Signaling and Regulation

AP-1 Regulates Cyclin D1 and c-MYC Transcription in an AKT-Dependent Manner in Response to mTOR Inhibition: Role of AIP4/Itch-Mediated JUNB Degradation

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Abstract

One mechanism by which AKT kinase-dependent hypersensitivity to mammalian target of rapamycin (mTOR) inhibitors is controlled is by the differential expression of cyclin D1 and c-MYC. Regulation of posttranscriptional processes has been demonstrated to be crucial in governing expression of these determinants in response to rapamycin. Our previous data suggested that cyclin D1 and c-MYC expression might additionally be coordinately regulated in an AKT-dependent manner at the level of transcription. Under conditions of relatively quiescent AKT activity, treatment of cells with rapamycin resulted in upregulation of cyclin D1 and c-MYC nascent transcription, whereas in cells containing active AKT, exposure repressed transcription. Promoter analysis identified AKT-dependent rapamycin responsive elements containing AP-1 transactivation sites. Phosphorylated c-JUN binding to these promoters correlated with activation of transcription whereas JUNB occupancy was associated with promoter repression. Forced overexpression of JunB or a conditionally active JunB-ER allele repressed cyclin D1 and c-MYC promoter activity in quiescent AKT-containing cells following rapamycin exposure. AIP4/Itch-dependent JUNB protein degradation was found to be markedly reduced in active AKT-containing cells compared with cells harboring quiescent AKT. Moreover, silencing AIP4/Itch expression or inhibiting JNK mediated AIP4 activity abrogated the rapamycin-induced effects on cyclin D1 and c-MYC promoter activities. Our findings support a role for the AKT-dependent regulation of AIP4/Itch activity in mediating the differential cyclin D1 and c-MYC transcriptional responses to rapamycin. Mol Cancer Res; 9(1); 115–30. ©2010 AACR.

Introduction

Inhibitors targeting the mTOR kinase are in clinical development as potential chemotherapeutic agents. mTOR is a regulatory kinase that integrates signals linking the ability of cells to undergo cell-cycle transit to the availability of nutrients (1, 2). Treatment with mTOR inhibitors typically results in a starvation response and cell-cycle arrest, however cells of hematopoietic origins undergo apoptotic death following exposure (3). The mTOR kinase is a member of the serine/threonine phosphatidylinositol 3-like family of kinases and is found in at least two separate complexes (4). The mammalian target of rapamycin complex 1 (mTORC1) is composed of Raptor, mLST8, PRAS40, Deptor and the catalytic subunit mTOR. MTORC2 components include Rictor, mSIN1, Protor, Deptor, as well as mLST8 and mTOR. MTORC1 is sensitive to rapamycin and is known to regulate several cell functions including growth, size, cap-dependent mRNA translation and ribosomal component biogenesis (5). MTORC2 has signaling outputs regulating cell growth and motility and is generally assumed to be insensitive to rapamycin, but the macrolide does seem to inhibit mTORC2 assembly in some cell lines following prolonged exposure (6, 7). The signaling effectors of mTORC1 include the p70S6 kinase, the translational repressor 4EBP1 and the recently described activation of SGK (8). MTORC2 is the major kinase responsible for full activation of AKT via phosphorylation of serine 473 (9).

Several studies have described the hypersensitivity of different tumor types to mTOR inhibitors in cells whose AKT activity is elevated due to gene amplification or loss of function mutations in the tumor suppressor PTEN (10–12). We have demonstrated that differential sensitivity can be explained, in part, by the differential regulation of cyclin D1 and c-MYC gene expression at the levels of mRNA translation initiation and stability (13, 14). Continued internal ribosome entry site (IRES)-dependent translation initiation and enhanced

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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doi: 10.1158/1541-7786.MCR-10-0105

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mRNA stability of cyclin D1 and c-MYC mRNAs is sufficient to overcome rapamycin-induced G1 arrest. Our data however, also suggested coordinate regulation of cyclin D1 and c-MYC transcription in addition to the posttranscriptional control exerted by AKT in the face of mTOR inhibition (12). How AKT activity may regulate the transcriptional responses of cells to mTOR inhibitors is unknown.

