Oncostatin M Induces Growth Arrest by Inhibition of Skp2, Cks1, and Cyclin A Expression and Induced p21 Expression

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Abstract
Oncostatin M has been characterized as a potent growth inhibitor for various tumor cells. Oncostatin M–treated glioblastoma cells cease proliferation and instigate astrocytial differentiation. The oncostatin M–induced cell cycle arrest in G1 phase is characterized by increased level of the cyclin–dependent kinase (CDK) inhibitory proteins p21 Cip1/Waf1/Sdi1 and p27Kip1. Induction of p21 protein corresponds to increased mRNA level, whereas p27 accumulates due to increased stability of the protein. Interestingly, stabilization of p27Kip1 occurs even in S phase, showing that p27 stabilization is a direct consequence of oncostatin M signaling and not a result of the cell cycle arrest. Degradation of p27 in late G1 and S phase is initiated by the ubiquitin ligase complex SCF-Skp2/Cks1. Oncostatin M inhibits expression of two components of this E3 ligase complex (Skp2 and Cks1). Although combined overexpression of Skp2 and Cks1 rescues p27 degradation in S phase, it can not override p27 accumulation in G1 phase and cell cycle arrest by oncostatin M. In addition to increasing Cdk inhibitor level, oncostatin M also impairs cyclin A expression. Cyclin A mRNA and protein level decline shortly after oncostatin M addition. The accumulation of two CDK inhibitor proteins and the repression of cyclin A expression may explain the broad and potent antiproliferative effect of the cytokine. (Cancer Res 2006; 66(13): 6530–9)

Introduction
Oncostatin M is a member of the interleukin-6 (IL-6) family cytokines that modulate differentiation, proliferation, and apoptosis of a wide variety of cell types (1). The cytokine exerts its biological activities through heterodimeric receptors composed of gp130 and either the oncostatin M–specific OSMRβ or the LIFR (1). Ligand-induced dimerization of the receptor components initiates the activation of members of the Janus family of protein kinases (Janus-activated kinase, JAK). Receptor-associated JAK kinases phosphorylate and thereby activate members of the signal transducer and activator of transcription (STAT) family of transcription factors, which translocate into the nucleus and regulate expression of cytokine-specific target genes (1). The response to oncostatin M depends on the cellular context, and it has been shown that oncostatin M inhibits proliferation of many tumor derived cells (1). In other cell types (e.g., fibroblasts), oncostatin M can stimulate proliferation (1). Previously, we observed that oncostatin M induces a potent growth arrest in human glioblastoma cells (2) and efficiently prevents tumor formation in a mouse glioblastoma model (3).

Progression through the cell cycle is controlled by oscillating activities of cyclin-dependent kinases (CDK; refs. 4, 5). The catalytic activity of CDK/cyclin complexes can be controlled by various mechanisms, including cyclin synthesis and degradation, activating or inhibitory phosphorylation, localization, or by binding of CDK inhibitory proteins (6, 7). During G1 progression, two families of CDK inhibitory proteins play a key role in regulating CDK activity of mammalian cells: INK4 proteins specifically bind to CDK4 and CDK6, whereas members of the Cip/Kip family (p21 Cip1/Waf1/Sdi1, p27Kip1, and p57Kip2) bind to and regulate a broader spectrum of CDK complexes (8). Although Ink4 proteins always inhibit CDK kinase activity, Cip/Kip proteins may also act as assembly factors for cyclin D/CDK4/6 complexes (6).

Whereas expression of p21 is regulated at the level of mRNA expression (9), p27 levels are frequently regulated posttranscriptionally. p27 plays a central role in regulation of the unperturbed cell cycle by regulating Cdk activity during the G0-G1-S progression. Its expression is regulated by diverse mitogenic or antiangiogenic signals (8, 10). Changes in p27 frequently involve regulation of its synthesis and proteolysis (11, 12). At least two distinct pathways regulate p27 stability at different stages of the cell cycle (13), which both involve the ubiquitin/proteasome pathway. p27 becomes increasingly unstable as cells approach S phase. Proteolysis of p27 is initiated at this time by a CDK-dependent phosphorylation of p27 at Thr187. Phosphorylated p27 is subsequently ubiquitinated by the SCF-Skp2 complex. This E3 ubiquitin ligase is composed of Skp1, Cul1, p53Skp2, and ROC1/Rbx1 and requires association with Cks1 for its ubiquitin ligase activity towards p27 (14, 15). Cks1 is a member of the highly conserved Suc1/Cks protein family originally identified as subunits of CDK complexes and associates with p27 and Skp2 (16). To efficiently degrade p27, the Cul1 subunit needs to be modified with the ubiquitin-related protein Nedd8 (17). This modification dissociates the inhibitory subunit p120-CAND1 from Cul1 (18), and deneddylation of Cul1 by the COP9 signalosome inhibits p27 degradation (19, 20). Although the COP9 signalosome complex can stabilize p27, overexpression of its subunit CSN5/Jab1 can stimulate p27 degradation through p27 nuclear export (21). A second pathway of p27 elimination during G0-G1 progression is independent of T187 phosphorylation but also involves the ubiquitin/proteasome pathway (22, 23). Monomeric p27 is ubiquitinylated by the KPC1/KPC2 ligase independently from T187 phosphorylation and subsequently degraded by the proteasome (24, 25).

