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MM-1 facilitates degradation of c-Myc by recruiting proteasome and a novel ubiquitin E3 ligase

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Abstract. We have reported that a novel c-Myc-binding protein, MM-1, repressed the E-box-dependent transcription activity of c-Myc by recruiting the HDAC1 complex via TIF1ß/KAP1, a transcriptional corepressor. We have also reported that a mutation of A157R in MM-1, which is often observed in patients with leukemia or lymphoma, abrogated all of the repressive activities of MM-1 toward c-Myc, indicating that MM-1 is a novel tumor suppressor. In this study, we found that MM-1 was bound to a component of proteasome and stimulated degradation of c-Myc in human cells. Knockdown of endogenous MM-1 in human HeLa cells by introduction of siRNA against MM-1 stabilized the endogenous c-Myc. To identify proteins that participate in c-Myc degradation by MM-1, *in vivo* and *in vitro* binding assays were carried out. The results showed that MM-1 directly bound to Rpt3, a subunit of 26S proteasome, and that c-Myc directly bound to Skp2, which recruited ElonginC, ElonginB and Cullin2, thereby forming a novel ubiquitin E3 ligase. Knockdown of endogenous Cullin2 stabilized the endogenous c-Myc. Thus, MM-1 is a factor that connects c-Myc to the ubiquitin E3 ligase and the proteasome.

Introduction

The proto-oncogene product c-Myc plays pivotal roles in cell proliferation, differentiation and apoptosis induction, and its levels are tightly regulated at several steps, including transcription, translation and protein stability (1-3). It is known that the half-life of c-Myc is approximately 30 min (4,5) and that its rapid proteolysis is catalyzed by the ubiquitin-proteasome system (6,7). It has been reported that the F-box protein Skp2 participated in c-Myc proteosomal degradation and acted as a cofactor for c-Myc-regulated transcription (8,9) and that phosphorylation-dependent degradation of c-Myc was mediated by the F-box protein Fbw7 (10). Since the expression of c-Myc is thought to be regulated by many pathways, it is possible that there exist other mechanisms or proteins that target c-Myc to the proteasome.

We have reported that MM-1, a novel protein binding to the myc box II located in the N-proximal region of c-Myc, suppressed transcription and transformation activities of c-Myc and that A157R mutation of c-Myc, which is observed at high frequency in patients with lymphoma or leukemia, abrogated all of the functions of MM-1 toward c-Myc, indicating that MM-1 is a novel tumor suppressor (11,12). Regarding the MM-1-dependent transrepression pathway of c-Myc, we have shown that MM-1 recruited the HDAC complex to c-Myc via TIF1ß, a corepressor (13), and that the *c-fms* gene was identified as a target gene for this pathway (14). In this study, we found another role of MM-1 in connecting c-Myc to the proteasome and a novel ubiquitin ligase that stimulates c-Myc degradation.

Materials and methods

Plasmids. pcDNA3-FLAG-Elongin B, pcDNA3-FLAG-Rpt3, pcDNA3-FLAG-Cullin2, pcDNA3-T7-Rpn12 and pcDNA3-T7-Skp2, corresponding cDNAs starting from the first ATG, were inserted in-frame into the *Eco*RI-*Xho*I sites of pcDNA3-FLAG and pcDNA3-T7, respectively. Plasmids of various kinds of c-Myc and MM-1 were described previously (11-13).

Antibodies. Catalog numbers and dilutions of commercially available antibodies used in this study are shown in Table I.

Cell culture and transfection. Human HeLa and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Transfection of plasmid DNAs into cells was carried out by the calcium phosphate precipitation method. The nucleotide sequences of the upper and lower strands for siRNA targeting various genes were as follows: MM-1, 5'-UGCUCAAGAACCAGCUGGAdTdT-3' and 5'-UCCAGCUGGUUCUUGAGCAdTdT-3'; Cullin 1, 5'-GGUUUGCCUGCAAUGAGAUUU-3' and 5'-AUCUCAUUGCAGGCAAACCCG-3'; Cullin 2, 5'-GCCGUGGUCAUGUU

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Key words: c-Myc, MM-1, degradation, ubiquitin-proteasome system

Table I. Details of antibodies used in this study.

Target protein	Company	Catalog number	Species	Dilution
Flag-tag	Sigma, St. Louis, MO	F3165	Mouse	5000
HA-tag	MBL, Ina, Japan	561	Rabbit	2000
T7-tag	Novagen, San Diego, CA	69522-3	Mouse	5000
HA-tag	Bethyl, Montgomer, TX	A190-108A	Rabbit	2000
T7-tag	Bethyl, Montgomer, TX	A190-116A	Rabbit	2000
c-Myc	Santa Cruz Biotech., Santa Cruz, CA	sc-764	Rabbit	1000
c-Myc	Santa Cruz Biotech., Santa Cruz, CA	sc-42	Mouse	1000
prefoldin 5/MM-1	Santa Cruz Biotech., Santa Cruz, CA	sc-19843	Goat	1000
actin	Chemicon, Temecula, CA	MAB1501R	Mouse	4000
Cullin1	Neomarkers, Fremont, CA	RB-042-P0	Rabbit	1000
Cullin2	Neomarkers, Fremont, CA	RB-14802-P0	Rabbit	1000
ElonginB	BioLegend, San Diego, CA	613001	Rabbit	500
ElonginC	BioLegend, San Diego, CA	613002	Rabbit	500
Skp1	Zymed, Carlsbad, CA	32-3800	Mouse	500
Skp2	Zymed, Carlsbad, CA	32-3400	Mouse	500
IRDye800-mouse IgG	Rockland, Philadelphia, PA	610-732-124	Donkey	5000
Alexa680-rabbit IgG	Molecular Probe, Temecula, CA	A-21076	Goat	5000

