Targeted Destruction of c-Myc by an Engineered Ubiquitin Ligase Suppresses Cell Transformation and Tumor Formation

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Abstract

Given that expression of c-Myc is up-regulated in many human malignancies, targeted inactivation of this oncoprotein is a potentially effective strategy for cancer treatment. The ubiquitin-proteasome pathway of protein degradation is highly specific and can be engineered to achieve the elimination of undesirable proteins such as oncogene products. We have now generated a fusion protein (designated Max-U) that is composed both of Max, which forms a heterodimer with c-Myc, and of CHIP, which is a U-box-type ubiquitin ligase (E3). Max-U physically interacted with c-Myc in transfected cells and promoted the ubiquitylation of c-Myc in vitro. It also reduced the stability of c-Myc in vivo, resulting in suppression of transcriptional activity dependent on c-Myc. Expression of Max-U reduced both the abundance of endogenous c-Myc and the proliferation rate of a Burkitt lymphoma cell line. Furthermore, expression of Max-U but not that of a catalytically inactive mutant thereof markedly inhibited both the anchorage-independent growth in vitro of NIH 3T3 cells that overexpress c-Myc as well as tumor formation by these cells in nude mice. These findings indicate that the targeted destruction of c-Myc by an artificial E3 may represent an effective therapeutic strategy for certain human malignancies. (Cancer Res 2005; 65(17): 7874-9)

Introduction

The proto-oncogene product c-Myc, a basic helix-loop-helix/leucine zipper (bHLH/LZ)–type transcription factor, is a master regulator of cell proliferation. c-Myc forms a heterodimeric complex with the smaller bHLH/LZ protein Max, and the c-Myc-Max complex binds to a specific DNA sequence (CACGTG) known as the E-box motif. This motif is located in the promoter region, introns, or translated or untranslated exons of genes that are activated by the c-Myc-Max complex. Such transcriptional activation contributes to the growth-promoting properties of c-Myc. Whereas Max is expressed constitutively, the expression of c-Myc is transient and is directly related to the proliferative potential of cells. c-Myc is thus virtually undetectable in quiescent cells, but its expression is rapidly induced as cells enter the G1 phase of the cell cycle in response to stimulation with serum or specific mitogens. The abundance of c-Myc subsequently decreases gradually to a low steady-state level at which it remains for as long as the cells continue to proliferate.

In many human malignant tumors, the expression level of c-Myc is increased as a result of amplification or mutation of the c-MYC gene.

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composed of Max and CHIP specifically interacted with c-Myc and induced its degradation, resulting in suppression of the oncogenic activity of c-Myc in vivo. This approach thus represents a potential new strategy for gene therapy in individuals with malignancies that overexpress c-Myc.

**Materials and Methods**

**Cell culture.** HEK293T and HeLa cells were cultured under an atmosphere of 5% CO₂ at 37° C in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen). NIH 3T3 cells were cultured under the same conditions in DMEM supplemented with 10% bovine serum.

**Figure 1.** Specific interaction of Max-U with c-Myc in vivo and Max-U-dependent ubiquitylation of c-Myc in vitro. A, schematic representation of the structure of Max-U. Max-U comprises full-length Max fused at its COOH terminus with the charged region and U-box domain of CHIP. B, interaction between Max-U and c-Myc in vivo. HEK293T cells were transfected with expression vectors for FLAG-tagged c-Myc and HA-tagged Max-U. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA, and the resulting precipitates were subjected to immunoblot (IB) analysis with anti-FLAG. HA-tagged Cdk2 and HA-Max were used for negative and positive controls, respectively. A portion of cell lysates corresponding to 5% of the input for immunoprecipitation was also subjected directly to immunoblot analysis with anti-FLAG and anti-HA. C, interaction between Max-U and Mad1 in vivo. HEK293T cells were transfected with expression vectors for FLAG-tagged c-Myc (positive control) or Mad1 and HA-tagged Max-U. Immunoprecipitation-immunoblotting analysis was performed as in (B). D, promotion of c-Myc polyubiquitylation in vitro by Max-U. An in vitro ubiquitylation assay was done with the indicated combinations of ATP, GST-ubiquitin (Ub), E1, E2 (UbC), His6-HA-tagged Max-U, and His6-c-Myc. Reaction mixtures were subjected to immunoblot analysis with anti-c-Myc (top) or anti-HA (bottom). The positions of His6-c-Myc or His6-HA-Max-U modified by various numbers of GST-ubiquitin moieties are indicated.

