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Skp1 stabilizes the conformation of F-box proteins

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ABSTRACT

The SCF ubiquitin ligase complex consists of four components, Skp1, Cul1, ROC1/Rbx1, and a variable subunit F-box protein, which serves as a receptor for target proteins. The F-box proteins consist of an N-terminal ~40 amino acid F-box domain that binds to Skp1 and the C-terminal substrate-binding domain. We have reported previously that Fbs1 and Fbs2 are *N*-linked glycoprotein-specific F-box proteins. In addition, other three F-box proteins, Fbg3, Fbg4, and Fbg5, show high homology to Fbs1 and Fbs2, but their functions remain largely unknown. Here we report that Skp1 assists in correct folding of exogenously expressed F-box proteins. Fbs2 as well as Fbg3, Fbg4, and Fbg5 proteins formed SCF complexes but did not bind to *N*-glycoproteins when exogenously expressed alone. However, co-expression of Fbs2 and Fbg5 with Skp1 facilitated their binding to glycoproteins that reacted with ConA. Furthermore, Skp1 increased the cellular concentrations of F-box proteins by preventing aggregate formation. These observations suggest that Skp1 plays an important role in stabilizing the conformation of these F-box proteins, which increases their expression levels and substrate-binding.

1. Introduction

Ubiquitin-mediated proteolysis is the major selective proteolytic pathway in the cell and regulates many diverse cellular processes. In this system, the formation of polyubiquitin chain on the substrates is catalyzed by a series of enzymes, such as E1, E2 and, E3 [1]. During the cascade reaction for ubiquitylation, the E3 ubiquitin ligase is responsible for the selection of target proteins. The SCF complex is a multi-protein E3 ubiquitin ligase complex, which consists of four components, the invariable subunits Skp1, Cul1 and RING-finger protein ROC1/Rbx1, and a variable F-box protein that serves as a receptor for the target protein [2,3]. Cul1 functions as a scaffold of the SCF complex, by recruiting ROC1 and E2 to its C-terminus and the Skp1–F-box protein dimer to its N-terminus [4]. The human genome encodes 69 F-box proteins, most of which seem to function in specific ubiquitylation of a wide range of substrates [5]. The F-box proteins are divided into three classes according to the type of substrate-binding domains. The FBXW and FBXL families have WD40 repeats and leucine-rich repeats in the substrate-binding domains, respectively. The third class of F-box proteins is termed the FBXO family, which does not contain any of these domains [6,7].

The Fbs1 (F-box protein that recognizes sugar chain 1) is a glycoprotein-specific F-box protein, which was identified as a member of the endoplasmic reticulum-associated degradation (ERAD)-linked E3 ubiquitin ligase component [8]. It has been reported that Fbs1 belongs to a subfamily under the FBXO family consisting of at least five F-box proteins containing a highly homologous C-terminal substrate-binding domain [7,9]. Among them, Fbs1/Fbg1/FBXO2/NFB42 and Fbs2/Fbg2/FBXO6 have been demonstrated to recognize glycoproteins modified with high-mannose type oligosaccharides, while the binding activities of other F-box proteins, Fbg3/FBXO44, Fbg4/FBXO17, and Fbg5/FBXO27 to *N*-glycan have not been reported [10–12]. Distinct from general F-box proteins, the majority of Fbs1 is present as a Fbs1–Skp1 heterodimer, rather than forming the canonical SCF complex [13–16]. We reported previously that Fbs1 acts also as a chaperone by suppressing aggregation of glycoproteins in the cytosol of neuronal cells, independent of E3 ubiquitin ligase activity [15].

In this study, we first examined the ability of these five F-box proteins to form the SCF complex. During this analysis, we found that co-expression with Skp1 stabilized each F-box protein and potentiated its substrate-binding ability. Biochemical and imaging analyses showed that the exogenously expressed F-box protein without Skp1 was unstable and sensible to be degraded or form aggregates. These results suggest that Skp1 regulates cellular function and levels of F-box proteins by stabilizing their active form.