GGAAUAC-3' and 5'-AUUCCAACAUGACCACGGCUU-3'; ElonginB, 5'-GGCCCAUUUCCCCCAAUAAAA-3' and 5'-UUAUUGGGGGAAAUGGGCCUC-3'; Elongin C, 5'-CGG GACUGACGAGAAACUACU-3' and 5'-UAGUUUCUCGU CAGUCCCGCA-3'; Skp2, 5'-CCGCUGCCCACGAUCAUU UAU-3' and 5'-AAAUGAUCGUGGGCAGCGGAA-3'; Skp1, 5'-CUAUAACAUGCCAGCAAAAGA-3' and 5'-UUUUGC UGGCAUGUUAUAGGU-3'; and Luciferase, 5'-CGUACGC GGAAUACUUCGATT-3' and 5'-UGCAAGUAUUCCGCG UACGTT-3'. Various amounts of siRNA were transfected into HeLa cells using Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the supplier's manual.

In vitro binding assay. 35S-labeled ElonginB, Rpt3, Rpn12 or c-Myc was synthesized *in vitro* using the reticulocyte lysate of the TnT-transcription-translation coupled system (Promega, Madison, WI). Labeled proteins were mixed with GST or GST-MM-1 expressed in and prepared from *E. coli* at 4˚C for 60 min in a buffer containing 150 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 8.0), and 0.5% NP-40. After washing with the same buffer, the bound proteins were separated on a 12% polyacrylamide gel containing SDS and visualized by fluorography.

In vivo binding assay. Two μg of pcDNA3-FLAG-Elongin B or pcDNA3-FLAG-MM-1 together with 2 μg of pcDNA3-HA-MM-1 or pcDNA3-T7-Rpn12 were transfected into 60% confluent human 293T cells in a 10-cm dish by the calcium phosphate precipitation technique. Forty-eight hours after transfection, the whole cell extract was prepared by the procedure described previously (11). Approximately 2 mg of the 293T cell proteins was first immunoprecipitated with a mouse anti-Flag antibody (Sigma) or with non-specific mouse IgG under the same conditions as those of the *in vitro* binding assay as described above. After washing with the same buffer, the precipitates were separated on a 12% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, and reacted with a rabbit anti-HA antibody (MBL), with a mouse anti-T7 antibody (Novagen) or with the mouse anti-FLAG antibody. To examine the association of endogenous DJ-1 with c-Myc, proteins from HeLa cells were immunoprecipitated with an anti-c-Myc polyclonal antibody (Santa Cruz Biotech.) or with non-specific rabbit IgG, and the precipitates were subjected to Western blot analysis with anti-prefoldin 5/MM-1 (Santa Cruz Biotech.) and anti-c-Myc monoclonal (Santa Cruz Biotech.) antibodies. Immunoprecipitated proteins were then reacted with an IRDye800 (Rockland, Philadelphia, PA) or Alexa680-conjugated second antibody (Molecular Probe) and visualized by using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE).

RT-PCR analysis. Total RNAs were prepared from HeLa cells by the acid guanidine thiocyanate-phenol-chloroform method, and cDNA was synthesized using the oligo dT primer and BcaBEST polymerase (Takara, Kyoto, Japan). The first strand of cDNA products was amplified with specific primers for the first 5 min at 94˚C and then for 30 cycles of 1 min at 94˚C, 2 min at 55˚C and 3 min at 72˚C. The nucleotide sequences of the sense and antisense primers were as follows: MM-1α sense, 5'-GAATTACTCGTCCCACTGAC-3'; and antisense, 5'-GCCCAGCTGGCACATTTATTGGCATTA-3'. The nucleotide sequences of the sense and antisense primers for c-myc and GAPDH genes were described previously (15). The amplified products were separated on a 2% agarose gel and stained with ethidium bromide.

Effect of siRNA on c-Myc. HeLa cells were transfected with 10 and 20 nM siRNA by Oligofectamine reagent. Forty-eight