**Figure 2.** Destabilization of c-Myc by Max-U in vivo. A, HEK293T cells were transfected with an expression vector for FLAG-c-Myc with or without (Mock) a vector for HA-Max-U. Forty-eight hours after transfection, the cells were cultured in the presence of cycloheximide (50 μg/mL) for the indicated times. Cell lysates were then subjected to immunoblot analysis with anti-FLAG, anti-HA, and anti-Hsp90. B, intensity of the FLAG-c-Myc bands in (A) was normalized by that of the corresponding Hsp90 bands and was then expressed as a percentage of the normalized value for time 0. C, NIH 3T3 cell lines were infected retroviral vectors encoding FLAG-tagged c-Myc either alone or with that encoding HA-tagged Max-U. Immunoblot analysis were done as in (A). D, intensity of the FLAG-c-Myc bands in (C) was normalized and expressed as in (B).
anti-FLAG (1 Products, Berkeley, CA or N262, Santa Cruz Biotechnology, Santa Cruz, CA), the mixture, which was then rotated for 1 hour at 4°C following primary antibodies: anti-c-Myc (16B12, Covance Research Products), and anti-Hsp90 (1640 (Invitrogen). Namalwa cells were cultured under the same conditions in RPMI 1640 (Invitrogen) supplemented with 10% bovine serum.

Cloning of cDNAs and plasmid construction. A cDNA encoding hemagglutinin epitope (HA)-tagged Max-U (full-length human Max fused at its COOH terminus with amino acids 129-304 of mouse CHIP and at its NH2 terminus with the HA tag) was generated by the PCR, sequenced, and subcloned into pcDNA3 (Invitrogen), pFASTBAC HTa (Invitrogen), or pMX-puro. To generate a mutant (P270A) of Max-U, we did site-directed mutagenesis with a Quick Change kit (Stratagene, La Jolla, CA) and corresponding oligonucleotide primers. cDNAs for human c-Myc and Mad1 were amplified by PCR, sequenced, and subcloned into pcDNA3 for expression of these proteins with a FLAG tag at its NH2 terminus.

Baculovirus expression system. The plasmid pFASTBAC HTa containing the relevant cDNA (c-Myc or HA-Max-U) was subjected to recombination with the baculoviral genome in HB10BAC, and the resulting recombinant viral genome was introduced into Sf9 cells by transfection to generate recombinant baculoviruses. The infected Sf9 cells were lysed, and the recombinant hexahistidine (His6)-tagged proteins were purified as described previously.

Transfection, immunoprecipitation, and immunoblot analysis. Cells (HEK293T) were transfected by the calcium phosphate method and lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, leupeptin (10 μg/mL), 1 mmol/L phenylmethylsulfonyl fluoride, 400 μmol/L Na3VO4, 400 μmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L NaF, and 10 mmol/L sodium pyrophosphate. The lysate was centrifuged at 16,000 × g for 10 minutes at 4°C, and the resulting supernatant was incubated with antibodies for 2 hours at 4°C. Protein G-Sepharose (Amersham Biosciences, Piscataway, NJ) that had equilibrated with the same solution was added to the mixture, which was then rotated for 1 hour at 4°C. The resin was separated by centrifugation, washed four times with lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was done with the following primary antibodies: anti-c-Myc (1 μg/mL; 9E10, Covance, Research Products, Berkeley, CA or N262, Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG (1 μg/mL; M5, Sigma, St. Louis, MO), anti-HA (1 μg/mL; HA.11/16B12, Covance Research Products), and anti-Hsp90 (1 μg/mL; 68, Transduction Laboratories, Lexington, KY). Immune complexes were detected with horseradish peroxidase–conjugated antibodies to mouse or rabbit immunoglobulin G (1:10,000 dilution, Promega, Madison, WI) and an enhanced chemiluminescence system (Amersham Pharmacia). In vitro ubiquitylation assay. An in vitro ubiquitylation assay was done as described. In brief, reaction mixtures (20 μL) containing 1 μg of recombinant Max-U, 0.1 μg of recombinant rabbit E1 (Boston Biomedica, West Bridgewater, MA), 1 μL of recombinant human UbC1 (15), 0.5 unit of phosphocreatine kinase, 1 μg of a glutathione S-transferase (GST) fusion protein of bovine ubiquitin (MBL, Woburn, MA), 25 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 2 mmol/L ATP, 1 mmol/L MgCl2, 0.3 mmol/L DTT, and 1 mmol/L creatine phosphate were incubated for 2 hours at 30°C. The reaction was terminated by the addition of SDS sample buffer containing 4% 2-mercaptoethanol and heating at 95°C for 5 minutes. Samples were resolved by SDS-PAGE on a 6% gel and then subjected to immunoblot analysis with mouse monoclonal antibodies to c-Myc (N262) and to HA (16B12). Luciferase assay. HeLa cells were transfected with expression vectors encoding FLAG-c-Myc, HA-tagged Max, or HA-Max-U, together with pRL-Tk (Promega) as an internal control and p4xE-SVP-Luc (kindly provided by H. Ariga, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan), with the use of FuGENE6 (Roche, Branchburg, NJ). The cells were harvested 48 hours after transfection, lysed, and assayed for luciferase activity with a Dual-Luciferase Reporter Assay System (Promega).

