PAX3-FKHR Transformation Increases 26 S Proteasome-dependent Degradation of p27\(^{\text{Kip1}}\), a Potential Role for Elevated Skp2 Expression* 

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PAX3-FKHR is an oncogenic form of the developmental regulator Pax3 transcription factor. PAX3-FKHR results from a t(2;13) chromosomal translocation, a unique genetic marker of alveolar rhabdomyosarcoma. In this study, we showed that ectopic expression of PAX3-FKHR, but not Pax3, in fibroblasts altered cell cycle control and accelerated G\(_1\)/G\(_2\) to S cell cycle transition. PAX3-FKHR-expressing cells had reduced expression of p27\(^{\text{Kip1}}\) protein, a key cell cycle regulator. The reduction in p27\(^{\text{Kip1}}\) levels by PAX3-FKHR resulted from destabilization of p27\(^{\text{Kip1}}\) as shown by cycloheximide treatment and in vivo pulse-chase labeling experiments. The reduced p27\(^{\text{Kip1}}\) protein level in PAX3-FKHR-expressing cells was restored to the level of control cells by treatment with chemical inhibitors that specifically blocked 26 S proteasome activity. Along with the reduction in p27\(^{\text{Kip1}}\) protein, PAX3-FKHR-expressing cells exhibited elevated expression of F-box Skp2 protein, a substrate-specific component of SCF (Skp1-Cullin-F box protein) ligase involved in the cell cycle-dependent control of p27\(^{\text{Kip1}}\) ubiquitination and 26 S proteasome dependent degradation. Finally, we showed that ectopic expression of p27\(^{\text{Kip1}}\) in PAX3-FKHR-expressing cells significantly reduced the proliferation and colony-forming potential of these cells, implicating that down-regulation of p27\(^{\text{Kip1}}\) protein played an active role in the PAX3-FKHR-directed cell transformation.

Rhabdomyosarcomas are the most common soft-tissue sarcoma of childhood (1–3). These are heterogeneous groups of malignant skeletal muscle tumors with alveolar rhabdomyosarcoma (aRMS) as the more malignant subtype (4–8). Most of the alveolar tumors carry a characteristic t(2;13)(q35;q14) chromosomal translocation (9, 10), and a minor group of aRMS carries a variant t(1;13)(p36;q14) chromosomal translocation (11). Both chromosomal translocations result in the same in-frame fusion of Pax genes (Pax3 gene from chromosome 2 and Pax7 gene from chromosome 1) to the FKHR gene located on chromosome 13 (11–13).

Pax3 and Pax7 genes belong to a nine-member gene Pax transcription factor gene family (14). All Pax gene members share a common 128-amino acid DNA binding domain termed the paired box domain. Many members of the Pax family, including Pax3 and Pax7, contain a second DNA binding domain of the homeodomain class. The Pax3 and Pax7 transcription activation domain is localized within a serine-, glycine-, and threonine-rich region at the COOH terminus. Both Pax3 and Pax7 genes have been shown to play critical roles in the development of myogenic cell lineage (15–17). FKHR gene is one of the three FKHR transcription factor gene family members (FKHR, FKHR-L1, AFX) that are closely related to the HNF3 gene family (18–21). FKHR protein is characterized by an NH\(_2\)-terminal winged-helix DNA binding domain and a COOH-terminal proline-rich acidic transcriptional activation domain. The FKHR family, like the Pax family, has also been implicated in developmental regulation (22, 23). Recently, the FKHR family members have been indicated to play a role in IGF-dependent cell survival (24, 25).