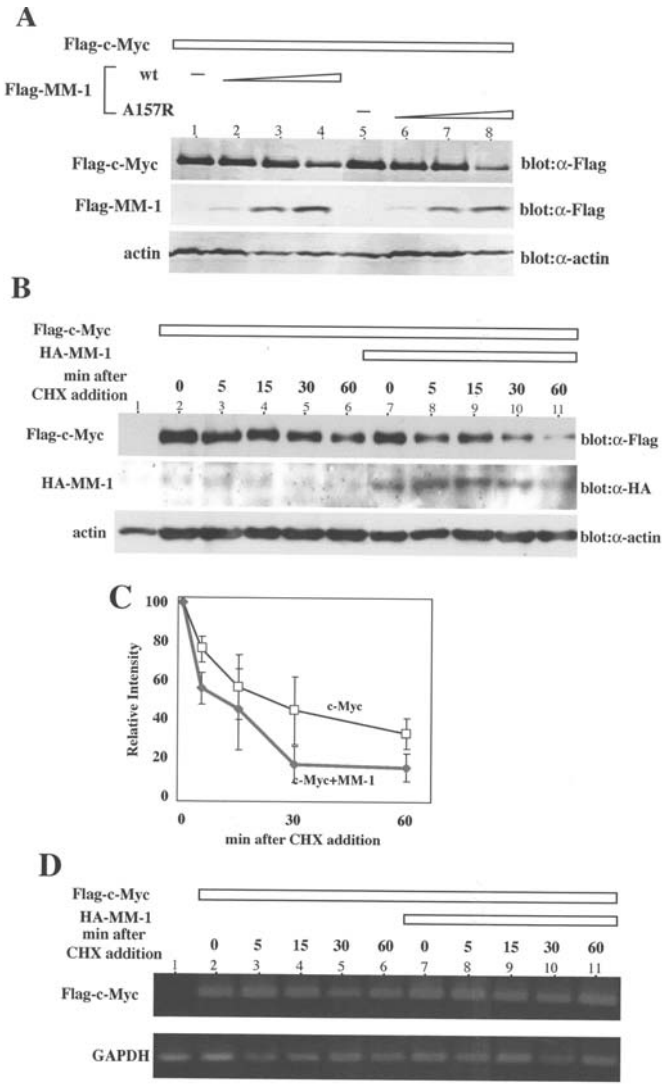

Figure 1. Stimulation of c-Myc degradation by MM-1. (A) HeLa cells were cotransfected with 1 μg of pcDNA3-Flag-c-Myc and various amounts of pcDNA3-Flag-MM-1 or pcDNA3-Flag-MM-1(A157R) by the calcium phosphate precipitation method. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were separated in a 10% or 15% polyacrylamide gel and blotted with an anti-Flag antibody (Sigma) or an anti-actin-antibody (Chemicon). The amounts of pcDNA3-Flag-MM-1 and pcDNA3-Flag-MM-1(A157R) used for transfection were 0, 0.5, 1, and 2 μg in lanes 1-4 and lanes 5-8, respectively. (B) HeLa cells were transfected with 1 μg of pcDNA3-Flag-c-Myc without (lanes 2-6) or with 2 μg of pcDNA3-Flag-MM-1 by the calcium phosphate precipitation method. Forty-eight hours after transfection, 25 μg/ml of cyclohexyimide (CHX) was added to the cells (lanes 7-11). At various times after addition of CHX, cell extracts were prepared and the proteins in the extracts were analyzed as described in A. (C) Intensities of the bands in Western blotting in B were measured by a densitometer, and intensities of Flag-c-Myc were normalized to those of actin. The intensity of Flag-c-Myc in cells transfected with Flag-c-Myc alone was set at 100, and relative intensities of Flag-c-Myc at various times after CHX treatment are shown. (D) HeLa cells were transfected with Flag-c-Myc without and with pcDNA3-Flag-MM-1 and treated with CHX as in B. Total RNAs were extracted from cells at various times after CHX treatment, and the expression of Flag-c-Myc and GAPDH was analyzed by RT-PCR with specific primers for Flag-c-Myc and GAPDH on total RNA extracted as substrates.

hours after transfection, proteins extracted from transfected cells were analyzed by Western blotting as described above. Antibodies used were anti-c-Myc rabbit polyclonal antibody (Santa Cruz Biotech.), anti-Cullin1 rabbit polyclonal antibody (Neomarkers), anti-Cullin2 rabbit polyclonal antibody (Neomarkers), anti-ElonginB rabbit polyclonal antibody (BioLegend), anti-ElonginC rabbit polyclonal antibody (BioLegend), anti-Skp1 mouse monoclonal antibody (Zymed), and anti-Skp2 mouse monoclonal antibody (Zymed, Carlsbad).

Statistical analyses. Data are expressed as means ± S.D. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's t-test.

Results and discussion

Degradation of c-Myc by MM-1. In order to determine the functions of MM-1, a two-hybrid screening of MM-1-binding proteins was carried out using a library of human K562 cell cDNA and MM-1 as a bait as described previously (13). In addition to TIF1ß, three proteins related to protein degradation systems, Rpt3, Rpn12 and ElonginB, were obtained in this screening, suggesting that MM-1 participates in c-Myc degradation. To examine this possibility, Flag-tagged c-Myc was cotransfected with various amounts of Flag-tagged wild-type or A157R mutant of MM-1 into human HeLa cells, and the levels of Flag-c-Myc and Flag-MM-1s were measured by Western blotting with an anti-Flag antibody (Fig. 1A). While the level of the endogenous actin was hardly changed, the levels of Flag-c-Myc were found to be decreased by the introduction of Flag-MM-1 and Flag-MM-1(A157R) in a dose-dependent manner (Fig. 1A, lanes 1-4). To further confirm this effect of MM-1 on c-Myc, the half-life of c-Myc was determined by transfection of Flag-c-Myc with or without HA-tagged MM-1 in the presence of cyclohexyimide (CHX), an inhibitor of protein synthesis, and the amounts of c-Myc were calculated after normalization to that of actin (Fig. 1B and C, respectively). The results clearly showed that while the half-life of Flag-c-Myc without introduced MM-1 was approximately 30 min as reported previously (4,5), MM-1 reduced the half-life of c-Myc to 15 min. Under these experimental conditions, it was found that Flag-MM-1 did not affect the mRNA levels of c-Myc (Fig. 1D). These results indicate that MM-1 stimulates c-Myc degradation.

To exert the effect of MM-1 on c-Myc degradation under physiological conditions in cells, siRNA targeting MM-1 was applied to HeLa cells. Since the *MM-1* gene is located on human chromosome 12q12-12q13 and produces fusion-type MM-1 and four splicing variants, MM-1α, -1ß, 1γ, and -1δ (12,16), the nucleotide sequence of siRNA corresponding to that in exon 1 was chosen to knock down all of the MM-1s (Fig. 2A). Forty-eight hours after transfection of the siRNA into HeLa cells, expression levels of mRNAs of MM-1, c-Myc and GAPDH were measured by RT-PCR using specific primers (Fig. 2B). The results clearly showed that while the levels of mRNAs of c-Myc and GAPDH were not changed, that of MM-1 was decreased by the siRNA against MM-1 in a dose-dependent manner, indicating that this siRNA knocks down endogenous MM-1. The endogenous levels of c-Myc, MM-1 and actin in HeLa cells transfected with siRNA against MM-1 were then measured by Western blotting with anti-c-Myc, anti-MM-1 and anti-actin antibodies, and the levels of c-Myc and MM-1 were normalized to that of actin (Figs. 2C, right and left panels, respectively). The results showed that a reverse