Retroviral infection. Retroviral expression vectors for HA-Max-U or HA-Max, c-Myc, or Myc epitope-tagged Bcl2 amplified by PCR, were constructed with pMX-puro, pMX-neo, and pMX-hyg (all kindly provided by T. Kitamura, The Institute of Medical Science, University of Tokyo, Tokyo, Japan), respectively. For retrovirus-mediated gene expression, NIH 3T3 cells were infected with retroviruses produced by Plat-E packaging cells and were then cultured in the presence of puromycin (1 μg/mL), G418 (2 mg/mL), or hygromycin B (0.2 mg/mL); Sigma, respectively.

Figure 3. Suppression of c-Myc-dependent transcriptional activity by Max-U. HeLa cells were transfected with the p4xE-SVP-Luc reporter plasmid (and pRL-Tk) either with or without expression vectors for c-Myc, Max, or Max-U, as indicated. Cell lysates were assayed for luciferase activity, and firefly luciferase activity normalized by Renilla luciferase activity was expressed as a percentage of that in cells transfected with p4xE-SVP-Luc (and pRL-Tk) alone. Values from each of two independent experiments.

Figure 4. Down-regulation of endogenous c-Myc expression by Max-U and its effect on cell growth in a Burkitt lymphoma cell line. A, suppression of endogenous c-Myc expression by Max-U. Namalwa cells infected either with a retroviral vector encoding HA-tagged Max-U (Nam.Max-U) or with the corresponding empty vector (Nam.puro) were lysed and subjected to immunoblot analysis with antibodies to c-Myc, to Hsp90, and to HA. The relative ratio of the intensity of the c-Myc band to that of the Hsp90 band is indicated. B, inhibition of cell proliferation by Max-U. Nam.Max-U or Nam.puro cells (2 × 104) were seeded in 6-cm dishes and harvested for determination of cell number at the indicated times thereafter. Points, means of values from three independent experiments; bars, ±SD.
Results

Interaction of an engineered ubiquitin ligase with c-Myc. To target the oncoprotein c-Myc for destruction, we designed a fusion protein that would theoretically interact with and mediate the ubiquitylation of c-Myc. We thus fused Max, a binding partner of c-Myc, to the COOH-terminal portion (including the charged region and U-box domain) of the U box-type ubiquitin ligase CHIP (Fig. 1A). This engineered E3 was designated Max-U. To examine the potential physical interaction between c-Myc and Max-U, we transfected HEK293T cells with expression vectors for FLAG-tagged c-Myc and HA-tagged Max-U. Immunoprecipitates prepared from lysates of the transfected cells with antibodies to (anti-) HA were subjected to immunoblot analysis with anti-FLAG (Fig. 1B). As controls, we confirmed that HA-Max, but not HA-Cdk2, interacted with FLAG-c-Myc in this system. Like Max, the Max-U fusion protein bound to c-Myc in the transfected cells. Unexpectedly, Max-U did not interact with Mad1, another Max-heterodimerization partner, probably due to structural constraints (Fig. 1C). These results showed that Max-U interacted physically with c-Myc in vivo, and that the charged region and U-box domain of CHIP did not interfere with the ability of Max to bind to c-Myc.

Promotion of the ubiquitylation and degradation of c-Myc by Max-U. We next examined the E3 activity of Max-U. An in vitro ubiquitylation assay was done with ATP, GST-ubiquitin, E1, E2 (Ubc4), His6-HA-tagged Max-U, and His6-tagged c-Myc as substrate (Fig. 1D). Max-U markedly enhanced the polyubiquitylation of c-Myc, and removal of any of the other components of the reaction mixture prevented c-Myc polyubiquitylation. These data thus suggested that Max-U functions as an E3 for c-Myc in collaboration with E1 and E2 and in an ATP-dependent manner. In addition, Max-U exhibited self-ubiquitylation activity.