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p53 is a member of a large family of F-box proteins and rate limiting for p27 degradation at the G1-S transition. A complex of Skp2 and Cks1 interacts with Thr187-phosphorylated p27, leading to p27 ubiquitination by SCF-Skp2 complex (26). Although SCF-Skp2 has been reported to ubiquitinate a number of additional substrates, including hOrc1p, E2F-1, or p130, its role in p27 degradation is central for its function as an oncogene (26).

The antagonistic relationship between p27 and Skp2/Cks1 is reflected in knockout animals and human tumors. Although mice lacking p27 are larger than wild-type animals due to multiorgan hyperplasia (27, 28), mice nullizygous for Skp2 or Cks1 are smaller than their littermates and accumulate elevated levels of p27 (14, 29). Many phenotypes of the Skp2 knockout animals are reverted to normal by loss of the single substrate p27 (30, 31), suggesting that p27 is the central substrate of this ubiquitin ligase, and that Skp2 plays a nonredundant role in p27 degradation. This hypothesis is supported by the observation that elevated expression of Skp2 correlates with low level of p27 in various human tumors (26). Skp2 itself is regulated during the cell cycle and degraded by the ubiquitin-proteasome pathway (32). Ubiquitin-dependent degradation of Skp2 in G1 phase is mediated by the APCCdc20 complex (33, 34). Skp2 regulation does also involve transcriptional mechanisms. Androgen treatment of human prostate carcinoma cells or detachment can lead to reduced Skp2 mRNA expression and p27 stabilization (35, 36).

The p27-related Cdk inhibitor p21 is also an unstable protein and degraded by the 26S proteasome. However, in contrast to p27, ubiquitination was reported to be not essential for p21 proteasomal degradation (37, 38). Ubiquitination of p21 can also involve the SCF-Skp2 pathway during the S phase (39) and switches from the basal turnover to SCF-Skp2 dependency upon DNA damage (40).

The aim of this study was to elucidate molecular mechanisms that allow for the broad antimitogenic function of oncostatin M. We found that oncostatin M induces the Cdk inhibitors p21 and p27. Although p21 induction involves increased mRNA expression, p27 accumulates due to stabilization of the protein. Decreased degradation of p27 is based on reduced Skp2 and Cks1 expression. Whereas Skp2 overexpression is usually sufficient to eliminate p27 and promote S-phase entry, ectopic expression of Skp2 in oncostatin M–treated cells could surprisingly neither destabilize p27 nor rescue cell proliferation. Combined overexpression of Skp2 and Cks1 is capable of reducing p27 induction but fails to abolish the cell cycle arrest. The sustained inability of cells to enter S phase despite overexpression of Skp2 and Cks1 can be explained by reduced expression of cyclin A. These redundant pathways converging in inhibiting Cdk kinase activity can explain the strong antiproliferative potency of oncostatin M and its broad spectrum of susceptible tumor cells.

Materials and Methods

Cell culture. Maintenance and treatment of the human glioblastoma cell line 86HG39 was done as recently described (3). For stable expression of Skp2, glioblastoma cells were transfected with a pCDNAS3-mycSkp2 (32). Single clones were analyzed for Skp2 expression after selection with 400 µg/ml neomycin. Stable cell lines expressing Skp2 and Cks1 were generated by transfecting Skp2 expressing clones with a Cks1 cDNA cloned into pIRE2a vector (Stratagene, Heidelberg, Germany) containing a hygromycin resistance gene. Stable expression of cyclin A was achieved by cloning the cDNA of human cyclin A in the pIRE2a/hygroR vector (Stratagene), and after transfection, cells were selected with hygromycin, and single clones were expanded.

Cell cycle analysis. 86HG39 glioblastoma cells or its derivatives cells expressing Skp2, Cks1, and Skp2 or cyclin A were cultured for 12 hours in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 5% FCS (Sigma, Deisenkirchen, Germany)/1% penicillin/streptomycin before oncostatin M was added to a concentration of 25 ng/ml. Cell cycle distribution was determined after bromodeoxyuridine (Brdu) incorporation by two-dimensional fluorescence-activated cell sorting (FACS) analysis. Cells (3 × 10⁶) were seeded in 6-cm cell culture dishes. Before harvesting, cells were incubated in the presence 30 µg/ml BrdUrd (Sigma) for 45 minutes. Cells were prepared and stained for BrdUrd incorporation using FITC-labeled anti-BrdUrd antibody according to manufacturer’s instructions (Becton Dickinson, Mountain View, CA) and for DNA content with propidium iodide, and cells were analyzed using FACSCalibur and Cell Quest Software (Becton Dickinson). The thymidine-induced S phase arrest was confirmed by staining of the fixed cells with propidium iodide.