The aRMS-associated chromosomal translocations join the paired and homeodomain DNA binding motifs of the Pax3 and Pax7 genes to the bisected DNA binding domain and COOH-terminal transactivation domain of FKHR gene, leading to the expression of PAX3-FKHR and PAX7-FKHR fusion transcription factors, respectively (11–13). Much information on the oncogenic potential of the PAX-FKHR fusion proteins has come from studies that compare the functional differences between PAX3-FKHR and wild type Pax3 proteins since the bisected DNA binding domain of FKHR has not been shown to bind DNA. These studies have revealed two major differences in the activity of the fusion protein as compared with wild type counterpart. One, PAX3-FKHR is a much more potent transcription activator than the wild type Pax3 toward Pax3-responsive DNA elements (26), and second, PAX3-FKHR is able to target gene sequences that are not normally regulated by the wild type Pax3 (27). The increased transcription potency in PAX3-FKHR is thought to result from the inability of the Pax3 NH\(_2\)-terminal cis-acting repressor domain and of the trans-acting repressor proteins that interact with Pax3 DNA binding domains to repress the FKHR transactivation domain (28–30). The increased gene-targeting range in PAX3-FKHR is proposed to result from uncoupling of the functional interdependency between the paired and homeodomain DNA binding motifs (31). The transactivation activity of the wild type Pax3 depends on the cooperative interaction between the two DNA binding motifs. As a result of fusion process, PAX3-FKHR is now able to bind and transactivate DNA sequences through its homeodomain alone. Recent reports indicate that the homeodomain, but not the paired domain, is critical for its cellular transformation function (31–33), suggesting that the importance of identifying those genes whose expression/or function is selectively disrupted by the oncogenic form of Pax3 in understanding the mechanistic links between PAX3-
FKHR and oncogenic transformation and, ultimately, the pathogenesis of rhabdomyosarcoma. In this study, we present evidence to show that the oncogenic form of Pax3 disrupts cell cycle regulation in fibroblast cells by negatively regulating the expression of p27Kip1 protein. The p27Kip1 protein is a member of the CIP-KIP family of cell cycle regulators (34, 35). The level of p27Kip1 protein increases when cells exit the cell cycle in response to signals such as serum deprivation, exposure to growth inhibiting and differentiation factors (36–38), and loss of adhesion to extracellular matrix (39). By contrast, p27Kip1 levels decline dramatically in late G1, relieving the inhibition of cyclin E- and cyclin A-Cdk2 activities, thus promoting G1/S-transition and S-phase progression. The concentration of p27Kip1 protein in cells is regulated at multiple levels including transcription (44, 45), translation (46–48), and post-translational modifications (49–52). The present study provides evidence that one mechanism involved in PAX3-FKHR transformation is through a decrease in p27Kip1 stability due to increased action of Skp2-mediated 26 S proteasome degradation.

MATERIALS AND METHODS

Reagents—Constructs containing mouse-human PAX3-FKHR hybrid cDNA and mouse Pax3 cDNA have been previously described (27, 53). The retrovirus-producing Phoenix cell line was obtained from Dr. Gary Dolan. The constructs containing wild type mouse p27Kip1 cDNA, human mutant p27Kip1 cDNA, and human p45Skp2 were gifts from Dr. Hiroshi Kyokawa (Department of Molecular Genetics, University of Illinois at Chicago), Dr. Koseichi Nakayama (Medical Institute of Biological Regulation, Kyushu University, Fukuoka, Japan), and Dr. Hideyo Yasuda (Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan), respectively. Antibodies against p27Kip1 (C-19), p45Skp2 (H-435), cyclin A (H-432), cyclin E (M-20), cyclin D (H-20), cyclin B1 (H-433), Cdk2 (H-298), p16 (P-12), p19Skp1 (C-20) were from Santa Cruz; anti-cyclin E-specific antibody for E2F-activated transcription factor 4 (TTF-4) was from Chemicon. Antibodies against p27Kip1 were from Pharmingen; antibody against Pax3 (ab-2) was from Geneka; body against Cullin1 was from NeoMarker; antibody against Rb was from Neomarker; antibody against p27Kip1 was from NeoMarker; antibody against α-tubulin (ab-1) was from Oncogene. In vitro translated p27Kip1 protein was generated by the use of the T7-coupled TNT in vitro transcription-translation kit under conditions recommended by the manufacturer (Promega). Total RNA and protein extract from human skeletal muscle were purchased from Stratagene.

Cell Culture—Murine NIH3T3 fibroblast cell line and its derivative cell lines were maintained in Dulbecco’s modified Eagle’s high glucose medium supplemented with 200 units/ml penicillin, 50 µg/ml streptomycin, 10% fetal calf serum, and 0.1% nonessential amino acids. Human ARMS cell lines RH4 and RH30 were maintained in 10% (v/v) fetal calf serum-supplemented Dulbecco’s modified Eagle’s medium. NIH3T3 cells stably expressing vector, Pax3, or PAX3-FKHR cDNA were established by retroviral infection method as previously described (31). All experiments were repeated, and results were confirmed using both clonal cell lines and the pooled cell population. In this report, with the exception of Fig. 9, only data obtained from pooled cells are presented.

