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c-Cbl Mediated Ubiquitination and Degradation of hSef*

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Abstract Sef (similar expression to *fgf* genes) was identified as a feedback antagonist of FGF signaling in zerbrafish, mouse and human. Sef has been reported to function in different ways, however the regulation of Sef stability remains unknown. The possible role of c-Cbl in the regulation of Sef protein degradation was investigated. Results from coimmunoprecipitation and immunostaining assays reveal that hSef colocalizes and interacts with c-Cbl. Data suggest that the interaction between hSef and c-Cbl results in the ubiquitination and subsequent degradation of the hSef protein. It was proposed that c-Cbl may serve as a modulator to regulate Sef protein stability during FGF signal transduction.

Key words hSef, c-Cbl, ubiquitination, degradation

Binding of growth factors to their cognate receptors triggers intracellular signaling pathways through receptor-tryrosine kinases (RTKs) that ultimately determine the cellular destinations such as differentiation, proliferation, and apoptosis [1]. Simultaneously, negative receptor signaling pathways are also initiated by activated receptors to decrease the amplitude and/or the duration of positive signals [2]. During the last few years, multiple feedback inhibitors of the RTKs mediated signaling pathways have been identified[3]. Sprouty family proteins were identified as ligand-induced antagonists of RTKs signaling in both vertebrates and invertebrates [4~6]. Most recently, Sef (similar expression to *fgf* genes) was found as a negative regulator to the RTK signaling in the vertebrates but not invertebrates[7, 8].

Sef is expressed in a synexpression group in early stages of embryos and functions similarly to Sprouty, which regulates FGF signals, in the development of the early embryo by inhibiting the RTK/Ras/MAPK pathway [7, 8]. The expression pattern of Sef is similar to that of Sprouty in the zebrafish development. Both Sef and Sprouty are co-expressed with FGF at the margin of the gastrula in the early zerbrafish embryo[7, 8]. In mouse, Sef and Sprouty are expressed in similar regions along the anterior-posterior axis of the embryo at 8.75 days post-coitum (dpc) [9]. Similarly, the expression of Sef and Sprouty are induced by FGF signaling during zebrafish development [7, 8]. Sef and Sprouty are also induced upon FGF and EGF stimulation in cultured cell lines [10~12]. In addition, it has been reported that Sef and Sprouty function synergistically to regulate *Gbx2* expression in the anterior hindbrain in mouse model[13]. These observations suggest that Sef may have similar regulatory partners to Sprouty during the FGF induced Ras/MAPK signaling.

A common regulatory partner in Ras/MAPK signaling is the multi-adaptor protein Casitas B-lymphoma (Cbl) [14], which was first identified as a retroviral transforming gene product that induces pre-B lymphoma and myeloid leukemia[15]. Cbl was reported

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to mediate the multi-ubiquitination of receptors including epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and colony-stimulating factor 1 receptor (CSF-1R), and to induce degradation of these receptors *via* the proteasomal/lysosomal pathway [16~18]. Interestingly, c-Cbl, one of the Cbl proteins, also regulates the ubiquitination and degradation of the negative regulators of RTKs. For example, c-Cbl has been reported to form a complex with Sprouty, and regulates the stability of Sprouty, depending upon the EGFR-mediated phosphorylation status of Sprouty[19, 20]. This mechanism may provide positive signaling facilitated by the release of negative regulators. On the other hand, the association of c-Cbl with negative regulators (e.g. Sprouty) may sequester c-Cbl (which is an E3 ligase) thus prevent binding to the EGFR and subsequent ubiquitination, internalization and degradation of the receptor [19, 21].

In this study, we provide evidence that c-Cbl associates with Sef and mediates Sef ubiquitination and degradation. Our results suggest that the degradation of hSef, mediated by c-Cbl, might be a mechanism by which the antagonist role of Sef in RTKs signaling pathway could be down regulated.