2. Materials and methods

2.1. Plasmid construction

The pcDNA3 vectors expressing amino-terminal Flag epitope fused to Fbs1, Fbs2, Fbg3, or Fbg4 and amino-terminal HA epitope fused to Cul1 or Skp1 have been described previously [8,15].

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Flag-tagged Fbg5 was generated from Myc-Fbg5 (from K. Iwai, Osaka University). Plasmids for the expression of fluorescently labeled F-box proteins were generated by inserting the DsRed-monomer as an amino-terminal tag into the pDsRed-Monomer C1 vector (Clontech, Mountain View, CA). All the clones generated by PCR were verified by sequencing.

2.2. Cell culture and immunologic analysis

293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), and transfected as described previously [8]. Cells were transiently transfected with a Flag-tagged F-box protein expression plasmid with or without a HA-tagged Skp1 plasmid. Immunoprecipitation from whole cell extracts was performed by using an anti-Flag antibody (M2, Sigma) with Dynabeads-protein G (Invitrogen Life Technologies, Carlsbad, CA). For Western blots or lectin blots analyses, immune complexes were eluted by using the Flag peptide (Sigma) and loaded on SDS-PAGE. Antibodies to Flag, Cul1, Skp1, and ROC1 and ConA-HRP have been described previously [8].

2.3. Immunofluorescence microscopy

293T and HeLa cells cultured onto glass-bottomed 35 mm dishes were transiently transfected to express Ds-Red monomer-fused F-box proteins or those in combination with HA-Skp1.48 h after transfection, cells were visualized with a laser-scanning microscope (LSM510; Carl Zeiss, Jena, Germany) equipped with a Plan-Apochromat 63× NA 1.4 oil differential interference contrast objective lens.

3. Results

3.1. Skp1 promotes substrate-binding ability of F-box proteins

We reported previously the inefficiency of SCF complex in the formation of Fbs1, and that the intervening segment (26 amino acids) between the F-box domain and the sugar-binding domain of Fbs1 suppresses the formation of the SCF complex [15]. To examine the ability of other Fbs family members to form the SCF complex, we expressed Flag-tagged F-box proteins alone in 293T cells (5 μg each plasmid for 10^6cells) and analyzed the immunocomplex precipitated with an anti-Flag antibody by Western blotting using anti-Cul1 and ROC1 antibodies (Fig. 1A). All F-box proteins tested, with the exception of Fbs1, formed the SCF complex under normal conditions, and the Cul1 complexed with Fbs2 and Fbg5 was prominently modified with NEDD8, which were detected due to a mobility shift in Cul1 blot. Previous studies reported that the cullin family proteins, Cul1, 2, 3, 4A, 4B, 5, and 7, can be modified with NEDD8 and that this neddylation increases the ubiquitylation activity of cullins [17]. This result suggests that the SCF complex containing Fbs2 or Fbg5 has enough ubiquitin ligase activity.