Immunohistochemical analysis. For anti-BrdUrd immunocytochemistry, 2 × 10⁴ cells were seeded on glass coverslips in 48-well plates. Treatment was done as described above. Before harvest cells were incubated with 3 ng/ml BrdUrd for 60 minutes. Fixation and staining was done according to 5-Bromodeoxyuridine Labeling and Detection Kit I instructions (Roche, Mannheim, Germany). Before mounting on microscope slides, cell nuclei were stained with Hoechst 33258 (Molecular Probes, Eugene, OR).

Samples were analyzed on Leitz DMRX fluorescence microscope, and pictures were digitally acquired with a Spot CCD camera (Visitron, München, Germany). Quantification of BrdUrd-positive cells was done using ANALYSIS software (Soft Imaging System, Münster, Germany).

Northern blot analysis. Total RNA was isolated from 1 × 10⁶ glioblastoma cells using the Trizol reagent (Life Technologies). Northern blotting was done as described (41). Polyadenylated RNA was purified from total RNA using magnetic beads from Miltenyi Biotech (Bergisch-Gladbach, Germany) according to the manufacturer’s recommendation. The membranes were hybridized consecutively with 32P-labeled DNA probes consisting of a 700-bp BamHI fragment of the human Skp2 gene, a 870-bp PvuII-SphI fragment of the human cyclin A gene, and a 450-bp PCR fragment from the human GAPDH gene.

p27 degradation assay. 86HG39 cells were cultured for 24 hours in the presence or absence of oncostatin M. For determination of p27 protein stability, 2 × 10⁵ cells were seeded on 6-cm dishes. The following day, cycloheximide (Sigma) was added to the culture medium (final concentration, 100 µmol/L) to inhibit protein synthesis. At times indicated following the addition of cycloheximide, cells were lysed, and p27 protein levels were determined by Western blot analysis.

Western blot analysis. 86HG39 cells or derivatives thereof were seeded for most experiments in subconfluent cultures on 6-cm dishes and were treated as indicated. Cell lysates and Western blotting were done as described recently (42).

The antibodies used in this study are as follows: anti-p27 (clone 57), anti-cyclin A (C88020), anti-cyclin D1 (DCS-6), anti-pRb (R68320), and p21 (clone 70) were obtained from BD PharMingen (San Diego, CA); anti-Cdk2 (sc-163), anti-Cdk4 (sc-260), anti-Cdk6 (sc-177), anti-cyclin E (sc-481), and anti-Cks1 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Skp2 (2B12) and anti-cullin-1 (RB-042) were from Neomarkers (Fremont, CA); anti-phospho-T187-p27 (06-996) was obtained from Upstate (Lake Placid, NY); and anti-actin (clone Ac-15) and anti-β-tubulin (clone SAP. 4G5) were from Sigma (Deisenkirchen, Germany). Antibodies against cyclin A (T310) and cyclin E (HE 172) were described earlier (11, 12).

For the quantitative expression analysis of p27, Western blots were processed as described before. The quantification of the blots was achieved by directly recording chemiluminescence emission with a ChemiDoc XRS System (Bio-Rad, Munich, Germany), and the quantitative analysis was done using the Quantity One software (Bio-Rad).

In vitro kinase assay and immunoprecipitation. Preparation of lysates for immunoprecipitation was done as described (42). Immunoprecipitates were washed thrice with lysis buffer and once with kinase reaction buffer [40 mmol/L HEPES (pH 7), 20 mmol/L MgCl₂, 10 mmol/L EGTA, 1 mmol/L DTT] and resuspended in 35 µL reaction buffer containing 20 µmol/L ATP.
5 µCi [γ-32P]ATP (NEN, Boston, MA), 2 µg histone H1 (Calbiochem, La Jolla, CA), or 2 µg pRb-GST (Santa Cruz Biotechnology). The reactions were incubated for 30 minutes at 30 °C and terminated by addition of 8× SDS loading buffer and heating to 95°C for 5 minutes. The samples were separated on a 12% SDS-PAGE gel. The gel was transferred on Whatman 3MM paper (Schleicher & Schuell, Dassel, Germany), dried, and phosphorylated proteins were visualized by autoradiography.

Immunoprecipitation for the detection of phospho-T187-p27 was done as described above. Lysates were prepared from 5 × 10⁵ cells on a 10-cm dish either treated with oncostatin M or left untreated. The lysates were incubated with antibodies recognizing p27 and analyzed by Western blot according to Western blot recommendation. Forty-eight hours after transfection, the cells were split into six-well dishes (10⁵ per well), so that one well could be treated with 25 ng/mL oncostatin M, and the others were left untreated. The cells from the six-well dishes were lysed after 48 hours for Western blot analysis as described above. The cells on the glass discs were further treated for the detection of BrdUrd incorporation by immunohistochemical analysis as described above.