Cell Extract Preparation—Whole cell extract was prepared from cells by either lysing cells in high salt extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 25% glycerol, 0.4 M NaCl, and 0.15 M sodium citrate) and then cleared of cell debris by brief centrifugation, and clarified supernatant was stored at −80 °C until use. Cytoplasmic cell extract was prepared from cells by first swelling in low salt hypotonic buffer (20 mM HEPES, pH 7.5, 1.5 mM MgCl2, 5 mM KCl, 1 mM DTT) for 60 min on ice. Routinely, 0.2 ml of a 32P-labeled E2F DNA probe prepared by Klenow labeling was added to the electrophoretic mobility shift assay reaction mixture (final volume of 20 µl) and allowed to form DNA-protein complexes during a 20-min incubation at room temperature. The complex was analyzed on a 7% non-denaturing polyacrylamide gel. Electrophoresis was carried out in 0.25× Tris borate-EDTA buffer at 120 V at 4 °C until the bromphenol blue dye reached the bottom, and the gel was dried and autoradiographed.

In Vitro Protein Degradation Assay—The in vitro p27Kip1 degradation set up was as previously described (54). In vitro degradation reaction was set up by incubating 55–100 µg of hypotonic cell extract with 0.3 fmol of 32P-labeled in vitro translated p27Kip1 in the presence of ATP-regenerating system (1 mM ATP, 10 µg/ml creatine kinase, 20 mM creatine phosphate) and 1/25 (v/v) rabbit reticulocyte lysate at 30 °C for the indicated times. The reaction was terminated by the addition of Laemmli sample buffer, analyzed by SDS-PAGE, and autoradiographed.

RESULTS

Inactivation of PAX3-FKHR-mediated activity in rhabdomyosarcoma cells significantly impairs the growth of the tumor cells, implying that the fusion protein actively participates in the pathological progression of the disease (55). Previous studies clearly demonstrate that PAX3-FKHR is a dominant oncogene in that it is sufficient to induce transformation in primary fibroblast cells (56) and immortalized murine embryonic fibroblast cells (31, 33); however, the mechanisms and pathways involved in PAX3-FKHR-initiated transformation have not been characterized. In this report, we focused on examining the effect of PAX3-FKHR on cell cycle control using NIH3T3 embryonic fibroblast cell line as a study model.

PAX3-FKHR Promotes G1/S Transition of Cell Cycle in NIH3T3 Fibroblast Cells—In our previous studies, we have shown that ectopic expression of Pax3 protein, even at a level severalfold higher than PAX3-FKHR, is unable to transform NIH3T3 cells (31). To better understand the changes in growth caused by PAX3-FKHR transformation, we examined the effect
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Fig. 1. PAX3-FKHR induces hyperproliferation in NIH3T3 fibroblast cells. NIH3T3 cell lines that ectopically expressed vector (open circles), PAX3-FKHR (filled squares), and Pax3 (crosses) gene were seed at 1 × 10⁴ cells per well in 12-well plates at day 0. At a six-day experimental period, cells were harvested each day into phosphate-buffered saline and stained with trypan blue. Only viable cells were counted. The experiment was carried out more than three times on different days using different lots of pooled cell lines.

of PAX3-FKHR on cell proliferation using NIH3T3 cells that stably expressed PAX3-FKHR. As controls we used NIH3T3 cells that were stably transfected with the expression vector or Pax3 expression vector. As shown in Fig. 1, Pax3-expressing cells displayed a comparable growth rate as the control cells, whereas PAX3-FKHR-expressing cells exhibited a greater proliferation rate than control cells.

PAX3-FKHR Overexpression Results in Reduced Expression of Cyclin Kinase Inhibitor, p27Kip1—To assess the effect of PAX3-FKHR on cell cycle behavior, we investigated the expression pattern of a number of key cell cycle regulatory proteins by Western blot analysis (Fig. 2). Although there were subtle variations in a few cell cycle regulators such as cyclin D1 and cyclin A, the most notable and reproducible changes were the decreased level of cyclin kinase inhibitor p27Kip1 protein and the increased level of hyperphosphorylated form of Rb. To verify that our observation with the p27Kip1 down-regulation was not a consequence of selecting the fastest-growing cells during establishment of stable cell lines, we carried out additional experiments to examine the status of p27Kip1 in NIH3T3 cells shortly after PAX3-FKHR expression was induced by retroviral infection. Proliferating NIH3T3 cells were infected with retroviral stock at increasing multiplicities of infection for 12 h and harvested at 12 h post-infection for Western blot analysis. As shown in Fig. 2B, we observed that p27Kip1 protein was specifically decreased in response to increasing amounts of PAX3-FKHR expression. Furthermore, the decrease in p27Kip1 protein expression was specific for the oncogenic form of Pax3 because Pax3-expressing cells had a similar level of p27Kip1 as control cells. This result suggested that suppression of p27Kip1 protein expression was likely to play a critical role in the oncogenic function of PAX3-FKHR fusion protein.