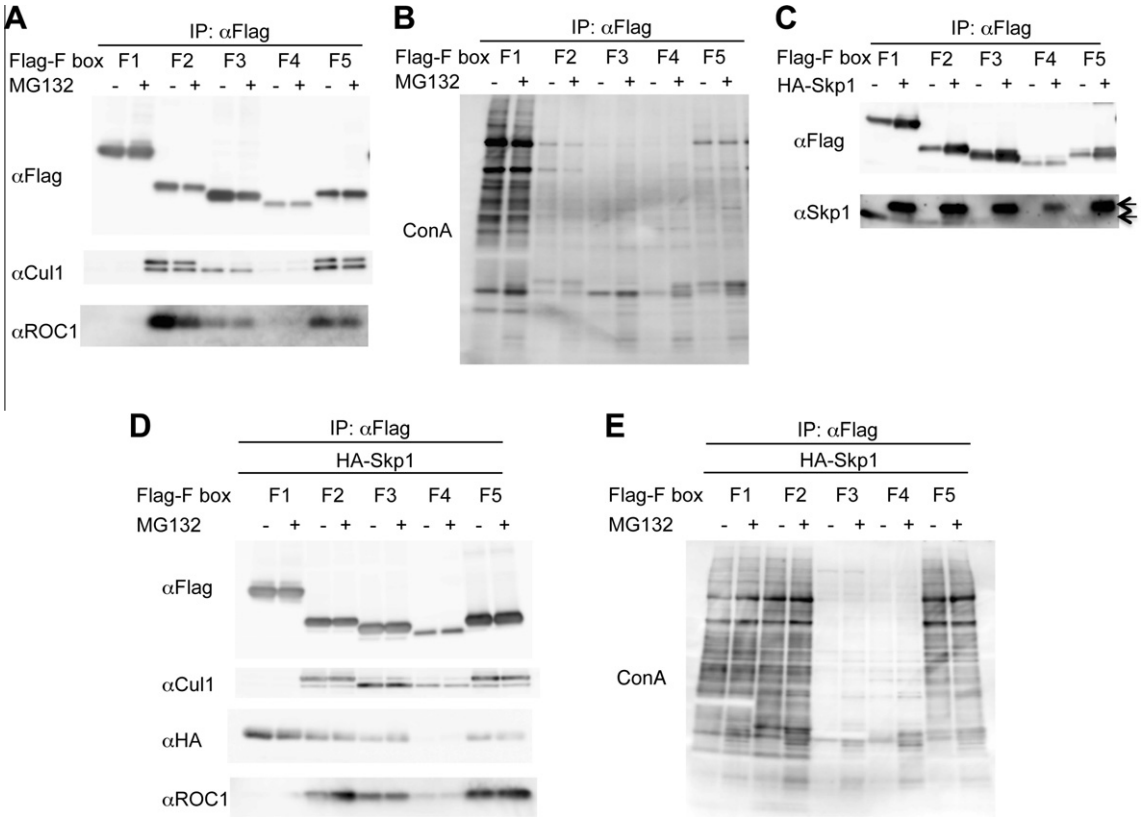

Fig. 1. Effects of exogenous Skp1 on SCF complex formation and binding to glycoprotein substrates of Fbs family member F-box proteins. (A and B) 293T cells were transfected with 5 μg plasmid encoding Flag-tagged Fbs1 (F1), Fbs2 (F2), Fbg3 (F3), Fbg4 (F4), or Fbg5 (F5). Forty-six hours after transfection, the cells were treated with or without 50 μM of MG132 for 90 min. Whole cell lysates were subjected to immunoprecipitation with an anti-Flag antibody. One third of the Flag peptide eluates from resulting precipitates were analyzed by immunoblotting with antibodies to Flag, Cul1, and ROC1 (A), and by lectin blotting with ConA. The higher molecular weight band that was reacted with anti-Cul1 antibody is neddylated Cul1. (C) 293T cells were transfected with a combination of 2.5 μg plasmid encoding Flag-tagged F-box protein and 2.5 μg plasmid encoding HA empty vector (-) or HA-tagged Skp1. In the blot of anti-Skp1 antibody, the upper and lower arrows show HA-Skp1 and endogenous Skp1, respectively. (D and E) 293T cells were transfected with 2.5 μg plasmid encoding Flag-tagged F-box protein and 2.5 μg plasmid encoding HA-tagged Skp1. One sixth of Flag peptide eluates from immunoprecipitates were analyzed by immunoblotting (D) and lectin blotting (E), as described in (A).