Results

Oncostatin M induces a cell cycle arrest in G0-G1 phase. Oncostatin M inhibits proliferation of glioblastoma cells. The cell cycle distribution of the 86HG39 cells was determined in a time course experiment after oncostatin M addition. Glioblastoma cells were treated with oncostatin M as indicated. Forty-five minutes before harvesting, cells were incubated with BrdUrd to label cells in S phase. The cell cycle phase distribution was determined by flow cytometry using the DNA content with propidium iodide and percentage of cells in S phase by a FITC-conjugated anti-BrdUrd antibody. The percentage of cells with a G0-G1 DNA content (2C) increased from 57% in the absence of the cytokine to 84% after 48 hours of oncostatin M treatment. At the

Figure 1. Oncostatin M (OSM) induces cell cycle arrest in G1 phase. A, 86HG39 cells arrest in G0-G1 phase after oncostatin M treatment. Glioblastoma cells were incubated with oncostatin M (25 ng/mL) for 0, 12, 24, and 48 hours as indicated. Before harvesting, cells were incubated with BrdUrd for 45 minutes. The distribution of cells in the different cell cycle phases was determined by flow cytometry using FITC-labeled anti-BrdUrd antibodies and propidium iodide. Top, two-dimensional FACS profiles. Bottom, percentage of cells in G0-G1, S, and G2-M phases were determined and plotted over time. B, CDK2/cyclin E and CDK6/cyclin D become inactivated in oncostatin M-treated cells. Kinase complexes were precipitated from 86HG39 cell lysates with anti-cyclin E and anti-CDK6 antibodies, and their associated kinase activities towards histone H1 (CDK2) or pRb-GST (CDK6) were determined. Incorporation of [γ-32P]ATP into the substrates was visualized by autoradiography after separation of the reaction mixtures by SDS-PAGE on 12% acrylamide gels. C, expression of G1-specific CDK and cyclin proteins is not reduced in oncostatin M–treated cells. Glioblastoma cells were treated with oncostatin M as indicated; 25 µg of total cell lysate were analyzed by Western blot using the respective antibodies as indicated. D, oncostatin M leads to hypophosphorylation of pRb; 25 µg of cell lysate was separated by SDS-PAGE after treating the cells with oncostatin M for the time indicated. Western blot analysis was done using antibodies recognizing both hyperphosphorylated and hypophosphorylated pRb.
same time, the percentage of cells in S phase declined from 30% to 3.7% (Fig. 1A). As cells ceased proliferation, we concluded that oncostatin M arrested the glioblastoma cell in G0-G1 phase. Sustained expression of cyclin E1 and cyclin D1 proteins suggests that cells accumulate in G1 phase rather then in G0 (Fig. 1C). Because cell numbers increased only slightly over time, and because no increase in apoptosis was observed (data not shown), we concluded that oncostatin M blocked the G1-S transition in human glioblastoma cells.

Oncostatin M induces p21 mRNA expression and enhances p21 and p27 protein level. Cell cycle progression depends on the sequential oscillation of CDK activities (44). To investigate whether the oncostatin M mediated G0-G1 arrest involves CDK inactivation, we compared G1 and G1-S phase-specific kinase activities from oncostatin M–treated and untreated cells. Oncostatin M led to a significant reduction of cyclin E–associated histone H1- and CDK6-associated pRb kinase activity, showing that oncostatin M leads to inactivation of these G1-S CDKs (Fig. 1B). One key substrate of G1 kinases in vivo is the retinoblastoma protein pRB. pRB becomes hyperphosphorylated as CDKs become activated. To confirm CDK inactivation in vivo, we investigated the phosphorylation status of pRB in oncostatin M–treated cells. Consistent with the lack of G1 CDK kinase activity, pRb became hypophosphorylated upon oncostatin M treatment (Fig. 1D). Interestingly, protein level of CDK subunits 2, 4, and 6 and levels of cyclins E and D1 protein were not reduced significantly by oncostatin M (Fig. 1C).

Loss of CDK activity might, therefore, result from increased CDK inhibitor level. Increased level of p27 has previously been observed in oncostatin M–treated human melanoma cells (45). Similarly, p27Kip1 increased steadily in 86HG39 cells to a plateau 24 hours after oncostatin M stimulation (Fig. 2A). This increase of p27 expression was posttranscriptional because p27 mRNA level remained largely unchanged (Fig. 2B). In addition to p27, p21 was also induced by oncostatin M with similar kinetics (Fig. 2A). In contrast to p27, this increase in p21 protein was paralleled by an increase of p21 mRNA (Fig. 2B). To investigate if the induction of p27 might be a consequence of the precedent p21 induction, we blocked the expression of p21 by small interfering RNA (siRNA) transfection. Knockdown of p21 was successfully achieved as shown in Fig. 2C, whereas a control siRNA oligonucleotide (SC) had no effect. The strong reduction of p21 expression had no significant effect on the induction of p27 by oncostatin M. This observation excludes that the p27 accumulation is a secondary consequence of elevated p21 expression.