1 Materials and methods

1.1 Plasmid constructs

The pcDNA3.1/Myc-His/hSef and pcDNA3/6 × Myc/hSef-S have been described previously [22]. The construct of pXJ/HA-c-Cbl was a gift from Dr. GR Guy (National University of Singapore, Singapore). For constructing the human c-Cbl siRNA expressing plasmid, we used the siRNA vector pBS/U6 that was provided by Dr. Yang Shi (Harvard University, Boston, USA) to construct a 22 bp hairpin siRNA according to published protocols [23]. The selected targeting sequences of the human c-Cbl siRNAs were 5' TAGGTCCAGAGTGTGACCTTCAAGAGAGGT-ACAACTCTGGACCTACTTTTTG 3' and 5' AA-TAGCCCACCTTATATCTTCAAGAGAGATATAA-GGTGGGCTATTCTTTTTG 3'. The synthesized complementary oligonucleotides were annealed to each other and subcloned into the *Apa* Ⅰ -*EcoR* Ⅰ sites of pBS/U6 vector, to generate two specific siRNAs that were subsequently named c-Cbl-siRNA1 and c-Cbl- siRNA2. The control siRNA for GFP (GFP-siRNA) was also a gift from Dr. Yang Shi (Harvard University, Boston, USA).

1.2 Cell culture and transfection

Cos7, HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 100 mg/L streptomycin. Cos7, HEK293T and HeLa cells were transfected for Western blot and immunostaining experiments with VigoFect (Vigorous).

1.3 Selection of stable clones

HeLa cells were grown in DMEM supplemented with 10% FBS (Invitrogen) and transfected with plasmid constructs encoding hSef-Myc or Vector using the VigoFect transfection reagent (Vigorous). 24 h later, cells were grown in medium supplemented with the antibiotic Genectin (G418, Life Technologies, GIBCO Invitrogen) at 600 mg/L, and selection of clonal cell lines was initiated. Individual G418-resistent clones were expanded and maintained in 300 mg/L G418. Overexpression of hSef-Myc was confirmed by Western blotting.

1.4 Co-immunoprecipitation and immunoblotting

The cells were washed twice with cold PBS and were lysed in lysis buffer as previous described[24]. The appropriate volume of protein was directly resolved by 8% ~10% SDS-PAGE or firstly subjected by co-immunoprecipiatation assay. The Western blot protocol and co-immunoprecipitation assays were previously described [24]. The ECF detection system (Amersham Pharmacia Biotech) was used to visualize protein bands. The secondary antibody and the third antibody for ECF were purchased from Amersham Pharmacia Biotech. Alternatively, the ECL detection system was also used for chemiluminescence of proteins, and the blots were then exposed to photo graphic films (Kadak, USA). The HRP-conjugated secondary antib odies for ECL were from Pierce. Monoclonal anti-Myc (9E10), monoclonal anti-Ubiquitin (P4D1) antibody and polyclonal anti-c-Cbl (C15) antibodies were purchased from Santa Cruz Biotechnology.

1.5 Immunostaining and confocal microscopy

The immunostaining assay was performed as previously described [24]. Essentially, cells growing on glass cover slips were washed with PBS, fixed for 15 min with 4% paraformaldehyde in PBS, and permeabilized for 10 min with 0.3% Triton X-100 in PBS followed by blocking with 10% FBS in PBS for 30 min at the room temperature. The primary antibodies diluted in PBS containing 0.1% Tween-20

were incubated for 1 h at room temperature and the bound antibodies were detected with FITC-, TRITC-conjugated goat anti-rabbit or anti-mouse IgG. The cover slips were mounted in glycerol and analyzed using a laser scanning confocal microscope with a 60× oil-immersion objective. FITC-, TRITC-conjugated goat anti-rabbit or anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories. Rabbit polyclonal anti-hemagglutinin (anti-HA) antibody were purchased from Santa Cruz Biotechnology.

2 Results

2.1 hSef co-localizes with c-Cbl in Cos7 cells

In our previous studies, we have observed that Sef localizes on cytoplasmic vesicles[24~26]. It has also been reported that c-Cbl is present in small punctuate cytoplasmic structures [16]. These observations suggest that Sef and c-Cbl may co-localize in cells. To examine this possibility, we co-expressed Sef-Myc and HA-c-Cbl in Cos7 cells and observed the localization of these two proteins. Our immunostaining results demonstrated that Sef and c-Cbl largely co-localizes to the cytoplasmic vesicles in transfected cells (Figure 1), indicating that Sef and c-Cbl localize together. Our data suggest the possibility that Sef and C-Cbl interact and that this interaction may affect the cellular function of Sef.