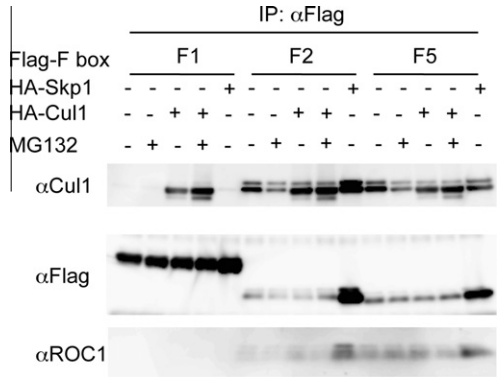

Fig. 2. Overexpression of Skp1 increases the amount of F-box proteins but does not inhibit SCF complex formation. 293T cells were transfected with 2.5 μg plasmid encoding Flag-tagged Fbs1 (F1), Fbs2 (F2), or Fbg5 (F5) combined with 2.5 μg plasmid encoding HA-tagged Cul1 or Skp1. Whole cell lysates were subjected to immunoprecipitaion with an anti-Flag antibody. One third of the immunoprecipitates were analyzed by immunoblotting with antibodies to Flag, Cul1, and ROC1.

We have reported that both Fbs1 and Fbs2 recognize *N*-linked high-mannose type oligosaccharides in their C-terminal sugar-binding domains [8,10]. To test the binding ability of *N*-glycan to Fbs member proteins, we examined the presence of glycoproteins in co-immunoprecipitates with these F-box proteins by ConA lectin blot, which detects proteins modified with high-mannose type oligosaccharides (Fig. 1B). Various glycoproteins known to react with ConA were included in co-immunoprecipitates with Fbs1, but Fbs2, in addition to other F-box proteins that scarcely interacted with glycoproteins containing high-mannose type oligosaccharides. The interaction of Fbs2 with glycoproteins did not increased even in the presence of MG132, suggesting that the lack of detectable interaction is not due to degradation by the ubiquitin–proteasome system.

We next expressed Flag-tagged F-box proteins simultaneously with HA-tagged Skp1 in 293T cells (2.5 μg plasmid for each F-box protein and 2.5 μg plasmid for Skp1), and the F-box proteins were immunoprecipitated with an anti-Flag antibody (Fig. 1C, D, and E). Since the expression of HA-tagged Skp1 increased the amount of exogenous F-box proteins (Fig. 1C), we applied half the amount of the immune complex onto SDS-PAGE in Fig. 1D and E compared with