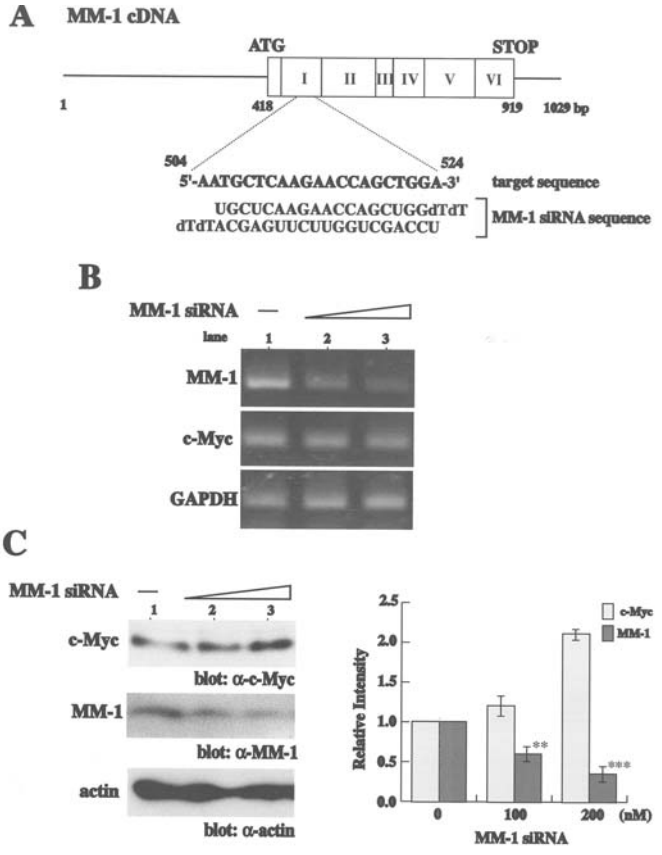

Figure 2. Stabilization of c-Myc in MM-1-knockdown cells. (A) The nucleotide sequence and location of siRNA targeting MM-1 corresponding to MM-1 cDNA are shown. (B) HeLa cells were transfected with 0, 100 and 200 nM of siRNA by Oligofectamine reagent (lanes 1, 2 and 3, respectively). Forty-eight hours after transfection, total RNAs were extracted from cells, and the expression of endogenous MM-1, c-Myc and GAPDH was analyzed by RT-PCR with specific primers for MM-1, c-Myc and GAPDH on total RNA extracted as substrates. (C) HeLa cells were transfected with 0, 100 and 200 nM of siRNA as described in B. Forty-eight hours after transfection, cell extracts were prepared, and the proteins in the extracts were blotted with an anti-c-Myc polyclonal antibody (Santa Cruz Biotech.), anti-MM-1 antibody (kindly provided by Dr A. Nakagawara) (26), or anti-actin-antibody (Chemicon). The proteins that reacted with primary antibodies were visualized with IRDye800-conjugated or Alexa Fluor680-conjugated secondary antibodies using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE) (Fig. 2C, right panel). Intensities of the bands in Western blotting shown in Fig. 2C (right panel) were measured by an Odyssey system, and intensities of c-Myc and MM-1 were normalized to those of actin. The intensities of c-Myc and MM-1 in cells not transfected with siRNA were set at 1.0, and relative intensities of c-Myc and MM-1 are shown (Fig. 2C, left panel). All the experiments were repeated more than 3 times. Asterisks indicate significant difference from the cells without siRNA; $^{**}p<0.01$, $^{***}p<0.001$.

correlation of the amounts of endogenous c-Myc and MM-1 was observed in cells transfected with siRNA in a dose-dependent manner. When the amount of MM-1 was reduced to 1/3 of that without siRNA, more than a two-fold amount of c-Myc was observed, suggesting that MM-1-knockdown stabilizes c-Myc at the protein level.

Association of MM-1 with ElonginB, Rpt3 and Rpn12. To identify the proteins that participate in the degradation reaction of c-Myc, associations of MM-1 with various proteins were investigated. Complex formation of endogenously expressed MM-1 with c-Myc was first examined. Proteins extracted from HeLa cells were immunoprecipitated with an anti-c-Myc antibody and the precipitates were analyzed by Western blotting with anti-MM-1 and c-Myc antibodies (Fig. 3A). The results showed association of endogenous MM-1 with c-Myc in cells. Since ElonginB, Rpt3 and Rpn12 were obtained as MM-1-binding proteins in yeast, associations of MM-1 with these proteins in cells were then examined. To do this, Flag-ElonginB, Flag-Rpt3 or Flag-MM-1 was cotransfected with HA-MM-1, HA-MM-1 or T7-Rpn12, respectively, into human 293T cells. Forty-eight hours after transfection, cell extracts were prepared from transfected cells, proteins in the extracts were immunoprecipitated with an anti-Flag antibody, and the precipitates were subjected to Western blotting with an anti-HA, anti-T7, or anti-Flag antibody (Fig. 3B). It was confirmed that the Flag antibodies precipitate Flag-tagged proteins (Fig. 3B, lower panels). The results also showed that HA-MM-1 or Flag-MM-1 was co-immunoprecipitated with Flag-ElonginB, Flag-Rpt3 and T7-Rpn12 (Fig. 3B, lanes 3, 6 and 9, respectively), indicating that MM-1 is associated with these proteins in 293T cells. To examine direct binding of MM-1 with these proteins, *in vitro* pull-down assays were then carried out. GST or GST-MM-1 expressed in and prepared from *E. coli* was mixed with ^{35}S-labeled c-Myc, ElonginB, Rpt3 and Rpn12, all of which were synthesized *in vitro* in a reticulocyte lysate, and bound proteins were separated in a gel and visualized by fluorography (Fig. 3C). It was confirmed that GST-MM-1, but not GST alone, bound to c-Myc as described previously (11) (Fig. 3C, lanes 1-3). The results showed that GST-MM-1 directly bound to Rpt3 but not to ElonginB and Rpn12 (Fig. 3C, lanes 9, 6 and 12, respectively). These results from *in vivo* and *in vitro* binding assays suggest that MM-1 forms complexes with a proteasome containing Rpt3, Rpn12 or a ubiquitin ligase containing Elongin B.