Oncostatin M stabilizes the p27 protein. To show oncostatin M regulation of p27 stability, we compared degradation of p27 in cycloheximide-treated cells in the presence or absence of oncostatin M. In untreated cells, the amount of p27 protein declined steadily and became undetectable at 8 hours of cycloheximide treatment, whereas p27 level declined much slower in oncostatin M–treated cells (Fig. 3A). This confirmed that p27 accumulates due to significantly increased stability of the protein.

P27 accumulation by oncostatin M is independent from the cell cycle position. Stabilization of p27 might be a consequence of the oncostatin M–induced cell cycle arrest because p27 is relatively stable in G1 and becomes increasingly unstable as cells progress into the S phase (11). To determine if p27 accumulation was merely a result of oncostatin M–induced growth arrest or mechanically involved in mediating the arrest, glioblastoma cells were arrested in S phase using a thymidine block. If p27 accumulation would be a consequence of the cell cycle arrest, no accumulation would be expected in S-phase cells, where p27 is very unstable (Fig. 3B; in the absence of oncostatin M). However, treatment of S phase–arrested cells with oncostatin M led to a keen increase of p27, without interfering with the S-phase arrest (Fig. 3B and C). These data showed that p27 accumulated independently from the cell cycle position and indicated that the increase in p27 protein is a primary consequence of oncostatin M signaling. Interestingly, the expression of p21 was also induced by oncostatin M in S phase–arrested glioblastoma cells (Fig. 7C).

Oncostatin M reduces Skp2 mRNA and protein level. Degradation of p27 in S phase requires its preceding phosphorylation on T187. To test whether oncostatin M prohibits T187 phosphorylation, we compared expression level of p27 with the abundance of the T187 phosphorylated isoform of the inhibitor. In the presence of oncostatin M, T187 phosphorylation was strongly increased (Fig. 4A). Lack of T187 phosphorylation is, therefore, not responsible for stabilization of p27. Because T187-phosphorylated p27 is a substrate of the SCF-Skp2 ligase, we next analyzed the expression of subunits of the SCF-Skp2 complex. The Cul1 protein was expressed at increased level in oncostatin M–arrested cells. A mobility shift of a Cul1 isoform that could be detected by SDS-PAGE was not inhibited by oncostatin M treatment (Fig. 4B).
This suggested that oncostatin M does not inhibit expression or modification of Cul1. In contrast, oncostatin M induced a dramatic decline of Skp2 protein (Fig. 4B). Declining Skp2 level corresponded well with the accumulation of p27 (Fig. 4B), suggesting that the decline in Skp2 protein is responsible for the accumulation of p27.

To investigate the mechanism of Skp2 repression by oncostatin M, we first determined Skp2 mRNA level. Although well detectable in proliferating cells, Skp2 mRNA expression was lost in cells treated with oncostatin M (Fig. 4D). Already 1 hour after oncostatin M addition, a decrease in Skp2 mRNA was detected, and the level remained low in oncostatin M–treated cells (Fig. 4D). The immediate decline of Skp2 mRNA after oncostatin M addition suggested that this was a primary consequence of the cytokine treatment and not a result from a cell cycle arrest. Reduced Skp2 protein expression might in addition involve regulation of the protein stability. As we observed that level of overexpressed myc-tagged Skp2 protein remained unchanged by oncostatin M addition (see below; Fig. 5A), we concluded that reduction in Skp2 protein is result of impaired mRNA expression. One mechanism of p27 stabilization is, therefore, consequence of lack of Skp2 expression.

Ectopic expression of Skp2 fails to destabilize p27 in oncostatin M–arrested glioblastoma cells. Skp2 is rate limiting for p27 degradation and proliferation (46). To investigate whether lack of Skp2 expression accounts for the oncostatin M–induced cell cycle arrest and the stabilization of p27, myc-tagged Skp2 protein was constitutively expressed in 86HG39 cells. Two different stable clones were selected for further analysis: one clone showed ectopic expression comparable with the endogenous Skp2 protein in untreated cells, whereas the other clone more severely overexpressed Skp2 (Fig. 5A). Addition of oncostatin M to both cell lines led to a decline in endogenous Skp2 protein (lower band, Fig. 5A) but did not reduce level of the slightly larger myc-tagged Skp2 protein.
expressions of basic SCF components like Skp1 or Cul1 was not inhibited by oncostatin M treatment, whereas expression of the Cks1 protein was strongly reduced by oncostatin M (Fig. 4C and Fig. 5C). The Cks1 protein is a cofactor of the SCF-Skp2 ubiquitin ligase complex and is required for degradation of p27. As overexpression of Skp2 did not rescue Cks1 expression (Fig. 5C), we speculated that loss of Cks1 protein was responsible for the accumulation of p27 even in the presence of overexpressed Skp2. As for Skp2, reduction of Cks1 protein corresponded to reduction of Cks1 mRNA expression (Fig. 4D). Reduced Skp2 and Cks1 mRNA level seems to be a characteristic for oncostatin M–resistant cell lines, as the oncostatin M–resistant glioblastoma cell line A172 (47) did not down-regulate either mRNA, whereas oncostatin M–resistant melanoma cells showed reduced Skp2 and Cks1 mRNA level upon oncostatin M treatment (data not shown). These observations suggested that inhibition of Skp2 and Cks1 expression is one redundant mechanism that could be responsible for the oncostatin M–induced cell cycle arrest.