To examine whether Max-U affects the stability of c-Myc in vivo, we transiently transfected HEK293T cells with an expression vector for FLAG-tagged c-Myc, either alone or together with a vector for HA-tagged Max-U, and then assessed the stability of FLAG-c-Myc by immunoblot analysis after various times of incubation of the cells with cycloheximide. Expression of Max-U promoted the degradation of c-Myc (Fig. 2A-B). We also examined the effect of HA-Max-U on the stability of FLAG-c-Myc that was stably expressed in NIH 3T3 cells. c-Myc was highly unstable in cells that expressed Max-U compared with that in mock transfectants (Fig. 2C-D). Our results thus suggested that Max-U mediates the ubiquitylation of c-Myc and thereby promotes its degradation in vivo.

We next examined the effect of Max-U expression on transcriptional activity dependent on c-Myc. HeLa cells were transfected with the pxeE-SVP-Luc reporter plasmid that contains the firefly luciferase gene under the control of the E box together with various combinations of expression vectors for c-Myc, Max, and Max-U. Measurement of luciferase activity of lysates of the transfected cells revealed that the expression of c-Myc or Max alone resulted in an ~50% increase in transcription of the reporter gene (Fig. 3). Expression of both c-Myc and Max increased transcriptional activity by >100%. In contrast, Max-U suppressed the increase in luciferase activity induced by c-Myc. Together, these observations suggest that the expression of Max-U reduced c-Myc-dependent transcriptional activity by inducing c-Myc degradation.

Figure 5. Inhibition by Max-U of the anchorage-independent growth of NIH 3T3 cells transformed with c-Myc. A, expression of ectopic proteins in NIH 3T3 cells infected with retroviral vectors. Lysates of NIH 3T3 cell lines expressing the indicated combinations of c-Myc, Myc epitope-tagged Bcl2, HA-tagged Max, and HA-Max-U (wild type or P270A mutant) were subjected to immunoblot analysis with antibodies to HA, to c-Myc, and to Hsp90. N.S., nonspecific band. B, colony formation by NIH 3T3 cell lines in soft agar. Cells (1 x 10^5) expressing the indicated ectopic proteins were seeded in 3.5-mm dishes containing 0.4% soft agar and cultured for 3 weeks. Bar, 0.1 mm. C, the total numbers of colonies with a diameter of >0.1 mm formed by NIH 3T3 cell lines in experiments similar to that shown in (B) were counted. Columns, means of values from three independent experiments; bars, ± SD. P values for the indicated comparisons were determined by Student’s t test.

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Suppression of endogenous c-Myc expression in a Burkitt lymphoma line by Max-U. Certain Burkitt lymphoma cell lines, including Namalwa cells, express c-Myc at a high level, and this deregulated expression of c-Myc is implicated in oncogenesis. We infected Namalwa cells with a recombinant retroviral vector encoding HA-tagged Max-U to examine the effect of the latter protein on the abundance of endogenous c-Myc. The amount of c-Myc in Namalwa cells stably expressing Max-U was about half (51.4%) of that in a Namalwa line infected with the corresponding empty virus (Fig. 4A). Furthermore, the growth rate of the cells expressing Max-U was markedly reduced compared with that of the control cells (Fig. 4B). These results indicate that Max-U induces the degradation of endogenous c-Myc in vivo, and that this effect results in a reduction in the rate of cell growth.

Suppression of the transforming activity of c-Myc by Max-U. To investigate whether Max-U affects the transformation activity of overexpressed c-Myc, we established NIH 3T3 cell lines stably expressing c-Myc by retroviral infection. Given that overexpression of c-Myc alone induced apoptosis in a substantial proportion of NIH 3T3 cells, we also infected the cells with a recombinant retrovirus encoding Myc epitope-tagged Bcl-2 to suppress c-Myc-induced apoptosis. The resulting cell line (NIH.Myc/Bcl-2) was further infected with recombinant retroviral vectors encoding HA-tagged Max, HA-Max-U, or HA-Max-U(P270A), in which the conserved residue proline-270 in the U-box domain is substituted with alanine. Expression of the recombinant HA-Max, HA-Max-U, or HA-Max-U(P270A), and Myc epitope-tagged Bcl-2 was confirmed by immunoblot analysis (Fig. 5A).

The various NIH 3T3 cell lines were then assayed for their ability to form colonies in soft agar as a means of assessing their ability to undergo anchorage-independent growth. Whereas cells infected with vector alone formed few colonies, those expressing c-Myc and Bcl-2 in the absence or presence of Max showed similar increases in the number of colonies formed (Fig. 5B-C). In contrast, Max-U greatly reduced the ability of cells expressing c-Myc and Bcl-2 to undergo anchorage-independent growth. The mutant Max-U (P270A) did not suppress the transforming activity of c-Myc, suggesting that the ubiquitin ligase activity of Max-U is required for this effect. This latter result also excludes the possibility that the inhibitory effect of Max-U was due to a dominant-negative action.