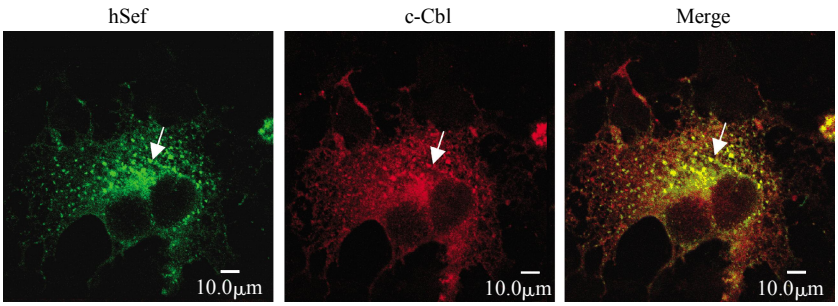

Fig. 1 Co-localization of hSef and c-Cbl

Cos7 cells were grown on cover slips, and were transfected with 1 μg of a hSef-Myc expression plasmid, together with 1 μg of a plasmid encoding HA-c-Cbl. After 24 h incubation, the cells were immunostained with anti-HA antibody, to detect c-Cbl, followed by TRITC-conjugated secondary antibodies (red) or anti-Myc, to detect hSef, antibody followed by FITC-conjugated secondary antibodies (green). The results were visualized by confocal microscopy. The solid arrows indicate a cytosolic vesicle.

2.2 hSef interacts with c-Cbl

To test whether Sef and c-Cbl physically interact, we performed a co-immunoprecipitation experiment. Sef-Myc was co-transfected into 293T cells with the HA-c-Cbl or control plasmid. When anti-Myc antibodies were used to precipitate hSef from the cell lysates, we observed that HA-c-Cbl was also present in the precipitated complexes (Figure 2), suggesting that Sef may constitutively associate with HA-c-Cbl (Figure 2) in the cell.

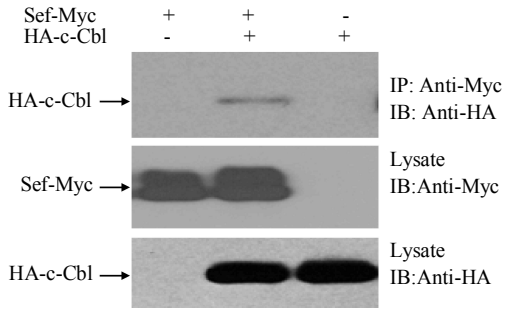

Fig. 2 hSef is immunoprecipitated with c-Cbl

HEK293T cells were co-transfected with 2.0 μg of Myc-tagged Sef and 2.0 μg of HA-tagged c-Cbl or control vector per 60 mm dish. After 24 h incubation, whole cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA (c-Cbl) antibody (upper panel) or were immunoblotted with anti-HA (c-Cbl) or anti-Myc (hSef) antibodies (lower panel). The immunoreactive bands were visualized by ECF.

2.3 hSef is ubiquitinated by c-Cbl

Proteins that interact with c-Cbl are often found to be ubiquitinated as a consequence of the c-Cbl ubiquitin ligase activity [16~18]. To determine whether the association of c-Cbl leads to the hSef ubiquitination, a Sef-Myc construct was co-transfected with the increasing amounts of c-Cbl expression vector in 293T cells. To observe the *in vivo* ubiquitination of Sef, we used anti-Myc antibodies to precipitate the cell lysates and detected the presence of smear ladders (indicative of ubiquitinated proteins) by using anti-ubiquitin antibodies (Figure 3, upper panel). When we co-expressed different amounts of c-Cbl-HA

in cells, we found that the ubiquitinated forms of the ectopically expressed Sef protein were increased in a dose dependent manner (Figure 3, lanes *3* to *5*), in parallel to the increased levels of c-Cbl. This result suggested that c-Cbl participates in hSef ubiquitination.

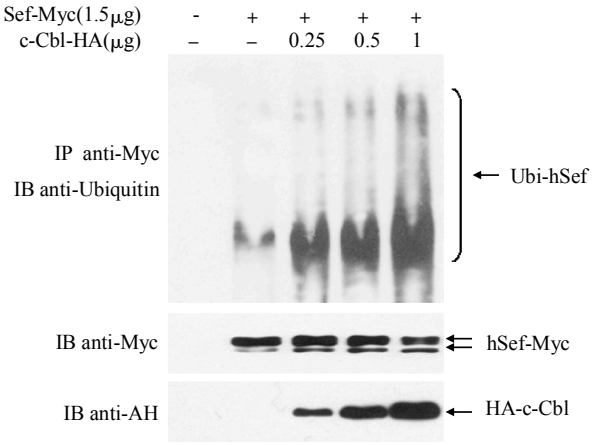

Fig. 3 hSef is ubiquitinated by c-Cbl

HEK293T cells were co-transfected with 1.5 μg of Myc-tagged hSef and an increasing amounts of HA-tagged c-Cbl plasmids (0μg, 0.25μg, 0.5μg, 1μg) of HA-tagged c-Cbl (control vector was added to yield equal amounts of added DNA) per 60 mm dish. After 24 h incubation, whole cell lysates were immunoprecipitated with anti-Myc (hSef) antibody and immunoblotted with anti-Ubiquitin antibody (upper panel) or were immunoblotted with anti-HA (c-Cbl) or anti-Myc (hSef) antibodies (lower panels). The immunoreactive bands were visualized by ECL.