In the current study, we have extended our previous analysis of AKT-dependent cyclin D1 and c-MYC posttranscriptional regulation to try and understand the mechanisms controlling gene transcription of these determinants following rapamycin exposure. Tumor cells containing active AKT were found to repress transcription of cyclin D1 and c-MYC, whereas in cells with relatively quiescent AKT activity transcription was induced. Subsequent deletion and mutational analysis of cyclin D1 and c-MYC promoter constructs identified rapamycin responsive promoter elements containing AP-1 transcription factor binding sites. JUNB binding to these promoter elements correlated with transcriptional repression of cyclin D1 and c-MYC promoter activity, whereas phosphorylated c-JUN binding strongly activated these promoters in an AKT-dependent manner upon rapamycin treatment. Moreover, the AKT-dependent regulation of promoter activity correlated with alterations in E3 ubiquitin ligase AIP4/Itch-mediated JUNB ubiquitination. These data support the involvement of differential AIP4/Itch-mediated JUNB degradation in regulating the transcriptional responses of cyclin D1 and c-MYC to mTOR inhibition in a manner dependent on cellular AKT activity.

Materials and Methods

Cell lines and transfections

The isogenic cell lines pairs used in this study differ significantly in their relative AKT activities virtue of either their PTEN status or forced expression of an activated allele of AKT1. These lines were kindly provided by Ingo Mellinghoff and Charles Sawyer and have been described previously (13). The isogenic Pten+/+ and Pten−/− MEF cells were kindly provided by Hong Wu and have also been described (15). Transient luciferase reporter transfections were performed using FUGENE 6 (Roche) as recommended by the manufacturer. To generate the JUNB and JunB-ER expressing lines cells were transfected similarly using FUGENE 6, and clones selected for G418 resistance.

Constructs and reagents

The CCND1 and c-MYC promoter constructs were provided by Drs. Anil Rusti (Department of Medicine, University of Pennsylvania) and Linda Penn (Ontario Cancer Institute, University of Toronto), respectively. Mutagenesis was performed using the QuikChange site-Directed Mutagenesis kit (Agilent Technologies) with the appropriate mutagenic primers according to the manufacturer. The minimal IRES sequences from the p27Kip1 5′ UTR were inserted immediately upstream of the luciferase ORF in all luciferase reporter constructs (13) and where indicated, native AP-1 sites in the cyclin D1 and c-MYC promoters were replaced with (TATTGTA). All mutagenesis was confirmed by sequencing. The pMV7JUNB and pMV7JunB-ER constructs were obtained from Drs. Latifa Bakiri and Moshe Yaniv (Institut Pasteur, Paris, France). The HA-ubiquitin construct was provided by Dr. Ted Dawson (Department of Neurology, Johns Hopkins University School of Medicine). Antibodies against the following proteins were used: anti-HA and control IgG were from Santa Cruz Biotechnology; phospho-S6K, S6K, phospho-AKT, AKT, cyclin D1, c-MYC, JUNB, c-JUN, and JNK and antibodies were from Cell Signaling; RNA PolII phospho-S2 CTD, phospho-c-JUN and AIP4/Itch antibodies were from Abcam; phospho-JUNB antibody was from Signalway and actin antibody from Sigma. Rapamycin was obtained from LC Laboratories, MG132 was purchased from Enzo Life Sciences, and the JNK inhibitor VIII was from EMD Biosciences. Rapamycin was used at 10 nmol/L for 24 hours for all treatments unless otherwise indicated.

Luciferase reporter assays

The indicated reporter constructs (200 ng DNA) were transiently transfected into U87 and U87Pten cells. Subsequently, cells were exposed to 10 nmol/L of rapamycin for 24 hours after which extracts were prepared and luciferase activity determined. Promoterless pGL3 plasmid coding for firefly luciferase was used to determine basal activity and phRG-Basic cotransfected for normalization (Promega). Luminescence was measured using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation, in vitro DNA-pull down, nuclear run on, and electrophoretic mobility shift assays