Suppression of c-Myc-induced tumor formation in nude mice by Max-U. Finally, we examined the effect of Max-U expression on tumor formation in nude mice with the use of the NIH 3T3 cell lines described above. Cells infected with vector alone failed to form tumors, whereas those expressing c-Myc and Bcl-2 formed large tumors both at the site of injection and at sites of metastasis (Fig. 6). Although coexpression of Max did not affect tumor formation by cells expressing c-Myc and Bcl-2, that of Max-U resulted in a significant reduction in tumor size. Consistent with the results of the colony formation assay, coexpression of the Max-U (P270A) mutant did not inhibit tumor formation by cells expressing c-Myc and Bcl-2. These findings suggest that Max-U suppresses tumor formation induced by overexpression of c-Myc and Bcl-2, likely as a result of enhanced degradation of c-Myc.

Discussion
Cancer is thought to be caused in large part by genomic events that result in the activation of oncogenes or the inactivation of tumor suppressor genes (26). Tumor cells have also been shown to be capable of reverting to a state of dormancy (27–29). Inactivation of activated oncogenes can result in the permanent loss of the neoplastic phenotype (30, 31). One of the most commonly activated oncogenes associated with the pathogenesis of human tumors is c-MYC. Animal models have confirmed that overexpression of c-Myc is able to induce many types of carcinoma, whereas inhibition of c-Myc expression in carcinoma cells results in a loss of neoplastic properties (32). A recent study with a conditional transgenic mouse model revealed that inactivation of c-Myc was sufficient to induce sustained regression of invasive liver cancer (33). Loss of c-Myc expression resulted in the differentiation of tumor cells and their eventual death. The elimination of oncoproteins such as c-Myc from tumors in humans is thus a goal of clinical oncology.

Given the high specificity and rapidity of protein degradation by the ubiquitin-proteasome system, engineering of this system has been considered a potentially effective approach to the elimination of undesirable proteins such as oncogene products. Attachment of a degradation signal based on the N-end rule to target proteins has been shown to increase their turnover rate (34), but this method cannot be applied to endogenous proteins. In an approach similar to that adopted in the present study, the F-box protein component (β-TrCP) of an SCF complex-type E3 has been fused to other proteins to target their binding partners for destruction in yeast or mammalian cells (18–23). However, the activity of the SCF complex...
is regulated by various processes such as Nedd-8 conjugation by Ubc12 and de neddylation by the COP9 signalosome. Moreover, formation of the multisubunit SCF complex requires the presence of components such as Cul1, Skp1, Rbx1, and the F-box protein in an appropriate molecular ratio, and complex formation is negatively regulated by binding of CAND1 (TIP120A) to Cul1, which serves as a scaffold for the SCF complex. We therefore attempted to develop a more simple system based on CHIP, a monomeric, constitutively active ubiquitin ligase of the U-box type. We fused Max to the charged region and U-box domain of CHIP to target the c-Myc oncoprotein for destruction. This approach can be applied in theory to the elimination of any intracellular protein for which a binding partner has been identified.

Further improvements in artificial proteolysis technology may be necessary to increase both the efficiency and specificity of proteolysis. This goal might be achievable by increasing the expression level of the engineered E3 to increase its affinity for the target protein, or by evaluation of a variety of E3s for fusion with the substrate recognition motif of the binding partner. Control of expression of the artificial E3 fusion protein will require the use of a tissue-specific or inducible promoter. Furthermore, targeting of the artificial enzyme to a specific subcellular localization, such as by attachment of a nuclear localization signal, may be an important consideration for some substrates.

Transgenic and gene knockout technology in the mouse has proven effective for determining the functions of specific genes, but application of these approaches on a genome-wide scale will be expensive in terms of both time and money. Protein knockout technology based on the elimination of specific cellular proteins in cultured cells or animals may represent a more economical and simpler approach. Characterization of the “interactome” by genome-wide screening in the yeast two-hybrid system will provide important information for the selection of proteins as fusion partners for CHIP to generate artificial ubiquitin ligases targeted to specific proteins.

Our present results show that elimination of c-Myc in cells by Max-U suppressed both colony formation in soft agar and tumor formation in nude mice. Clinical application of this approach will require the development of a delivery system such as an adoviral or lentiviral expression vector. Targeted destruction of specific proteins by an artificial E3 may prove of therapeutic benefit not only for the removal of oncoproteins in cancer but also for the elimination of other abnormal proteins including those that accumulate in protein storage disorders such as certain neurodegenerative or metabolic diseases.

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