In normal cycling cells, the level of p27Kip1 protein accumulates to maximum levels during G0/G1 and declines rapidly in late G1 before cells enter S phase. To determine whether PAX3-FKHR expression affected the rate of p27Kip1 protein disappearance during the transition from G0/G1 to S phase, we examined the p27Kip1 protein profile in the control and PAX3-FKHR-transformed cells under synchronized conditions. The PAX3-FKHR-transformed cells required a longer time to exit the cell cycle than the control cells upon serum withdrawal. As shown by the fluorescence-activated cell sorter analysis in Fig. 3A, it took close to 60 h of serum deprivation (0.5% serum) for PAX3-FKHR-transformed cells to achieve G0/G1 arrest, whereas it took 24 h to achieve a similar status for the control cells. Control and PAX3-FKHR-transformed cells were synchronized to G0/G1 by 60 h of serum deprivation (0.5% serum), whereas it took 24 h to achieve a similar status for the control cells. This result suggested that suppression of p27Kip1 protein expression was specific for the oncogenic form of Pax3 and PAX3-FKHR-expressing cells displayed early activation of both cyclin E- and cyclin A-associated kinase activities.

The Expression of p27Kip1 in PAX3-FKHR-transformed Cells Is Controlled at the Post-transcriptional Level—The change in p27Kip1 protein levels in normal cells can be regulated at multiple levels including transcription (44, 45), translation (46–48), and post-translational modifications (49–52). Because PAX3-FKHR acts as a transcription factor, we examined whether the change in p27Kip1 protein expression induced by PAX3-FKHR was reflected by changes in p27Kip1 mRNA content. As shown in Fig. 4, the Northern blot analysis revealed that the level of p27Kip1 mRNA was unaffected by PAX3-FKHR expression (Fig. 4A), whereas the level of p27Kip1 protein was increased (Fig. 4B). We next examined whether the reduced p27Kip1 pro-
tein level was due to changes in protein stability by carrying out cycloheximide treatment (Fig. 5A) and in vivo pulse-chase labeling (Fig. 5B) experiments to compare the stability of p27\textsuperscript{Kip1} protein between vector and PAX3-FKHR expressing NIH3T3 cells. Results from both experiments showed that the half-life of p27\textsuperscript{Kip1} protein was dramatically shortened in PAX3-FKHR-expressing cells. Collectively, these data suggested that altered regulation of p27\textsuperscript{Kip1} protein stability, not changes in transcription, translation, or mRNA stability, was a major cause in the reduced level of p27\textsuperscript{Kip1} protein expression in PAX3-FKHR-transformed cells.

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Fig. 3. Analysis of the effect of PAX3-FKHR on the expression of p27\textsuperscript{Kip1} protein and activities of cyclin E-Cdk2 and cyclin A-Cdk2 kinases in synchronous culture. A, fluorescence-activated cell sorter analysis of growing and synchronized control and PAX3-FKHR-transformed cells. B, duplicate plates of growth-arrested (60 h) serum starvation control or PAX3-FKHR-transformed NIH3T3 cells were stimulated with 10% bovine calf serum containing media to enter cell cycle. At the indicated times after serum stimulation, one set of cells was extracted with high salt buffer, and a total of 30 μg of extract was used to detect the expression of PAX3-FKHR and p27\textsuperscript{Kip1}. The second set of cells was extracted with immunoprecipitation buffer, and a total of 200 μg of cell extract was used in the immunoprecipitation reaction in the presence of either cyclin A or cyclin E antibody followed by the kinase reaction as described under "Materials and Methods."
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FIG. 4. Decreased p27Kip1 expression in PAX3-FKHR-expressing NIH3T3 cells is not due to changes in p27Kip1 mRNA content. Duplicate plates of NIH3T3 cells expressing either vector, PAX3-FKHR, or Pax3 with or without expression of Pax3 and PAX3-FKHR were used to prepare total RNA for Northern blot analysis (A) and Western blot analysis (B), respectively. For Northern blot analysis, a total of 15 μg was used to determine p27Kip1 mRNA content. For sample normalization, the blot was stripped and re-probed with 32P-labeled DNA corresponding to the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence. The membrane was scanned by phosphorimaging for quantitative analysis. The signal (corrected for glyceraldehyde-3-phosphate dehydrogenase) detected in control cells that were transfected with empty vector was assigned an arbitrary value of 1. Fold induction is the ratio of the signal in PAX3-FKHR-expressing cells to that of control cells. For Western blot analysis, a total of 25 μg of radioimmune precipitation assay buffer extract was used to detect the expression of Pax3, PAX3-FKHR, and p27Kip1 proteins.