2.4 hSef is degraded by c-Cbl

Proteins that are multi-ubiquitinated by c-Cbl (e.g. EGFR) are often targeted to the proteasome/ lysosome for degradation[16]. To test whether the ubiquitination of hSef results in its degradation, we co-expressed hSef-Myc with increasing amounts of HA-c-Cbl in the 293T cells. Results of our Western blot analysis show that c-Cbl decreases the hSef protein levels in a dose dependent manner (Figure 4a). To confirm that endogenous c-Cbl protein can mediate the degradation of Sef, we transfected siRNAs targeting c-Cbl to knock down c-Cbl expression in HeLa cells that stably express hSef-Myc. Western blot analysis show that the two siRNAs targeting c-Cbl significantly knocked down expression of the endogenous c-Cbl, and these same cells show increased levels of hSef protein (Figure 4b). In contrast, siRNA targeting GFP or the empty pBS/U6 vector did not show any effect on Sef protein levels (Figure 4b). These results indicated that knocking down c-Cbl levels prevents the degradation of the hSef protein.

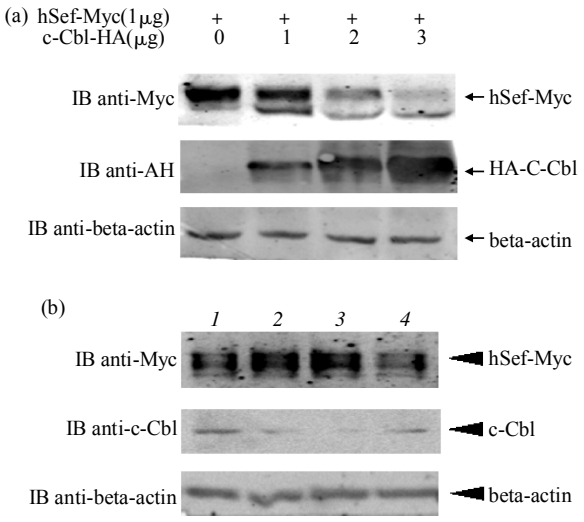

Fig. 4 hSef is degraded by c-Cbl

(a) c-Cbl decreases hSef levels in a dose dependent manner. HEK293T cells that co-expressed hSef-Myc (1μg) construct and increasing amounts of HA-tagged c-Cbl plasmids (0μg, 1μg, 2μg, 3μg control vector was added to yield equal amounts of DNA). After 24 h of incubation with the transfected plasmids, whole cell lysate was immunoblotted with anti-Myc (hSef) or anti-HA (c-Cbl) antibodies. The β-actin was also detected by immunoblotting to show equal loading. Immunoreactive bands were visualized by ECF. (b) The c-Cbl siRNA inhibits hSef protein degradation in the hSef-Myc expressing HeLa stable cells. The HeLa stable cells were transfected with siRNAs targeting c-Cbl (2 μg) or control plasmid pBS/U6 (2 μg) or siRNA targeting GFP (2 μg) as indicated. After 48 h incubation, whole cell lysate was immunoblotted with anti-Myc (hSef) and anti-c-Cbl antibodies. Immunoblotted β-actin as a loading control. Immunoreactive bands were visualized by ECL. *1*: pBS/U6; *2*: c-Cbl siRNA1; *3*: c-Cbl siRNA2; *4*: GFP-siRNA.

3 Discussion

Feedback modulators are important for RTK function and characterization of their mechanisms has been drawing an increasing attention. Sef and Sprouty proteins have been reported to be in the same synexpression group in the early stages of animal embryos. Both Sef and Sprouty have been identified to be antagonists of RTKs signaling, and their expression levels are regulated by FGF signaling during zebrafish development. In previous reports, Sprouty was demonstrated to be ployubiquitinated and degraded by c-Cbl, thus limiting its inhibitory effects [19, 20]. Given the similarities of these two antagonists, we speculated that Sef may also be regulated by c-Cbl. To determine whether c-Cbl has functional links with Sef, we performed an immunostaining assay and demonstrated

that Cbl colocalizes with Sef in Cos7 cells. We further showed that these two proteins interact together physically by using a coimmunoprecipitation assay. Finally, we observed that Sef is ubiquitinated and degraded by c-Cbl. Taken together, our data suggest that hSef is targeted to ubiquitin-proteasome pathway by c-Cbl, a mechanism that potentially regulates hSef protein levels during receptor tyrosine kinase signaling.