Chromatin immunoprecipitation (ChIP) assays and subsequent real-time PCR analysis was performed as described (16). Briefly, cells were washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 minutes. Cells were rinsed with ice-cold PBS twice and resuspended into 100 mmol/L of Tris-HCl (pH 9.4), 10 mmol/L of DTT and incubated for 15 minutes at 30°C. Cells were washed and resuspended in lysis buffer (1% SDS, 10 mmol/L of EDTA, 50 mmol/L of Tris-HCl, pH 8.1, 1× protease inhibitor cocktail (Roche) and sonicated 3 times for 10 seconds each pulse at the maximum setting (Fisher Sonic Dismembrator, Model 300), followed by centrifugation for 10 minutes. Immunoprecipitations were performed overnight at 4°C with specific antibodies. Precipitates were washed and heated at 65°C to reverse cross-linking and DNA fragments purified using QIAquick Spin Kit (Qiagen) for subsequent PCR. Detailed information regarding primer sequences is available upon request. In vitro DNA pull-down assays were performed as described (17) using a double-stranded oligonucleotide containing the AP-1 site (s) and immediately flanking sequences from either the cyclin D1 or c-MYC promoters attached to streptavidin-Sepharose beads via a 5′ biotinylated plus strand according to the manufactures recommendations (Invitrogen). Beads
with adsorbed proteins were analyzed by SDS-PAGE followed by immunoblotting. Nuclear run on assays were performed as previously described (18). Electrophoretic mobility shift assays (EMSA) were performed as described (19) and antibodies added to the binding reactions where indicated to visualize supershifted species.

**Immunoprecipitations, immunoblot and in vitro kinase and ubiquitination assays**

Immunoprecipitations and immunoblots were performed as described (13) and for displaying ubiquinated protein, 0.1% SDS and 5 mmol/L of N-ethylmaleimide were added to the lysis buffer to disrupt non-specific protein interactions. JNK activity was assessed via an in vitro kinase assay kit as described by the manufacturer (Cell Signaling). In vitro AIP4 ubiquitination reactions were performed using a ubiquitin–protein conjugation kit as described by the manufacturer (BostonBiochem) using immunoprecipitated AIP4 and GST-JUNB as the substrate.

**RNA interference analysis**

siRNA transfections targeting human AIP4 were performed using double-stranded RNAs directed at sequences within the coding region and 3′ UTR (ON-TARGETplus SMART-pool, Thermo Scientific). An siRNA with a scrambled sequence was used as a negative targeting control. Statistical analysis was performed by using Student’s t test and ANOVA models using SigmaStat 4 (Aspire Software International).

**Results**

**AKT-dependent differential regulation of cyclin D1 and c-MYC promoter activity by rapamycin**

The lines used in this study have been described (12) and are isogenic lines which differ in their degrees of constitutive AKT activity. The glioblastoma U87-MG cell line contains a PTEN-null mutation and has heightened AKT activity. This line was stably transfected with a wild-type PTEN construct which markedly downregulated AKT activity (10). Similarly, the LAPC-4 prostate cancer cell line, which possesses relatively quiescent AKT activity, was stably transfected with a myristylated AKT allele (or empty vector control, LAPC-4puro). The differential AKT/mTOR cascade activation of these paired lines has been described (10, 12). We also utilized murine embryonic fibroblasts (MEF) in which the Pten gene had been disrupted (15). The Akt activity in these MEFs is markedly higher as compared with Pten+/− MEFs (15, 20). Our previous studies (12) demonstrated prominent differences in the steady-state mRNA levels of the cyclin D1 and c-MYC transcripts in these cell lines in response to mTOR inhibition. To study potential transcriptional contributions, we analyzed RNA Pol II promoter occupancy of these promoters via chromatin immunoprecipitation assays (21). As shown in Figure 1A and B, both promoters demonstrated reduced Pol II association in lines with elevated AKT activity (U87, LAPC-4myrAkt, Pten−/− MEFs), whereas those lines with relatively quiescent AKT activity (U87Pten, LAPC-4puro, Pten+/− MEFs) displayed increased Pol II content following rapamycin exposure. We also performed nuclear run-on analysis and observed differential alterations in nascent transcription of cyclin D1 and c-MYC following rapamycin exposure, consistent with the Pol II occupancy results (Fig. 1C). The relative steady-state abundances of several proteins in these lines, both prior to and following rapamycin treatment are shown in Figure 1D. As can be seen, rapamycin exposure led to inhibition of TORC1 activity in all 3 paired lines and did not significantly affect TORC2 activities, as determined via phospho-S473-AKT levels. This also demonstrated that the alterations in cyclin D1 and c-MYC transcription are not due to increased AKT activation as a result of de-repressed AKT in response to rapamycin via blockade of the TORC1/S6K/IRS1 feedback loop. In addition, the rapamycin induced AKT-dependent alterations in cyclin D1 and c-MYC promoter activities correlated with our previously observed effects on cell-cycle arrest in these lines following drug treatment (12). Lines harboring active AKT (U87, LAPC4myrAkt, Pten−/− MEF) were markedly hypersensitive to rapamycin resulting in G1 arrest (Fig. 1E) and concomitant inhibition of cyclin D1 and c-MYC promoter activity (Fig. 1A–C), as compared with their isogenic quiescent AKT-containing counterpart lines (U87Pten, LAPC4puro, Pten+/− MEF) which were relatively resistant and displayed increased promoter activities following exposure. These data demonstrate that the relative degree of AKT activity differentially regulates the state of cyclin D1 and c-MYC transcriptional activity in cells displaying AKT-dependent hypersensitivity to rapamycin.