Association of c-Myc with a novel ubiquitin ligase complex. ElonginB was first identified as a component of a transcription elongation complex composed of ElonginA, ElonginB and ElonginC, in which ElonginB directly binds to ElonginC and ElonginC directly binds to the F-box region of ElonginA (17). It is known that there are two ubiquitin ligase complexes, an SCF complex composed of Cullin1, Skp1 and an F-box-containing protein such as Skp2 and a VBC complex composed of Cullin2, ElonginB, ElonginC and VHL (18-21). Skp2 and VHL recognize substrates to be ubiquitinated, and Skp1 directly binds to the F-box region of Skp2 (17,19,20). Since Skp1 and ElonginC mutually have homologous amino acid sequences (19), it is therefore possible that ElonginC, instead of Skp1, binds to Skp2, leading to formation of a complex among ElonginB, ElonginC, Skp2, MM-1 and c-Myc. To examine this possibility of association between Skp2, MM-1 and c-Myc, Flag-MM-1 or Flag-c-Myc was cotransfected with T7-Skp2 into 293T cells. Forty-eight hours after transfection, cell extracts were prepared from transfected cells, proteins in the extracts were immunoprecipitated with an anti-Flag antibody, and the precipitates were analyzed by Western blotting with an anti-T7 antibody or anti-Flag antibody (Fig. 4A). It was confirmed that the Flag antibodies precipitate Flag-tagged proteins, including wild-type MM-1, MM-1(A157R) and c-Myc (Fig. 4A, lower panels). The results also showed that T7-Skp2 was co-immunoprecipitated with Flag-wild-type

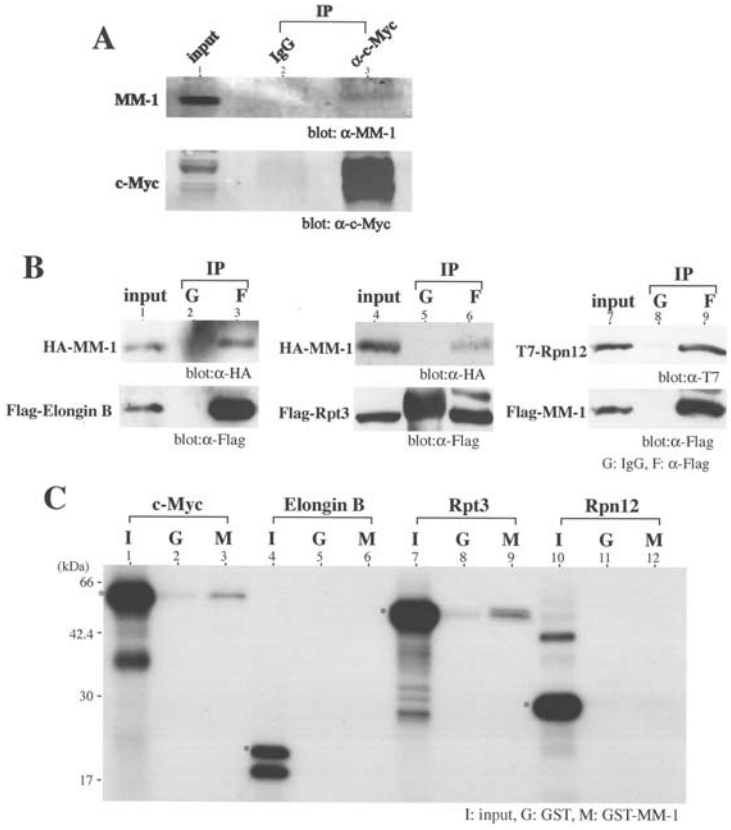

Figure 3. Associations of MM-1 with Elongin B, Rpt3 and Rpn12. (A) Proteins extracted from HeLa cells were immunoprecipitated with an anti-c-Myc antibody and the immunoprecipitates were then analyzed by Western blotting with anti-MM-1 and anti-c-Myc antibodies. (B) Expression vectors for Flag-Elongin B, Flag-Rpt3, Flag-MM-1, HA-MM-1 and T7-Rpn12 were transfected in various combinations into 293T cells. Forty-eight hours after transfection, cell extracts were prepared, proteins were immunoprecipitated (IP) with an anti-FLAG monoclonal antibody (Sigma), and precipitates were blotted with an anti-HA polyclonal antibody (MBL), anti-T7 monoclonal antibody (Novagen) or anti-FLAG antibody. (C) GST-MM-1 or GST was expressed in *E. coli* BL21(DE3) and applied to glutathione-Sepharose 4B. 35S-c-Myc, -Elongin B, -Rpt3, or -Rpn12 synthesized *in vitro* in a coupled transcription/translation system was then applied to the column. The proteins that had bound to the column were separated in a gel and visualized by fluorography.