Cbl proteins control multiple cellular processes by acting as ubiquitin ligases and multifunctional adaptor molecules. This family of proteins is involved in the control of cell proliferation, differentiation and cell morphology, as well as in pathologies such as autoimmune disease, inflammation and cancer [14, 27]. One of the well-studied functions of the Cbl proteins is its ubiquitin E3 ligase activity. In cells, Cbl protein E3 ligase activity is thought to be inactive until they encounter an activated kinase. Upon interaction with an active kinase, the E3 activity of mammalian Cbl proteins are induced, leading to the ubiquitination and degradation of the kinase[28, 29]. However, in our results, hSef was observed to be ubiquitinated and degraded by c-Cbl under normal cultural condition without the addition of growth factors. We have considered three possibilities to explain our observation. Firstly, there may be sufficient endogenous factors in cells that stimulate the activation of an unknown kinase. The second possibility is that the serum may contain enough growth factors to stimulate the activation of a kinase. The third possibility is that Sef may constitutively interact with c-Cbl and activates the c-Cbl E3 ligase activity itself. We propose to clarify this issue in future studies.

Given the fact that antagonists can be induced during RTKs signaling, we propose that a mechanism to degrade antagonists of the signals should exit in order to ensure the efficiency of next round of growth factor stimulation. To date, the expression of Sef, an antagonist for FGF signal, has been shown to be controlled at the transcriptional level [7, 8, 12]. In this report, we present evidence that the protein level of hSef is also regulated by the mechanism of ubiquitination and degradation mediated by c-Cbl. We envision that the degradation of antagonist is necessary for facilitation of the signal transduction. Thus, the levels of Sef can be controlled both at the transcriptional and post-transcriptional levels. Indeed, such temporal restriction is crucial for many developmental processes where cells need to respond repeatedly to the same ligand in order to become committed to specific fates [2, 30]. c-Cbl therefore may play a critical role on balancing the levels of inhibitors (e.g. Sef), yielding the ability to promptly tune MAPK signaling under appropriate cellular circumstances. However, more detailed studies are needed to conclusively address this issue.

The regulation of ubiquitin E3 activity is crucial for cell function and development [31]. The activity of c-Cbl towards the RTKs is regulated by both protein-protein interactions and tyrosine phosphorylation For example, Sprouty2 has been reported to attenuate EGFR ubiquitination and degradation by binding to the SH2 domain of c-Cbl and inhibiting its association with EGFR [19, 21]. Also, activated cdc42 binds to c-Cbl *via* p85cool-1/β-pix and inhibits the coupling of c-Cbl to the EGFR and thereafter receptor ubiquitination [32]. Previous studies have shown that Sef associates with FGFRs[8, 12, 22, 33] and EGFR (manuscript in preparation). Therefore, whether the interaction between Sef and c-Cbl could affect the function of c-Cbl on RTKs stability remains to be elucidated.

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c-Cbl 介导了 hSef 的泛素化和降解 *

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摘要 Sef (similar expression to *fgf* genes)作为 FGF 信号通路中可诱导的拮抗分子相继在斑马鱼，小鼠，和人类中被鉴定出来，并进行了相应的功能研究．目前对于 Sef 蛋白本身稳定性的研究还未见报道．对 c-Cbl 对 Sef 稳定性的影响进行了研究．免疫荧光实验表明 Sef 能够和 c-Cbl 蛋白在细胞中发生共定位，随后的免疫共沉淀实验证明 Sef 能够和 c-Cbl 发生相互作用．体内泛素化实验表明 c-Cbl 能够使 Sef 发生明显的泛素化作用．这种泛素化最终导致了 Sef 本身的剂量依赖性的降解．针对 c-Cbl 的 siRNA 表达也使 Sef 稳定细胞系的表达水平得到恢复．结果表明，c-Cbl 对 Sef 的泛素化及降解可能作为一种调控拮抗因子的蛋白质水平从而最终调节信号通路的一种机制．

关键词 hSef，c-Cbl，泛素化，降解
学科分类号 Q

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