p27Kip1 is a substrate of cyclin E-Cdk2 and cyclin A-associated Cdk2 activities after release from serum starvation. To examine whether Cdk2-dependent phosphorylation of p27Kip1 protein was critical to the PAX3-FKHR-induced effect on p27Kip1 protein in vivo, we utilized chemical inhibitor roscovitine to block Cdk2 kinase activity. Proliferating cells were treated with roscovitine for 12 h, and the level of p27Kip1 protein expression was determined by Western blot analysis. As shown in Fig. 6B, roscovitine treatment of PAX3-FKHR-transformed cells restored p27Kip1 protein to levels similar to the levels observed in control cells, suggesting that phosphorylation of p27Kip1 by Cdk2 kinase is involved in raising the p27Kip1 protein level in PAX3-FKHR-expressing cells during the late G1 phase of cell cycle.

FIG. 5. PAX3-FKHR induces destabilization of p27Kip1 protein. A, measurement of p27Kip1 protein degradation after inhibition of protein synthesis by cycloheximide (CHX). Proliferating NIH3T3 cells (2 × 10^6 cells/60-mm dish) were infected with the vector or PAX3-FKHR-expressing retrovirus (PF). Twenty-four hours after infection, cells were treated with 10 μg/ml cycloheximide for the indicated times, and p27Kip1 protein levels were followed by Western blot analysis. B, measurement of p27Kip1 protein stability by an in vivo pulse-chase labeling method. Proliferating control and PAX3-FKHR-transformed NIH3T3 cells were pulse-labeled in media containing [35S]Met and later chased with media containing unlabeled Met for the indicated times as described under “Materials and Methods.” Radiolabeled p27Kip1 and α-tubulin from the radioimmune precipitation assay buffer cell extracts were immunoprecipitated with anti-p27Kip1 and anti-α-tubulin antibodies, and the immunocomplexes were pulled down with protein G-Sepharose beads. The immunocomplexes were separated by SDS-PAGE, and radioactive protein products were visualized by autoradiography.

FIG. 6. Inhibition of 26S proteasome (A) or Cdk2 (B) activities increases p27Kip1 levels in PAX3-FKHR-transformed cells. Proliferating control and PAX3-FKHR-transformed (PF) NIH3T3 cells were treated with Me2SO (DMSO) solvent or indicated inhibitors for 6 h (A) or 12 h (B) before harvest. High salt extracts (25 μg) of each sample were analyzed by Western blot. Inhibitors used in A were 26S proteasome inhibitors (10 μM MG132, 10 μM lactacystin, 100 μM N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN)), and caspase inhibitor (Inh.) III mixture (50 μM).

The phosphorylation of p27Kip1 by Cdk2 kinase is critical for the decreased stability of the p27Kip1 protein by PAX3-FKHR, we developed an in vitro degradation assay. In this assay, S35-radiolabeled protein was translated in vitro, and then treated with Cdk2 kinase. The translated p27Kip1 protein was then immunoprecipitated from the reaction mixture and analyzed by Western blot analysis. As shown in Fig. 6A, PAX3-FKHR-induced destabilization of p27Kip1 protein is not due to changes in p27Kip1 mRNA content.
substrate was incubated with cytoplasmic extracts prepared from either control or PAX3-FKHR-transformed cells for 1 h at 30 °C. Under this reaction condition, we were able to show that the wild type p27Kip1 substrate was more rapidly degraded by PAX3-FKHR cell extract than control cell extract (Fig. 7 A). This enhanced degradation activity in PAX3-FKHR cell extract appeared to specifically involve the 26 S proteasome pathway since the activity was blocked when the non-hydrolyzable form of ATP (ATP[S]) was used in place of the ATP-regenerating system (Fig. 7 B), and the activity was specifically blocked in the presence of Me2SO (DMSO), MG132, lactacystin, and caspases inhibitor mixture (C).  