We speculated that combined expression of Skp2 and Cks1 might overcome the oncostatin M–induced growth inhibition. To investigate this hypothesis, we generated stable cell lines that simultaneously overexpressed Skp2 (myc tagged) and Cks1 (HA tagged; Fig. 6A). Whereas p27 level increased within 24 hours after oncostatin M addition in wild type and Skp2-overexpressing cells, combined overexpression of Skp2 and Cks1 clearly delayed the accumulation of p27. There was no detectable change in p27 level in 86HG39-SKP2-Cks1 cells 24 hours after oncostatin M addition (Fig. 6A). Still, at 48 hours after oncostatin M treatment, these cells accumulated p27 to level that were only slightly reduced compared with those of wild-type cells or cells expressing exclusively Skp2 and Cks1. To further investigate if the S phase–dependent degradation of p27 was restored by combined expression of Cks1 and Skp2, we examined the oncostatin M–dependent accumulation of p27 in S phase–arrested cells. Oncostatin M treatment over 24 hours was now unable to accumulate p27 in S phase–arrested cells (Fig. 6C).

Consistent with the observation that overexpression of Skp2 and Cks1 only delayed the oncostatin M–induced p27 accumulation in asynchronous cells, oncostatin M–treated glioblastoma cells were still able to arrest in G1 phase, although somewhat less efficient than wild-type cells (Fig. 6B).

**Knockdown of p27 by siRNA could not affect the cell cycle arrest by oncostatin M in Skp2/Cks1–expressing glioblastoma cells.** Because the concurrent overexpression of Skp2 and Cks1 could not abrogate the oncostatin M–induced p27 expression, we investigated if a knockdown of p27 by siRNA might abolish the G1 arrest in Skp2/Cks1–overexpressing cells. 86HG39 glioblastoma cells and two cell lines derived from this parental line were transfected with siRNA molecules designed to knock down p27 gene expression. Significant reduction of p27 level was achieved by both different siRNA oligonucleotides in all three cell lines, whereas an unrelated control oligonucleotide (SC) did not affect gene expression. Significant reduction of p27 level was achieved by both different siRNA oligonucleotides in all three cell lines, whereas an unrelated control oligonucleotide (SC) did not affect gene expression. Significant reduction of p27 level was achieved by both different siRNA oligonucleotides in all three cell lines, whereas an unrelated control oligonucleotide (SC) did not affect gene expression. Significant reduction of p27 level was achieved by both different siRNA oligonucleotides in all three cell lines, whereas an unrelated control oligonucleotide (SC) did not affect gene expression.
Loss of p27 in Skp2 and Cks1 overexpressing cells is still not sufficient to rescue proliferation in the presence of oncostatin M. Oncostatin M induced down-regulation of cyclin A. Ubiquitination of p27 by the SCF-Skp2 complex requires its preceding phosphorylation on the Thr187. Cyclin A–, cyclin E–, and cyclin B1–associated Cdk complexes can phosphorylate p27 at this residue (48), and recent studies proposed an additional noncatalytic function of cyclin A in the ubiquitination of p27 (49). Although cyclin E level remained unchanged after oncostatin M treatment, we found severely reduced expression of cyclin A in oncostatin M–treated glioblastoma cells (Fig. 8A). Similarly to Skp2 and Cks1, the reduction of cyclin A protein was preceded by a decrease in cyclin A mRNA level that clearly preceded the cell cycle arrest (Fig. 8B): already within 3 hours of oncostatin M treatment, a decline of cyclin A mRNA level could be observed (Fig. 8B).

Oncostatin M treatment of S phase–arrested cells still resulted in a decline of the S-phase cyclin (Fig. 8C), suggesting that the decline in cyclin A is not a consequence of the G1 phase arrest by oncostatin M but rather cell cycle phase independent.

Stable overexpression of cyclin A could not overcome the oncostatin M–mediated accumulation of p21 and p27 (Fig. 8D) nor could it rescue the growth inhibition by oncostatin M (data not shown). This suggests that stabilization of CKIs and inhibited expression of cyclin A act independently on inhibiting cell cycle progression. Reduced cyclin A expression is unlikely limiting p27 arrested in the absence of p27 (Fig. 7B). Loss of p27 in Skp2 and Cks1 overexpressing cells is still not sufficient to rescue proliferation in the presence of oncostatin M.