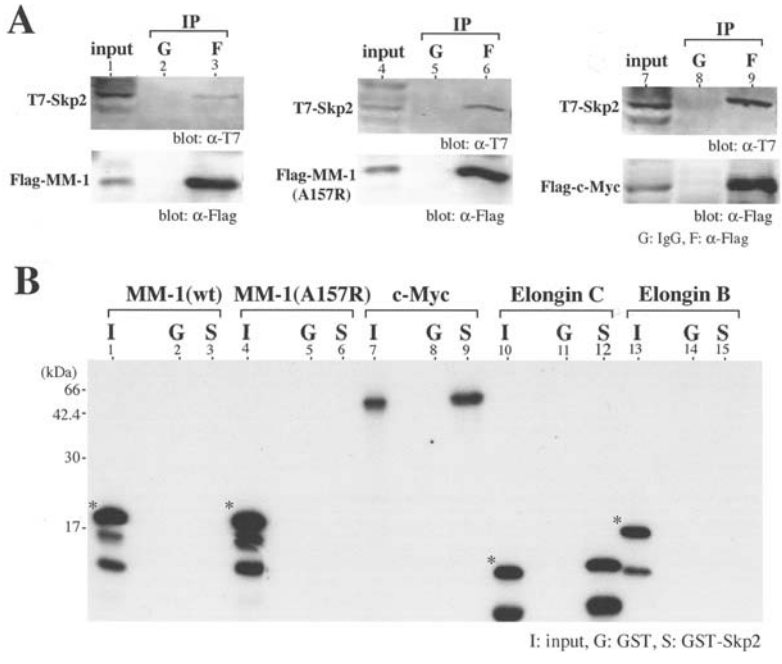

Figure 4. Association of MM-1 or c-Myc with Skp2. (A) Expression vectors for Flag-MM-1, Flag-MM-1(A157R), Flag-c-Myc and T7-Skp2 were transfected in various combinations into 293T cells. Forty-eight hours after transfection, cell extracts were prepared, proteins were immunoprecipitated (IP) with an anti-FLAG monoclonal antibody (Sigma), and precipitates were blotted with an anti-T7 monoclonal antibody (Novagen) or anti-FLAG antibody. (B) GST-Skp2 or GST was expressed in *E. coli* BL21(DE3) and applied to glutathione-Sepharose 4B. 35S-MM-1, -MM-1(A157R), -c-Myc, -ElonginC, or -ElonginB synthesized *in vitro* in a coupled transcription/translation system was then applied to the column. The proteins that had bound to the column were separated in a gel and visualized by fluorography.

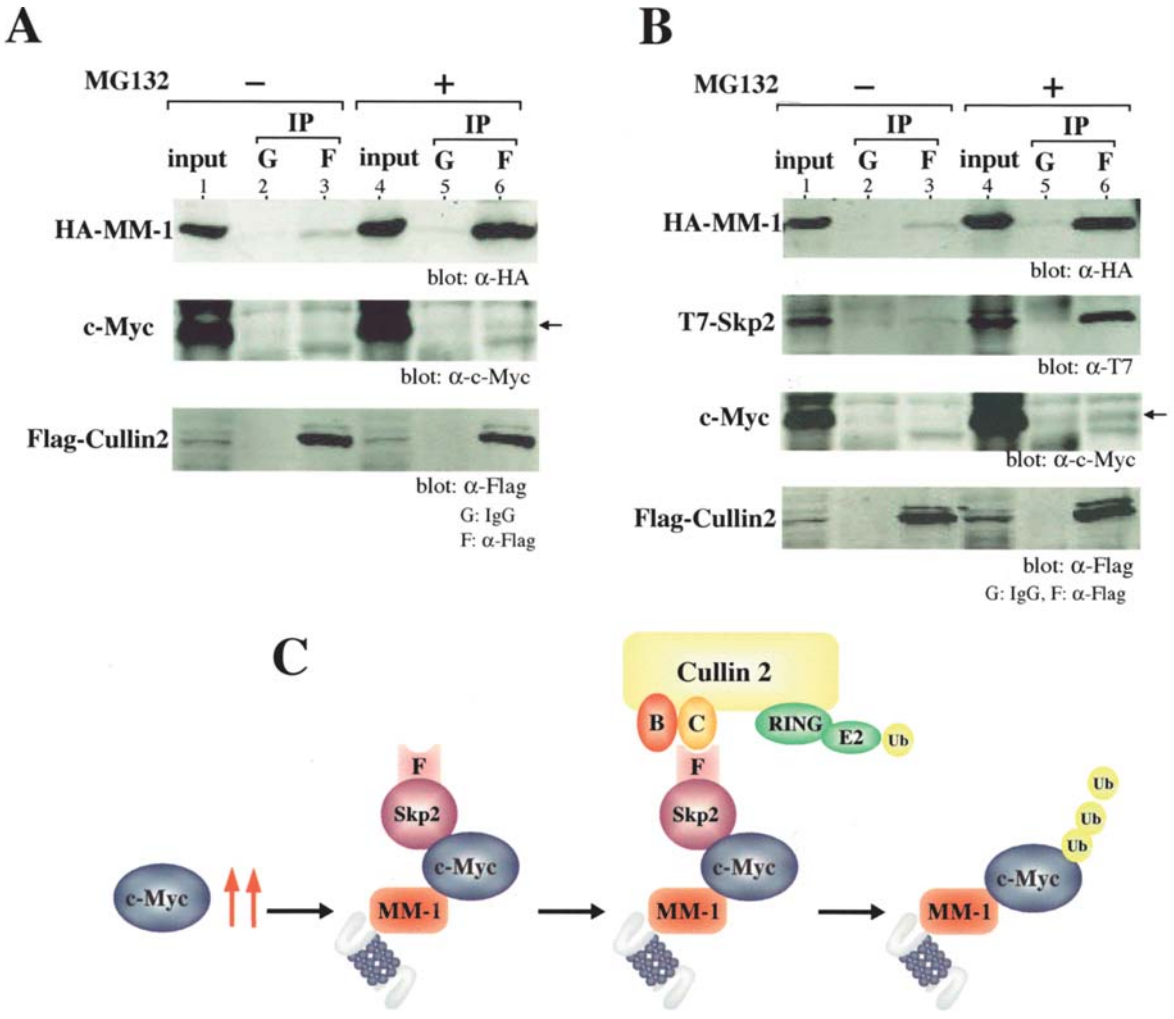

Figure 5. Association of MM-1 or c-Myc with Cullin2. (A and B) Expression vectors for HA-MM-1, Flag-Cullin2 and T7-Skp2 were transfected in various combinations into 293T cells. Forty-eight hours after transfection, cell extracts were prepared, proteins were immunoprecipitated (IP) with an anti-FLAG monoclonal antibody (Sigma), and precipitates were blotted with an anti-c-Myc polyclonal antibody (Santa Cruz Biotech.), anti-T7 monoclonal antibody (Novagen) or anti-FLAG antibody. (C) Schematic model of mutual interactions of c-Myc, MM-1, the proteasome and the ubiquitin ligase complex.