Recent studies show that phosphorylation of p27Kip1 protein on Thr187 provides a binding site for SCF (Skp1-Cullin-F box protein), an E3 ubiquitin ligase complex that is responsible for p27Kip1 polyubiquitination and its subsequent degradation by the 26 S proteasome pathway (57, 63). The F-box protein is the substrate-specific recognition component of the SCF complex that is used to target specific proteins for degradation. In the case of the p27Kip1 protein, this substrate-specific F-box protein is Skp2 (58, 66). We investigated whether PAX3-FKHR altered the expression of one or more subunits within the SCF complex that might lead to increased ubiquitination and subsequent degradation of p27Kip1 protein. As shown in Fig. 8A, although the protein levels of Skp1 and CulI remained unchanged between control and PAX3-FKHR-transformed cells, the level of Skp2 protein was significantly increased in PAX3-FKHR-transformed cells. Furthermore, we showed that increased expression of PAX3-FKHR achieved by varying the multiplicity of retroviral infection led to increased Skp2 expression that appeared to correlate with decreased levels of p27Kip1. This effect was not observed in control cells or in cells expressing increasing levels of Pax3 protein (Fig. 8B). Unlike p27Kip1 gene, the half-life of Skp2 was unchanged in PAX3-FKHR-expressing NIH3T3 cells (data not shown). Instead, the induction of Skp2 protein level by PAX3-FKHR was correlated with an increased accumulation of Skp2 mRNA (Fig. 8C). The altered expression of Skp2 and p27Kip1 levels was similarly observed in cells derived from aRMS tumors containing the t(2;13) chromosomal translocation (Fig. 8D).

Exogenous Expression of the p27Kip1 Gene Impairs the Hyperproliferative and Colony-forming Activities in PAX3-FKHR-expressing NIH3T3 Cells—Finally, we addressed the question of whether overexpression of p27Kip1 protein could affect the growth rate and colony formation of PAX3-FKHR-transformed cells. To this end, PAX3-FKHR-transformed cells were stably transfected with pcDNA3 encoding the mouse p27Kip1 gene or not. Transfection of the empty vector did not affect the p27Kip1 protein expression levels in PAX3-FKHR-transformed cells (third lane, Fig. 9A). We screened several p27Kip1 transfectant PAX3-FKHR-expressing clones to identify ones (an example of...
one such clone is shown in the second lane, Fig. 9A) that expressed p27\textsuperscript{Kip1} protein at a level comparable with that observed in untransformed NIH3T3 cells (first lane, Fig. 9A). We showed that increasing the p27\textsuperscript{Kip1} protein levels in PAX3-FKHR-transformed cells significantly slowed their growth rate (Fig. 9B) and drastically inhibited their contact inhibition-independent colony-forming activity (Fig. 9C). The biological assays were carried out in three independently isolated p27\textsuperscript{Kip1} overexpressing stable cell lines with similar results. These data suggested that suppression of p27\textsuperscript{Kip1} protein expression by PAX3-FKHR played an active role in oncogenic activity of the fusion protein.

**DISCUSSION**

Rhabdomyosarcoma is a soft tissue sarcoma of children and young adolescents. The most prevalent subtypes of RMS are the embryonal RMS and the alveolar RMS, with the latter closely associated with higher disease stage and a poor prognostic outcome (4–8). Unlike embryonal RMS tumors that do not contain consistent chromosomal aberrations, more than 90% of aRMS tumors contain a unique t(2;13)(q35;q14) chromosomal translocation. The molecular consequence of such translocation is the formation of a novel chimeric protein PAX3-FKHR. Several lines of evidence strongly suggest that the PAX3-FKHR fusion protein plays an active role in RMS tumor development. Ectopic expression of PAX3-FKHR induces accelerated cell proliferation in embryonic fibroblast cells (31–33, 56) and murine C2C12 myogenic cells\textsuperscript{2} and blocks terminal differentiation in myoblast cells (59). An antisense-induced block of PAX3-FKHR activity in rhabdomyosarcoma cells is shown to significantly impair the growth of these tumor cells (55). Clinically, aRMS tumors are a more aggressive subtype, often showing invasive metastatic behavior than embryonal tumors. This difference in clinical behavior may be explained by the presence of PAX3-FKHR fusion protein in the alveolar subtype. Anderson et al. (60) recently showed that ectopic expression of PAX3-FKHR into the embryonal RMS cell line increases cell proliferation and promotes the embryonal RMS cells to form faster growing, more invasive tumors (60). Taken together, these studies strongly support the notion that PAX3-FKHR fusion protein has a pleiotropic and dominant effect on tumor development. However, despite the overwhelming evidence supporting a dominant oncogenic role for PAX3-FKHR, little is known about the mechanisms involved in altering the cell cycle pathways that lead to uncontrolled growth phenotype.