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Oncostatin M induced down-regulation of cyclin A. Ubiquitination of p27 by the SCF-Skp2 complex requires its preceding phosphorylation on the Thr187. Cyclin A–, cyclin E–, and cyclin B1–associated Cdk complexes can phosphorylate p27 at this residue (48), and recent studies proposed an additional noncatalytic function of cyclin A in the ubiquitination of p27 (49). Although cyclin E level remained unchanged after oncostatin M treatment, we found severely reduced expression of cyclin A in oncostatin M–treated glioblastoma cells (Fig. 8A). Similarly to Skp2 and Cks1, the reduction of cyclin A protein was preceded by a decrease in cyclin A mRNA level that clearly preceded the cell cycle arrest (Fig. 8B): already within 3 hours of oncostatin M treatment, a decline of cyclin A mRNA level could be observed (Fig. 8B).

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Figure 6. Overexpression of Skp2 and Cks1 in 86HG39 cells. A, top, cell clones coexpressing Skp2 and Cks1 were generated and analyzed for expression of Cks1 (HA, indicating HA-tagged Cks1; endo, endogenously expressed Cks1) and Skp2 and p27 and p21. B, FACS analysis of Cks1 and Skp2 overexpressing 86HG39 cells. Black, cells in G1 phase; white, S-phase cells; gray, G2-M indicates the fraction of cells in G2-M phase. C, expression of p27 in Cks1 and Skp2 overexpressing 86HG39 cells. Stable clones of 86HG39 cells overexpressing Skp2 and Cks1 exhibit diminished p27 induction in response to oncostatin M (OSM). The cells were treated with thymidine for 24 hours. As indicated, oncostatin M was added, and cells were incubated for another 24 hours. Extracts of cells were investigated in Western blots for p27 and p-actin. D, coexpression of Cks1 and Skp2 prevents an accumulation of p27 in oncostatin M–treated S-phase cells. Quantification of p27 was done as described in Materials and Methods by direct measurement of the chemiluminescence of the Western blot.

Figure 7. siRNA-mediated knockdown of p27 in glioblastoma cells fails to rescue proliferation in the presence of oncostatin M (OSM), irrespective of Skp2 and Cks1 overexpression. A, p27 knockdown following siRNA transfection. 86HG39 glioblastoma cells and their derivates overexpressing either Skp2 or Skp2 and Cks1 were transfected with siRNA targeting p27. The p27–directed siRNA reduced p27 expression. SC, control siRNA. Level of p27 and p21 was determined by Western blot analysis. B, knockdown of p27 expression does not prevent the cell cycle arrest by oncostatin M. Following siRNA transfection as described in (A), glioblastoma cells were incubated with oncostatin M for 48 hours. One hour before harvesting, BrdUrd was added to determine the percentage of cells in S phase. Incorporation of BrdUrd was analyzed immunohistochemically using a FITC-labeled anti-BrdUrd antibody. Percentage of BrdUrd-positive cells is displayed. Silencing of p27 did not alter the ability of oncostatin M to prevent cell proliferation, even in the presence of overexpressed Skp2 and Cks1.
T187 phosphorylation, as we observed strong T187 phosphorylation in the presence of oncostatin M (Fig. 4A).

To determine the distribution of p21 and p27 in different Cdk/cyclin complexes, we immunoprecipitated p21 and p27 complexes and determined level of associated cyclins A, D1, or E by Western blotting. Significantly more cyclin E and cyclin D1 were associated with p21 and p27 after oncostatin M addition. Consistent with the reduced cyclin A protein level, less cyclin A was found in complex with p27 (Fig. 8E), whereas level of cyclin A bound to p21 remained largely unaltered.

Discussion

The cytokine oncostatin M can inhibit proliferation of a broad range of different human tumor cell lines (1). We aimed to investigate the molecular basis by which this cytokine prevents cell proliferation. Cell cycle arrest by oncostatin M coincides with inactivation of CDK activities. Reduced CDK activity results from reduced expression of cyclin A and increased expression of CDK inhibitors p21 and p27. Oncostatin M or the related cytokine IL-6 have previously been reported to induce transcription of CDK inhibitors; p21 transcription was induced in osteosarcoma and p27 transcription in melanoma cells (45, 50). In contrast, we found here that p27 mRNA level remained unchanged in glioblastoma cells. Instead, degradation of p27 was blocked by oncostatin M. To exclude that stabilization of p27 was a result of the cell cycle arrest in G1 phase where p27 is more stable (11, 12), we determined the stability of p27 in S phase–synchronized cells. Although p27 is usually very unstable in S phase (Fig. 3B; refs. 11, 12), oncostatin M dramatically increased p27 stability in S phase–arrested cells. This indicated that degradation of p27 in S phase was inhibited by oncostatin M. Consistently, we found that oncostatin M prevents expression of two subunits of the E3 ligase that triggers degradation of p27 in S phase.