**AP-1 sites in the cyclin D1 and c-MYC promoters are required for AKT-dependent effects on transcription**

To investigate these transcriptional responses we analyzed a series of promoter constructs shown in Figure 2. Towards this end, we performed luciferase reporter gene assays utilizing constructs under the control of either the human cyclin D1 or c-MYC promoter sequences. To avoid confounding effects of inhibiting cap-dependent translation of the reporter following rapamycin exposure, luciferase translation was driven via the p27Kip1 IRES sequences placed upstream of the luciferase open reading frame. This IRES promotes translation of downstream ORFs following mTOR inhibition irrespective of AKT activity (12, 13). As illustrated in Figure 2, transient transfection of U87 cells with either the full-length cyclin D1 or c-MYC constructs (−1,749wt and −2,052HBMwt, respectively) resulted in marked decreases in luciferase activities following rapamycin exposure in cells containing high levels of AKT activity. In U87Pten cells containing relatively quiescent AKT activity, these full-length constructs showed significant increases in luciferase activities following rapamycin treatment. Thus, these promoter constructs recapitulated the AKT-dependent responses of the native cyclin D1 and c-MYC promoters following mTOR inhibitor exposure (Fig. 1).

Progressive deletion of the cyclin D1 promoter revealed that an AKT-dependent rapamycin responsive element lies somewhere between −1,095 and −770 bp upstream of the transcription start site. Removal of this region abrogated the AKT-dependent response of cyclin D1 promoter activity to rapamycin in U87 and U87Pten cells. Similarly, deletion of
Figure 1. AKT-dependent alterations in cyclin D1 and c-MYC promoter activities following rapamycin exposure. A, cyclin D1 and (B) c-MYC AKT-dependent RNA polymerase II (PolII) association following rapamycin treatment. The indicated cell lines were treated without or with rapamycin and PolII occupancy determined. ChIP-quantitative PCR data are expressed as a ratio of cyclin D1 or c-MYC to tubulin. Mean ± SD are shown; n = 3. C, nascent transcription of cyclin D1 and c-MYC mRNAs from the indicated lines treated without or with rapamycin. Actin and pBlueScript KS probes are also shown. Band intensities were quantified by densitometry and fold differences relative to nontreated control values are shown in parentheses below. D, relative levels of the indicated proteins from the indicated cell lines treated without or with rapamycin. E, AKT-dependent effects on cell-cycle distributions of the indicated cell lines following rapamycin exposure. Mean ± SD are shown; n = 3.
a region between −1,846 and −1,256 bp upstream of the transcription start site within the c-MYC promoter resulted in a complete loss of AKT-dependent promoter activity in response to rapamycin in both cell lines. This demonstrated that a 325-bp fragment of the cyclin D1 and a 590-bp fragment of the c-MYC promoters were responsible for regulating the responses of these 2 promoters to rapamycin in an AKT-dependent manner.
Present within these promoter sequences were previously characterized AP-1 or AP-1–like binding sites, which we reasoned may play a role in the observed effects on promoter activity (22–24). Within the cyclin D1 promoter a single AP-1 site was mutated and within the c-MYC promoter 3 AP-1 sites were sequentially mutated (shown in red). These constructs were transiently transfected into U87 and U87PTEN cells and relative luciferase activity determined before and after rapamycin treatment. As shown, mutating the cyclin D1 AP-1 binding site (−954 bp) completely abolished the rapamycin-induced effects on promoter activity. Similarly, mutating all 3 AP-1 sites within the c-MYC promoter abrogated AKT-dependent promoter activity in response to rapamycin, while mutating either a single or 2 AP-1 sites resulted in partial responses. This suggested that AP-1 transcription factor binding was responsible for the AKT-dependent changes in cyclin D1 and c-MYC promoter activities following rapamycin exposure.