In this report, we have presented evidence to demonstrate that PAX3-FKHR-transformed cells proliferate much faster than control and Pax3-expressing cells. The PAX3-FKHR-expressing cells are more resistant to serum starvation-induced cell cycle arrest and are able to enter S phase much more quickly upon serum stimulation. At a molecular level, we have shown that ectopic PAX3-FKHR expression drastically suppresses the steady state level of p27\textsuperscript{Kip1} protein in exponentially growing NIH3T3 cells. The p27\textsuperscript{Kip1} protein is a member of the CIP-KIP family of Cdk2 inhibitors. It has been shown that the timed decrease in the level of p27\textsuperscript{Kip1} protein in late G\textsubscript{1} is crucial in promoting S-phase cell cycle transition from G\textsubscript{1}. Here, we demonstrate that upon serum-induced cell cycle reentry, the rate of decline of p27\textsuperscript{Kip1} protein is drastically increased in PAX3-FKHR-transformed cells as compared with control cells. We propose that by actively reducing the level of p27\textsuperscript{Kip1} protein in cells, PAX3-FKHR is able to effectively alleviate the inhibitory effect on its two major targets, the cyclin A/Cdk2 and cyclin E/Cdk2 complexes. As such, the increased cyclin/Cdk2 activities mediate and sustain the hyperphosphorylated form of Rb that is required to release its association with E2F complexes and allow transcription of several E2F-responsive genes essential for DNA synthesis and for early commitment of cells to S phase. This idea is supported by the

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\textsuperscript{2} C. Wang, unpublished data.
findings that PAX3-FKHR elevates the overall level of hyperphosphorylated form of Rb.

In addition to shortening of G1 phase, we have also observed that the PAX3-FKHR-transformed cells are less sensitive to serum starvation than the control cells in that they require nearly 60 h of low serum (0.5% serum) treatment to be properly arrested at G0/G1, whereas the control cells arrest within 24 h of serum starvation. These data suggest that ectopic expression of PAX3-FKHR does not induce mitogen independence in NIH3T3 cells but rather significantly delays the cell response to negative growth signal. Because PAX3-FKHR-expressing cells are able to accumulate p27Kip1 protein to the same level as in control cells upon prolonged serum deprivation, it is unlikely that PAX3-FKHR-transformed cells have a defective mechanism in p27Kip1 protein synthesis; rather, the transformed cells require a longer time to increase p27Kip1 levels due to the robust degradation of this protein. It is likely that additional mechanisms are also involved in delayed growth arrest response in PAX3-FKHR-transformed cells. For example, a recent study has shown that PAX3-FKHR can directly transcriptionally activate the promoter of IGF receptor gene (69). If so, the higher density of IGF receptor in these cells might be sufficient enough to carry cells through extra rounds of cell cycle at low serum condition.

Although the level of p27Kip1 mRNA stays relatively unchanged throughout cell cycle in normal cells, suppression of p27Kip1 transcription has been reported in cells expressing viral oncoprotein v-src (44). Moreover, we become intrigued by the possibility that PAX3-FKHR might affect p27Kip1 expression through altered gene transcription because 1) PAX3-FKHR normally acts as a transcription factor, 2) we have shown in previous study that PAX3-FKHR fusion protein has a
gain of function in that it can alter expression of genes that are not normally targeted by Pax3 (27), and 3) the normal FKHR counterpart of the fusion protein has been shown to play a role in transcriptional regulation of p27Kip1 after mitogen induction (45). However, we are unable to detect a change in the total amount of p27Kip1 mRNA in PAX3-FKHR-transformed cells, suggesting p27Kip1 is not a direct target for transcription activation by PAX3-FKHR. Instead, we find that the predominant effect of PAX3-FKHR on p27Kip1 is through an enhanced degradation of p27Kip1 protein. We have provided several lines of evidence to support this conclusion. First, experiments using the protein synthesis inhibitor cycloheximide as well as in vitro pulse-chase labeling studies show that the stability of p27Kip1 protein is dramatically reduced in PAX3-FKHR-expressing cells. Second, we show that protease inhibitors specific for 26S proteasome activity prevent degradation of p27Kip1 protein and restore p27Kip1 protein levels in PAX3-FKHR cells to the levels found in control cells. Third, we show that cytoplasmic protein extract prepared from PAX3-FKHR-transformed cells can degrade in vitro translated wild type p27Kip1 substrate much more efficiently than extracts prepared from the control cells. Similar to the in vivo result, 26S proteasome inhibitors specifically block the in vitro degradation activity. Collectively, these results suggest that p27Kip1 stability is decreased by PAX3-FKHR through increased ubiquitin-dependent degradation by the 26S proteasome pathway.