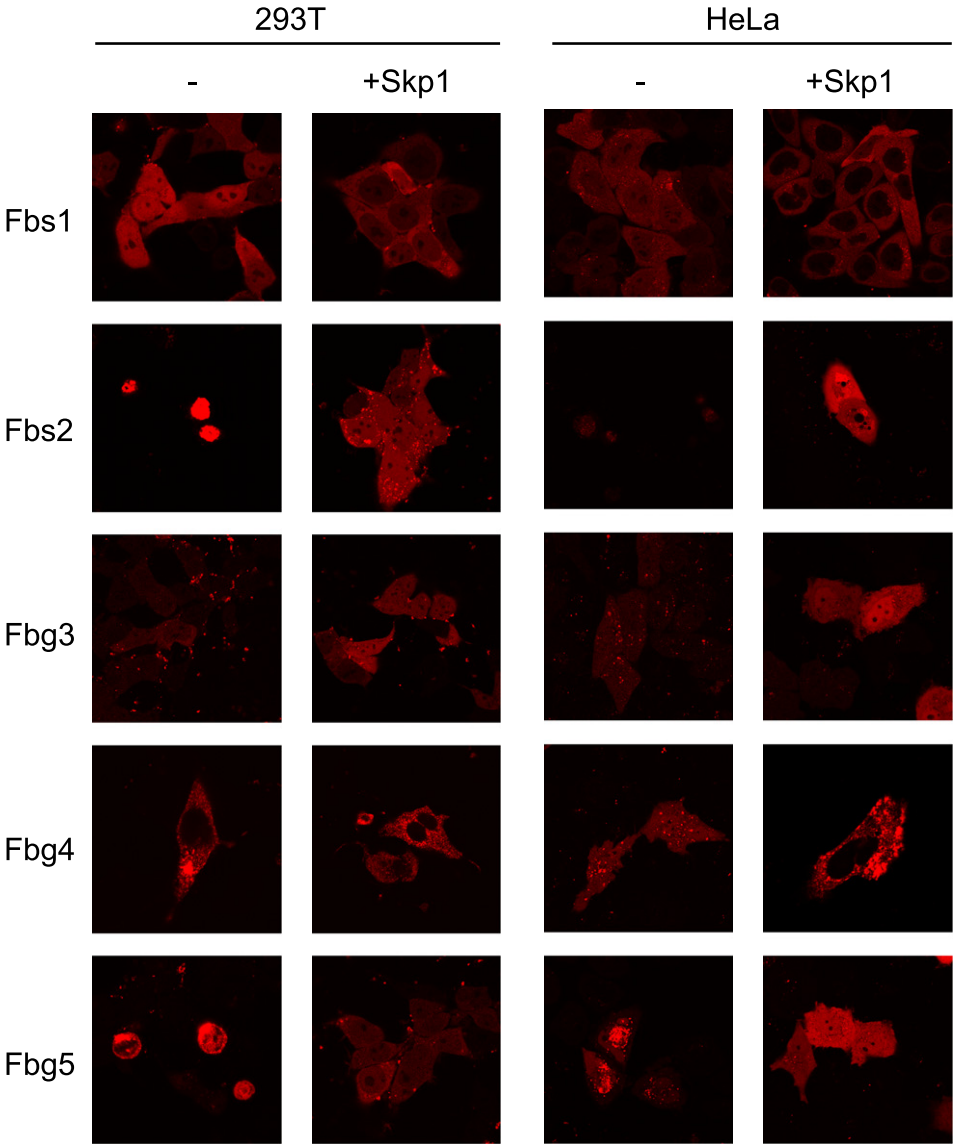

Fig. 3. Exogenous Skp1 expression suppresses aggregate formation and enhances expression of F-box proteins. 293T and HeLa cells were transfected with ds-Red-monomer-F-box protein alone or with HA-tagged Skp1. After 48 h, cells were visualized with a laser-scanning microscope.

those of Fig. 1A and B. The expression of HA-tagged Skp1 accelerated neddylation of Cul1 that interacted with Fbs2 and Fbg5, and increased the interaction of Cul1 with Fbg3 and Fbg4. However, Skp1 expression did not result in the formation of SCF-Fbs1 complex (Fig. 1D). Interestingly, various glycoproteins modified with high-mannose type oligosaccharides were detected in co-immunoprecipitates containing Fbs2 and Fbg5 as well as Fbs1, upon co-expression of HA-tagged Skp1 (Fig. 1E). The amounts of glycoproteins associated with Fbs1, Fbs2, and Fbg5 were almost the same in cells treated or untreated with MG132. On the other hand, both Fbg3 and Fbg4 did not interact with glycoproteins even in the presence of exogenous Skp1. These results suggest that Skp1 promotes the interaction of the exogenously expressed F-box proteins with their substrates.

3.2. Effect of Skp1 overexpression on SCF complex formation

It is possible that Skp1 overexpression impairs the formation of the SCF complex, thereby increasing the stable Skp1–F-box protein complex. Accordingly, we examined the effect of exogenous expression of Skp1 on SCF complex formation. To this end, we expressed exogenous Cul1 with F-box proteins (Fig. 2). The expression of HA-tagged Cul1 did not affect the intracellular concentrations of any F-box protein, but the amount of Cul1 complexed with F-box proteins, even Fbs1, increased. However, the expression of Cul1 was not associated with an increase in the amount of ROC1 and neddylated Cul1, suggesting that over expression of Cul1 hardly accelerated active SCF complex formation. In contrast, exogenous Skp1 rather increased the amount of ROC1 and neddylated Cul1 in immunocomplexes with Fbs2 and Fbg5. These results indicate that exogenous Skp1 promotes the substrate-binding ability of Fbs1, Fbs2, and Fbg5 without preventing the SCF complex formation.