c-JUN and JUNB bind differentially to the cyclin D1 and c-MYC promoters in an AKT-dependent manner following rapamycin exposure

Within the cyclin D1 promoter, the AP-1 site has been previously demonstrated to bind protein complexes containing activated c-JUN which promoted cyclin D1 transcription (25). Furthermore, activation of promoter activity via this AP-1 site had been shown to be driven by ERK phosphorylation of c-JUN (26). Since, we had previously demonstrated a differential activation of ERK in our paired cell lines following rapamycin exposure (13), we initially investigated whether these site(s) within the cyclin D1 and c-MYC promoters differentially bound c-JUN in an AKT-dependent manner following rapamycin exposure. As shown in Figure 3A, EMSAs utilizing the AP-1 sites from either the cyclin D1 (left) or c-MYC (right) promoters and adjacent sequences did differentially bind c-JUN in nuclear extracts prepared from either U87 or U87PTEN cells following rapamycin exposure. c-JUN binding was only observed in extracts prepared from quiescent AKT-containing U87PTEN cells, whereas in U87 cells containing active AKT no c-JUN binding was detectable. Similar analyses interrogating other AP-1 family members (FRA-1, FRA-2, FOS A, FOS B, JUN D, JAB1) demonstrated significant differential binding only for JUNB. Binding of JUNB to the cyclin D1 promoter has also been associated with the repression of transcriptional activity (25) and we observed significant binding of JUNB only in active AKT-containing U87 cells to the cyclin D1 and c-MYC AP-1 sites (Fig. 3B; cyclin D1, left; c-MYC, right) where promoter activity was inhibited following rapamycin treatment. We validated this EMSA data by chromatin immunoprecipitation experiments, followed by quantitative PCR analysis (16). Following rapamycin exposure, the AP-1 sites within the cyclin D1 and c-MYC promoters bound c-JUN in cells with quiescent AKT (U87PTEN) and demonstrated no appreciable binding in cells with elevated AKT activity (U87; Fig. 3C, left and right top). In a reciprocal fashion, JUNB was found associated with these AP-1 sites exclusively in cells containing elevated AKT activity.

![Figure 3. AKT-dependent c-JUN and JUNB differential binding to the cyclin D1 and c-MYC promoters following rapamycin treatment.](https://example.com/figure3.png)