MM-1, -MM-1(A157R) and -c-Myc (Fig. 4A, lanes 3, 6 and 9, respectively), indicating that Skp2 is associated with MM-1 and c-Myc in 293T cells. To examine the binding activity of Skp2 to ElonginC and the direct binding partner of Skp2 to MM-1 or c-Myc, *in vitro* pull-down assays were then carried out. GST or GST-Skp2 was mixed with ^{35}S-labeled wild-type MM-1, -MM-1(A157R), -c-Myc, -ElonginC and -ElonginB, all of which were synthesized *in vitro* in a reticulocyte lysate, and bound proteins were separated in a gel and visualized by fluorography (Fig. 4B). The results showed that GST-Skp2, but not GST, directly bound to c-Myc and ElonginC but not to MM-1s or ElonginB (Fig. 4B, lanes 9, 12, 3, 6 and 15, respectively). These results from *in vivo* and *in vitro* binding assays suggest that MM-1 binds to a proteasome complex via Rpt3 and to an ElonginB-ElonginC-Skp2 complex via c-Myc. Thus, the presence of a novel combination of a complex containing ElonginB, ElonginC and Skp2 was shown.

The VBC complex of E3 ligase is composed of Cullin2, ElonginB, ElonginC and VHL (20,21). In the present study, Skp2 was found to be a substitute for VHL in order to recognize a substrate, c-Myc. To examine the possibility that Cullin2 forms a complex with c-Myc and MM-1, Flag-Cullin2 was cotransfected with HA-MM-1 into 293T cells in the presence or absence of MG132, a proteasome inhibitor. Forty-eight hours after transfection, cell extracts were prepared from transfected cells, proteins in the extracts were immunoprecipitated with an anti-Flag antibody, and the precipitates were analyzed by Western blotting with an anti-HA antibody, anti-Flag antibody, or anti-c-Myc antibody (Fig. 5A). It was confirmed that the Flag antibody, but not non-specific IgG, precipitated Flag-Cullin2 (Fig. 5A, lower panels). The results also showed that Flag-Skp2 was co-immunoprecipitated with HA-MM-1 and a small amount of endogenous c-Myc in the absence of MG132 (Fig. 5A, lane 3 in the upper and middle panels, respectively) and that the amounts of co-immunoprecipitated HA-MM-1 and endogenous c-Myc increased in the presence of MG132, suggesting that abrogation of degradation of c-Myc or MM-1 by the proteasome system stabilizes the complex formation among Cullin2, MM-1 and c-Myc. Involvement of Skp2 in this complex was confirmed by the same experiment as that described above, in which T7-Skp2 was cotransfected (Fig. 5B). It was found that Flag-Cullin2 was co-immunoprecipitated with T7-Skp2 along with HA-MM-1 and endogenous c-Myc and that