3.3. Skp1stabilizes the expression of F-box proteins

We next assessed the expression and subcellular distribution of these F-box proteins in the presence and absence of exogenous Skp1 in 293T or HeLa cells by utilizing the Ds-Red-monomer, a fluorescent protein, which fuses to the F-box proteins at their amino-terminus (Fig. 3). Fbs1 was expressed strongly under both the presence and absence of exogenous Skp1, but its distribution switched to predominantly cytoplasmic by co-expressing with Skp1. In contrast, 293T cells expressing Fbs2 or Fbg5 in the absence of Skp1 were shrunk with large aggregates in the cytoplasm. However, co-expression of Skp1 resulted re-distribution of Fbs2 and Fbg5 into both the cytoplasm and the nucleus in 293T and HeLa cells. Moreover, the fluorescent signal of Fbg3 in the absence of Skp1 was weak in cells, but exogenous Skp1 enhanced Fbg3 expression. On the other hand, the aggregate formation by the expression of Fbg4 was restored partially by co-expression of Skp1, reflecting the inefficient immunoprecipitation of Fbg4 co-expressing Skp1. These fluorescence images suggest that Skp1 prevents aggregation formation of exogenously expressed F-box proteins. Consequently, Fbs2 and Fbg5 that interact with glycoproteins could be efficiently recovered by immunoprecipitation from cell lysates in the presence of sufficient amount of Skp1. In contrast, exogenously expressed Fbs1 can form an active conformation that efficiently recognizes glycoproteins even in the absence of Skp1. These results indicate that F-box proteins that tend to aggregate or to be degraded can be stabilized by Skp1 but not Cul1.

4. Discussion

While 69 F-box proteins have been discovered in the human genome and may be involved in a broad range of cellular functions, the available information about their function is still limited. One of the most important prerequisites of elucidating the function of F-box proteins is to identify their substrates. In this study, we demonstrate that overexpression of Skp1 stabilizes F-box proteins and promotes their substrate-binding activities probably by stabilizing their protein conformation. Therefore, overexpression of Skp1 could serve as an useful strategy to isolate new substrates of the SCF ubiquitin ligase complex.

As reported in previous studies, Fbs1 is expressed at higher levels than those of other Fbs family proteins and efficiently binds glycoproteins that can react with ConA lectin [11,14]. Our data indicated that Fbs1 is resistant to aggregate formation even in the absence of Skp1, but that other F-box proteins tend to aggregate in cells, reflecting their weak substrate-binding activity. It was recently reported that Fbs1 can interact with APC2 through the F-box domain [16]. In addition, Fbs1 has been shown to interact with co-chaperone/ubiquitin ligase CHIP through the N-terminal PEST domain [14]. It seems possible that these interacting proteins can stabilize Fbs1 instead of Skp1. In contrast, F-box proteins other than Fbs1 were markedly stabilized by exogenous Skp1, suggesting that Skp1 is the only protein that can interact with their F-box domains, although the effects on F-box proteins were not identical.

Importantly, Fbs2, Fbg3, and Fbs5 were re-localized to the nucleus, but Fbs1 and Fbg4 showed predominantly cytoplasmic distribution upon Skp1 overexpression. It has been reported that some F-box proteins contain a nuclear export sequence (NES) in their F-box domains, and competitive binding between Skp1 and CRM1 to the F-box domain controls their localization [18]. Both Fbs1 and Fbs2 include NES-like sequences in their F-box domains, and the nuclear accumulation of Fbs2 is consistent with this model. However, Fbs1 showed cytoplasmic localization upon Skp1 overexpression, suggesting non-functionality of the NES-like sequence in Fbs1. The increased substrate-binding of F-box proteins by overexpression of Skp1 may induce proper subcellular distribution of these proteins.

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