Degradation of p27 follows the ubiquitin-proteasome pathway (26). At least two distinct ubiquitin ligases regulate p27 degradation during the cell cycle. Although the degradation of p27 during G0-G1 progression is independent from T187 phosphorylation (22, 23) and depends on ubiquitination by the KPC complex rather than SCF-Skp2 (51), degradation of the inhibitor in S phase requires T187 phosphorylation of p27 and an active SCF-Skp2 ubiquitin ligase complex. This E3 ligase complex includes the cullin Cul1/Cdc53, Skp1, the F-Box protein Skp2, the Ring finger protein Rbx1/Roc1, and the Cks1 protein. Although Cul1, Skp1, and Rbx/Roc1 are mutual components of other SCF complexes, Skp2 and Cks1 act in substrate targeting and interact with T187-phosphorylated p27 (46). p27 stabilization by oncostatin M involves inhibition of Skp2 and Cks1 expression. Down-regulation of Skp2 and Cks1 mRNA was observed immediately after oncostatin M addition, strongly suggesting that inhibition of Skp2/Cks1 transcription or decreased Skp2/Cks1 mRNA stability...
is a primary consequence of oncostatin M signaling. Interestingly, oncostatin M treatment failed to reduce Skp2 and Cks1 expression in oncostatin M–resistant glioblastoma cell lines (i.e., glioblastoma cell lines that still initiate Jak/STAT signaling upon oncostatin M treatment but fail to cease proliferation; data not shown), suggesting that SCF-Skp2 regulation is a central component in the antiproliferative response to oncostatin M.

Stabilization of p27 in the absence of Skp2 was also observed in mice, as homozygous deletion of Skp2 resulted in increased p27 (29). Regulation of Skp2 has been observed after shifting cells from adherent growth into suspension (35), in androgen-treated prostate carcinoma cells (36), after ectopic expression of PTEN in glioblastoma cells or mouse embryonic stem cells, which lack the endogenous PTEN gene (52, 53). Interestingly, in all of these studies, overexpression of Skp2 was sufficient to reverse the p27 accumulation and rescued proliferation. 86HG39 glioblastoma cells investigated in this study express PTEN endogenously (and grow adherent); however, overexpression of Skp2 was not sufficient to rescue p27 degradation after oncostatin M treatment.

Ablation of the Cks1 gene in mice leads to a similar phenotype as deletion of the Skp2 gene, including a smaller body size, indicating impaired cell proliferation (14). A prominent role of Cks1 in controlling cell proliferation was suggested by the observation that Cks1 is induced in human tumors (54). Whereas Skp2 is regulated by multiple mechanisms, data on regulation of Cks1 by mitogenic or antimitogenic signals have been scarce. In mink epithelial cells, transforming growth factor-β treatment leads to down-regulation of Cks1 mRNA and protein followed by up-regulation of p27 protein (55). This suggested that decreasing Cks1 mRNA is sufficient for stabilization of p27 protein and inhibition of cell proliferation. These conclusions are consistent with our observation that Skp2 overexpression was not sufficient to rescue p27 degradation and proliferation of oncostatin M–treated cells. Only combined expression of Skp2 and Cks1 clearly delayed the accumulation of p27 in the presence of oncostatin M (Fig. 6.4). However, even the coexpression of Skp2 and Cks1 was not sufficient to stably override the oncostatin M–induced growth arrest and to prevent accumulation of p27 in asynchronous cells. It restored, however, p27 degradation in oncostatin M–treated S phase–arrested cells (Fig. 6C). As discussed below, alternative oncostatin M–induced pathways lead to a cell cycle arrest in G1 phase, which then would lead to p27 accumulation, as a result of the cell cycle arrest. This model would be consistent with the delayed accumulation of p27 in cells coexpressing Skp2 and Cks1.

This cell cycle arrest involves increased p21 and reduced cyclin A. Increased p21 protein may be sufficient to inactivate Cdks in G1 phase even if p27 is not stabilized. Reduced cyclin A leads to lack of S-phase CdK activity and a shift of CdK inhibitors to other CdK complexes. In addition, reduced cyclin A may also directly contribute to p27 accumulation. Cyclin A has been shown to form a complex with Skp1 and Skp2 (56), and the cyclin has been shown to present p27 to ubiquitination more efficiently than cyclin E (48). Recent reports indicate that cyclin A has an additional non-catalytic function in p27 degradation (49), suggesting that cyclin A mediates p27 degradation independently from its role in p27-T187 phosphorylation.

The broad potency of oncostatin M to arrest multiple tumor cells (where different growth inhibitory pathways may be impaired due to oncogenic transformation) may rely in part on the redundancy of pathways that lead to CDK inactivation. If one single pathway is inactivated in a specific tumor cell, the alternative pathways may be sufficient to sustain growth arrest by oncostatin M. It will be interesting to further explore the different signaling pathways triggered by oncostatin M that converge in inhibiting CdK kinase activity.

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Oncostatin M Induces Growth Arrest by Inhibition of Skp2, Cks1, and Cyclin A Expression and Induced p21 Expression

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