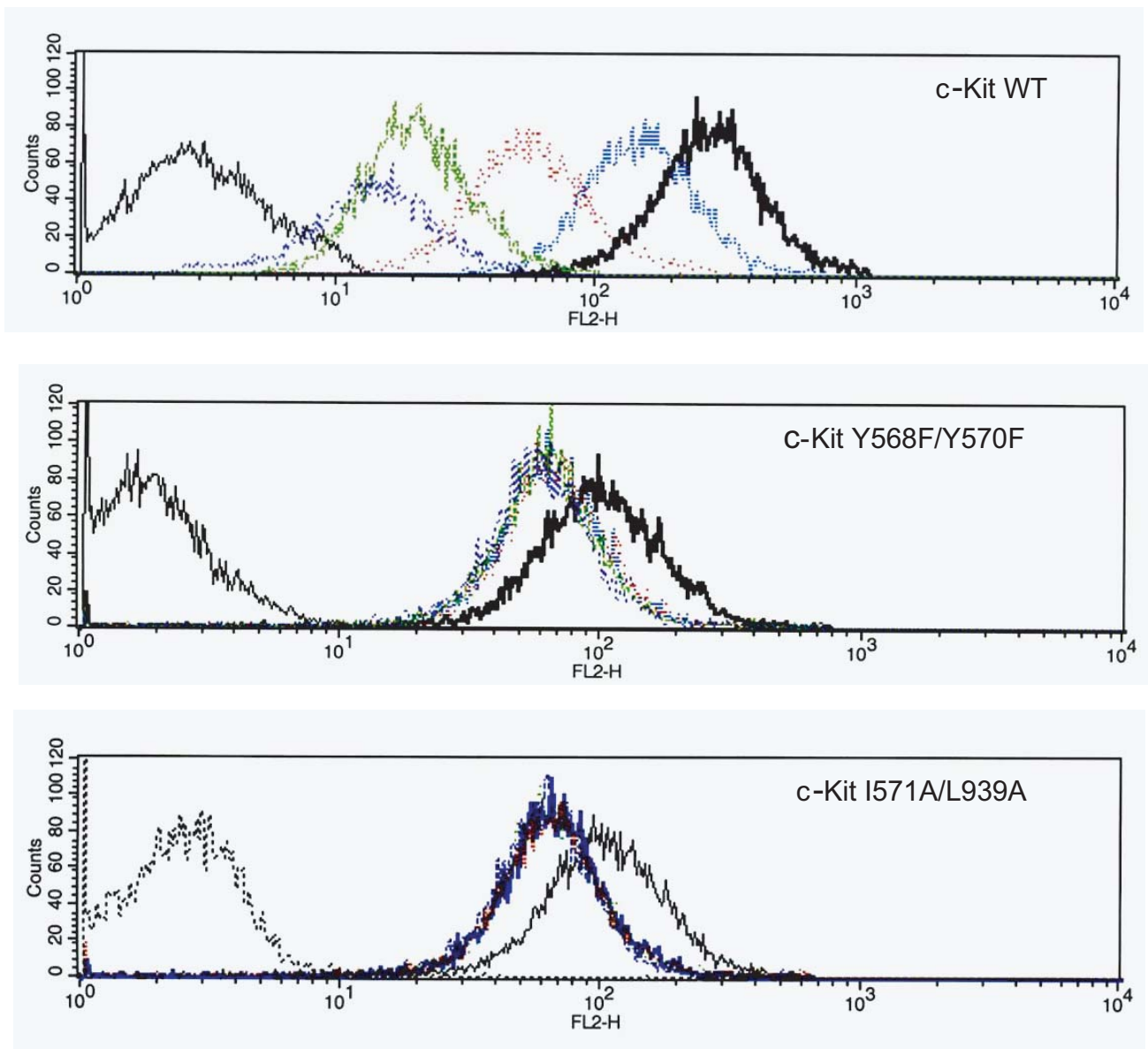


Fig. 6C

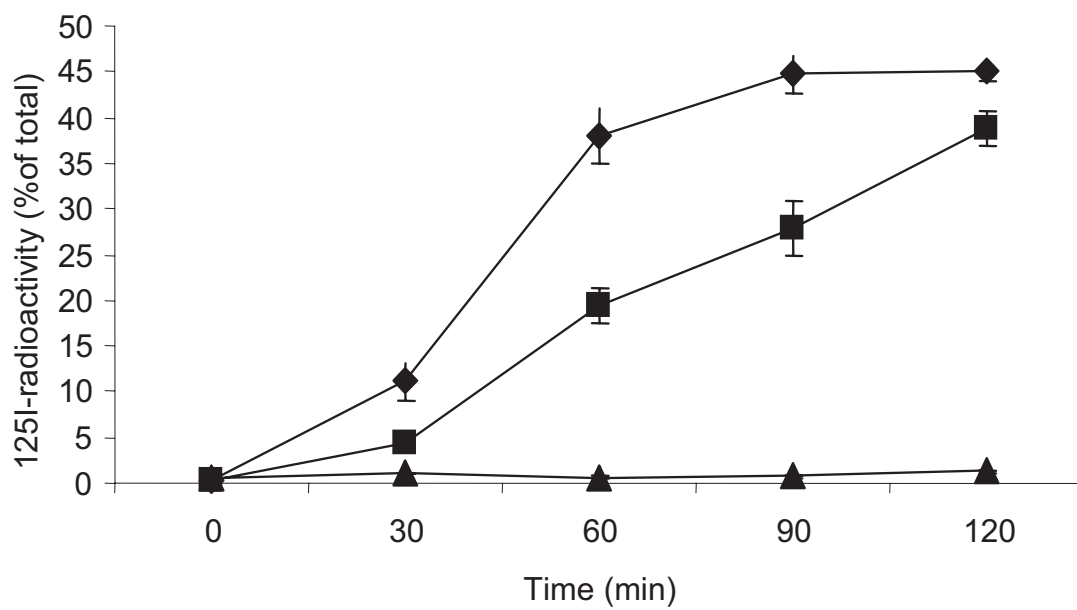


Fig. 7

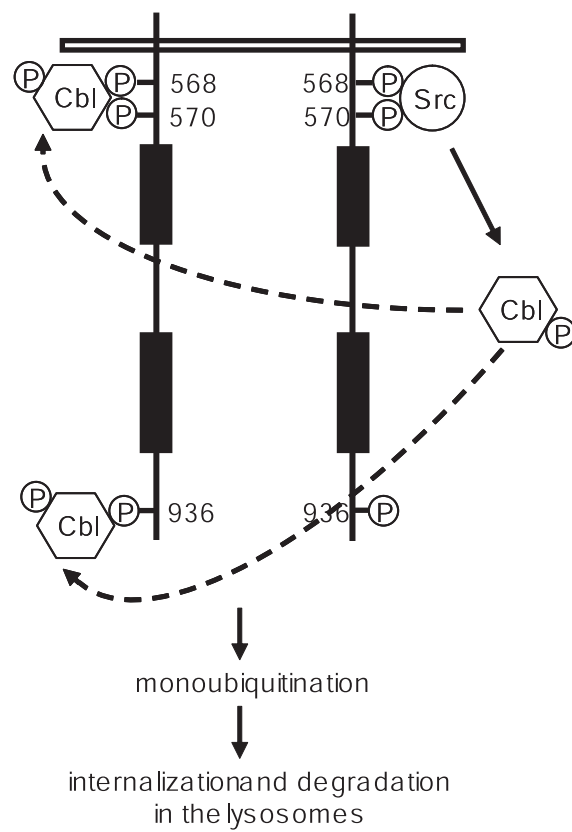


Table 1

LLQPNNYQFC	c-Fms
DSFLQRYSSDPT	EGFR
SVNSSRYKTEPF	Let23
TLNSDGYTPEPA	ZAP70
TVSFNPYEPELA	Syk
LIEDNEYTARQG	Src/Fyn
KGDGNLYSSLPL	P75 NTR
YNSVVLYSTPPI	VEGFR
IRGSNEYTEGPS	Sprouty2
SNESVDYRATFP	Met
RAVENQYSFY	APS
EEINGNYVYIDP	c-Kit Tyr 568
ESTNHIYSNLAN	c-Kit Tyr 936