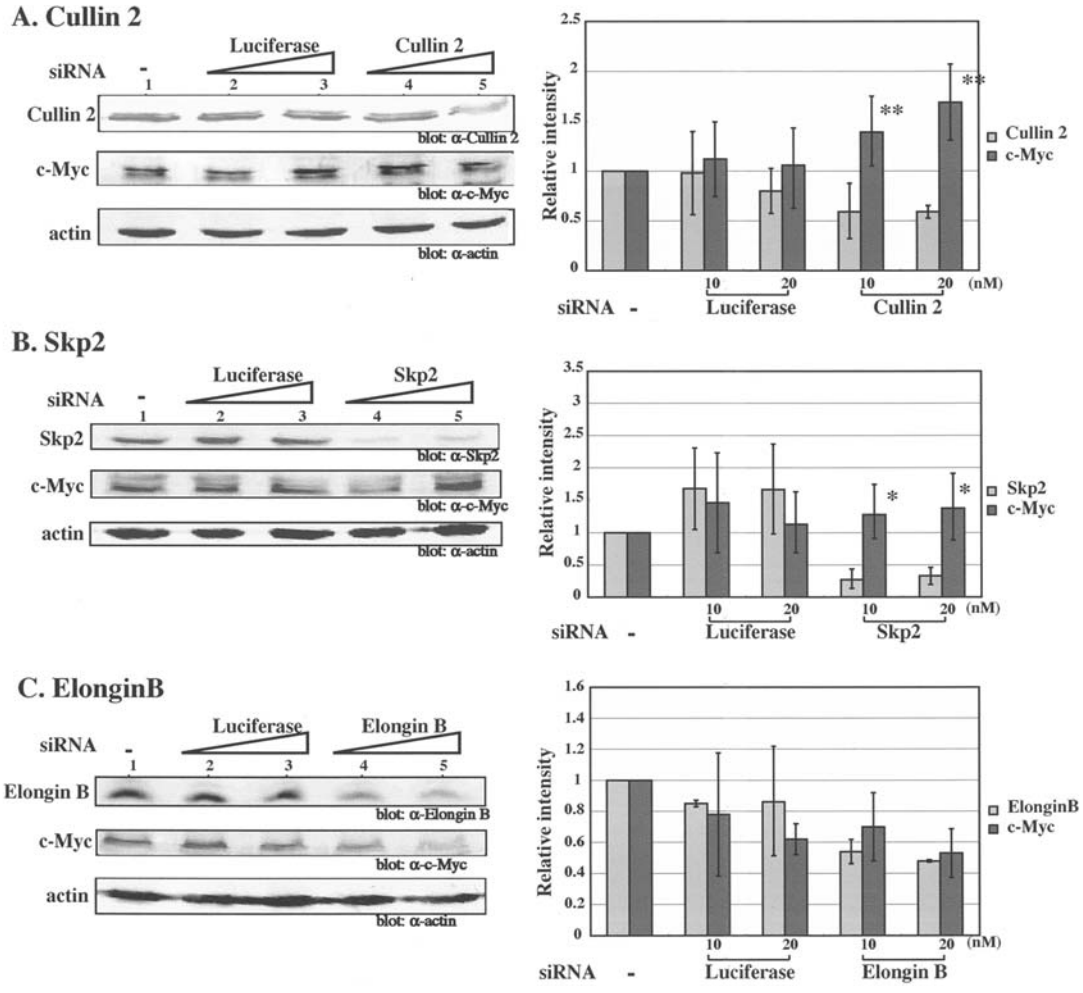

Figure 6. Stabilization of c-Myc by siRNAs targeting Cullin2 and Skp2. HeLa cells were transfected with various siRNAs against proteins of interest. Forty-eight hours after transfection, proteins were extracted from transfected cells and analyzed by Western blotting with corresponding antibodies. Intensities of the bands in Western blotting shown in Fig. 6 (right panel) were measured by an Odyssey system, and intensities of c-Myc and respective protein were normalized to those of actin. The intensities of c-Myc and respective protein in cells not transfected with siRNA were set at 1.0, and relative intensities of c-Myc and respective protein are shown (Fig. 6, left panel). All the experiments were repeated more than 7 times. Asterisks indicate significant difference from the cells without siRNA; $^{*}p<0.05$; $^{**}p<0.01$.

association of these proteins was enhanced by the presence of MG132 (Fig. 5B, lanes 3 and 6). These results suggest that MM-1 plays a role as a factor that connects c-Myc to the ubiquitin E3 ligase and the proteasome.

Stabilization of c-Myc by knockdown of cullin2 and Skp2. To investigate roles of a ubiquitin E3 ligase in c-Myc degradation under a physiological condition, siRNAs targeting genes encoding proteins described above were transfected into HeLa cells. Forty-eight hours after transfection, the endogenous expression levels of proteins extracted from transfected cells were analyzed by Western blotting, and the levels of c-Myc and various proteins were normalized to that of actin (Fig. 6). The siRNA against luciferase was also transfected into cells as a negative control. When siRNA against Cullin2 was transfected, a reverse correlation of the amounts of endogenous c-Myc and Cullin2 was observed in cells transfected with siRNA in a dose-dependent manner (Fig. 6A). Although siRNA against Skp2 also stabilized the level of c-Myc as described previously (18,19), its effect on stabilization of c-Myc was weak (Fig. 6B). The siRNAs against ElonginB and ElonginC, on the other hand, did not show stabilization of c-Myc, and only the results of transfection of siRNA against ElonginB are shown (Fig. 6C). The siRNAs against Cullin1 and Skp1 did not affect stabilization of c-Myc (data not shown). These results suggest that at least Cullin2 and Skp2 participate in a c-Myc-degradation reaction as components of the ubiquitin-proteasome system.

Regarding the ubiquitin-proteasome system that degrades c-Myc, two FCS complexes containing either Skp2 or Fbw7 have been reported. SCF^{Skp2} and SCF^{Fbw7} degrade c-Myc in c-Myc phosphorylation-independent and -dependent manners, respectively (8-10). In this study, we found that knockdown of MM-1 expression stabilized c-Myc. Furthermore, we found that a novel combination of protein complex, arising from SCF^{Skp2} and VBC complexes, would work as the ubiquitin E3 ligase for c-Myc. A hypothetical model of these interactions of proteins is shown in Fig. 6C; MM-1 binds to the proteasome and c-Myc, then recruits Skp2-containing Cullin2-ElonginB-ElonginC complex to facilitate or modulate ubiquitination of c-Myc, and modulated c-Myc is degraded by the proteasome. The validity of this hypothetical model was proved by results

showing that knockdown of Cullin2 and Skp2 by siRNAs stabilized endogenous c-Myc under physiological conditions. The siRNAs targeting Cullin1, Skp1, ElonginB and ElonginC were, on the other hand, found not to affect the stability of c-Myc. These findings suggest that expression levels of these proteins are high, thereby being sufficient for proteins to degrade c-Myc after knockdown by siRNAs. Since c-Myc is a key factor that regulates growth, differentiation and apoptosis of cells, stability and/or degradation of c-Myc may be regulated by several pathways that would be determined by cell conditions, including the cell cycle, growth and death signals.

In addition to the transcriptional repression function toward c-Myc, it has been shown that MM-1 is a subunit of prefoldin (PFD)/Gim complex, PFD5/Gim5 (22,23). PFD has been reported to play a role in delivering the newly synthesized unfolded proteins to TRiC/CCT, a chaperone that folds newly synthesized proteins (24). It has also been reported that TRiC/CCT, in cooperation with HSP70 or HSC70, mediates a complex formation of ElonginC, ElonginB and VHL of the VBC complex (24) and that HSC70 in this complex then facilitates the unfolding of its ubiquitin substrates, resulting in placement of the E3 ligase-binding region of ubiquitin substrates at the protein surface (25). Since MM-1 is the subunit of PFD, it is possible that MM-1 both recruits the protein-degradation machinery to c-Myc and mediates the unfolding of c-Myc. Collectively, the results suggest that MM-1 is an important player in the regulation of c-Myc both in transcription and degradation steps.

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