Down-regulation of p27Kip1 protein expression by PAX3-FKHR appears to be a relatively early event since this effect is observed within 12 h after PAX3-FKHR protein is induced in the cells (Fig. 3B). Because PAX3-FKHR is a transcription factor, it seems unlikely that PAX3-FKHR is directly involved in the increased increase in the degradation of p27Kip1 protein. In the in vitro degradation assay, we have determined that the addition of PAX3-FKHR protein to control cell extract is not sufficient to cause accelerated p27Kip1 protein degradation (data not shown). An alternative explanation would be that additional cellular components that are specifically activated in PAX3-FKHR-transformed cells are required to induce p27Kip1 protein degradation. As mentioned earlier, IGF receptor has been shown to be a direct target for transcription activation by PAX3-FKHR. Increased IGF receptor expression would lead to an activation of mitogen-induced signal transduction pathways that may alter the p27Kip1 level. However, in our PAX3-FKHR-transformed NIH3T3 cells, we were unable to detect an effect on p27Kip1 protein levels by chemical blockers that inhibit Akt/protein kinase B, extracellular signal-regulated kinase/mitogen-activated protein kinase (MAPK), p38 MAPK, and p70S6 kinase mediated activities (data not shown).

In this report, we have found that the expression of F-box protein Skp2, the substrate-specific E3 ubiquitin ligase of SCF complex (Skp1/Cul1/F-box), is selectively up-regulated in PAX3-FKHR-transformed cells. Both biochemical and genetic experiments have implicated that Skp2 is responsible for the ubiquitin-dependent degradation of p27Kip1 protein in vivo and in vitro. Binding of Skp2 to p27Kip1 protein requires phosphorylation of p27Kip1 on Thr-187 by cyclin-Cdk2 complexes (50, 65, 66). In our study, we have shown that inhibition of Cdk2 activity by roscovitine increases p27Kip1 protein levels in PAX3-FKHR-transformed cells in vivo (Fig. 7B) and the p27Kip1 with mutation to the Thr-187 residue is resistant to degradation by extracts from PAX3-FKHR cells in vitro (Fig. 8B). Presumably, elevation of Skp2 protein by PAX3-FKHR would permit accelerated ubiquitination of p27Kip1, which is a prerequisite step for targeted degradation of p27Kip1 protein by the 26S proteasome pathway. This notion is in line with the observation of a PAX3-FKHR dose-dependent, inverse correlation between the expression of Skp2 and p27Kip1 proteins. The finding that Skp2 RNA content is increased in the PAX3-FKHR-expressing NIH3T3 and aRMS tumor cells leads us to hypothesize that the direct target for PAX3-FKHR effect on p27Kip1 may be Skp2 transcription. It will be important in the future to test this idea by determining whether PAX3-FKHR can directly transactivate Skp2 promoter. Furthermore, the activation of Skp2 gene expression is likely to affect degradation of other proteins, and it will be important to identify additional targets and examine their role in PAX3-FKHR oncogenesis.

In summary, the present study has presented clear evidence to show that the PAX3-FKHR selectively accelerates p27Kip1 protein degradation by the ubiquitin-dependent 26S proteasome pathway. The targeted destabilization of p27Kip1 protein by PAX3-FKHR serves as an active mechanism in PAX3-FKHR transformation since restoration of p27Kip1 level through ectopic expression greatly reduces the hyperproliferation and contact inhibition-independent growth properties of PAX3-FKHR-transformed cells. Moreover, we have identified Skp2 as a potential regulatory link between PAX3-FKHR activation and p27Kip1 down-regulation. We have used NIH3T3 cells as our model system because it offers the opportunity to identify proximal targets whose expression/function is changed as a consequence of PAX3-FKHR expression, whereas in tumor cells it is likely that there have been extensive genetic mutations acquired during the progression from transformation to tumor. In this report, we are able to verify that both p27Kip1 and Skp2 are similarly dysregulated in two PAX3-FKHR-expressing aRMS cell lines as compared with normal skeletal muscle. In future work, it will be important to determine whether these alterations are common to all RMS or more specific to a particular RMS subtype. It is possible that the fusion process induces a conformational change within PAX3-FKHR, enabling it to regulate the expression and function of genes that are not regulated by its normal counterparts under normal physiological conditions. Thus, identification of genes whose expression and function are selectively disrupted by the oncogenic form of Pax3 is essential to gain a full understanding of the mechanistic links between PAX3-FKHR and oncogenic transformation.

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PAX3-FKHR Transformation Increases 26 S Proteasome-dependent Degradation of p27 Kip1, a Potential Role for Elevated Skp2 Expression
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