A, EMSA analysis using 32P-labeled cyclin D1 (left) or c-MYC (right) DNA probes containing native AP-1 binding sites were carried out using the indicated nuclear extracts in the absence or presence of control (IgG) or antibody against c-JUN. B, as in (A) except antibody to JUNB was used. Arrows indicate super-shifted species. Experiments were carried out 3 times with similar results.
relatively active AKT (U87) compared with cells containing quiescent AKT (U87\textsubscript{PTEN}; Fig. 3C, left and right, bottom). The phosphorylation states of functionally bound c-JUN and JUNB to the cyclin D1 and c-MYC AP-1 elements were also assessed in a series of \textit{in vitro} DNA binding pull-down assays (Fig. 3D). High levels of both unbound c-JUN and JUNB are present in nuclear extracts from both U87 and U87\textsubscript{PTEN} cells in the absence of rapamycin, however following rapamycin exposure markedly elevated levels of Ser\textsuperscript{63} phosphorylated c-JUN is associated with the cyclin D1 AP-1 promoter element linked to sepharose beads (D1-beads) in quiescent AKT-containing U87\textsubscript{PTEN} cells but not in active AKT-containing U87 cells (Fig. 3D, left). Consistent with the chromatin immunoprecipitation experiments, we observed high levels of JUNB bound to the cyclin D1 AP-1 promoter element linked beads, only from nuclear extracts from active AKT-containing U87 cells following rapamycin exposure. No detectable amount of Ser\textsuperscript{79} phosphorylated c-JUNB was found in U87 or U87\textsubscript{PTEN} nuclear extracts or associated with the cyclin D1 AP-1 element-linked beads irrespective of rapamycin exposure. Similar binding data were observed in DNA pull-down experiments using the AP-1 sites and flanking sequences from the c-MYC promoter (Fig. 3D, right; MYC-beads). Again, high levels of Ser\textsuperscript{63} phosphorylated c-JUNB were detected in nuclear extracts from active AKT-containing U87 cells following rapamycin exposure.

**Figure 3.** (Continued) C, extracts from U87 and U87\textsubscript{PTEN} cells untreated or treated with rapamycin were chromatin immunoprecipitated with the indicated antibodies (c-JUN, top; JUNB, bottom) and fragments were subjected to quantitative real-time PCR analysis using various primer sets spanning the promoter regions (cyclin D1, left; c-MYC, right) as indicated by the gray bars. Primer sets 1 correspond to the AP-1 binding site(s) in the cyclin D1 and c-MYC promoters. At least 2 independent experiments were performed and the mean ± SD are shown. D, sepharose beads were conjugated with cyclin D1 promoter element DNA (D1-beads, left) or c-MYC promoter element (MYC-beads, right) containing the native AP-1 binding site(s) or beads without linked DNA were incubated with nuclear extracts from U87 or U87\textsubscript{PTEN} cells treated without or with rapamycin as indicated. Following recovery by centrifugation and washing of the beads, bound material was analyzed by immunoblot for the indicated proteins. These experiments were performed twice with similar results.
c-JUN bound AP-1 promoter linked beads in quiescent U87PTEN extracts following rapamycin exposure, whereas JUNB specifically associated with AP-1 promoter linked beads in active AKT-containing U87 extracts under similar conditions. We further confirmed that JUNB was not significantly phosphorylated at other residues via phosphatase treatment of nuclear extracts from U87 and U87PTEN cells before and after rapamycin exposure and found no significant electrophoretic mobility changes following in vitro phosphatase treatment (not shown). In addition, we examined whether heterodimers containing c-JUN and JUNB were present following rapamycin exposure. Nuclear extracts from both cell line pairs, prior to and following rapamycin treatment, were coimmunoprecipitated using either c-JUN or JUNB antibodies followed by immunoblot analysis. As shown in supplementary Figure 1A, we were able to detect modest amounts of heterodimer formation prior to treatment in all lines, which increased in lines containing active AKT (U87, LAPC-4myrAKT) following rapamycin exposure. However, we were unable to detect stable co-complexes in lines with quiescent AKT activity (U87PTEN, LAPC-4puro) following treatment consistent with the absence of JUNB under these conditions (see Discussion). We also determined whether the differential binding of phosphorylated c-JUN to the AP-1 sites could be correlated with differential activation of JNK. Shown in Supplementary Figures 1B and 1C, JNK activity was determined by in vitro kinase analysis and was induced similarly following exposure to rapamycin in U87, U87PTEN, LAPC-4myrAKT, and LAPC-4puro cells. This was consistent with the findings of others (27) and suggested that additional mechanisms mediated the differential transcriptional responses to rapamycin. These results taken together demonstrate that c-JUN and JUNB differentially bind to AP-1 sites present in the cyclin D1 and c-MYC promoters in an AKT-dependent manner following rapamycin treatment.

**JUNB governs cyclin D1 and c-MYC promoter activities in an AKT-dependent manner in response to rapamycin**

In our in vitro DNA pull-down experiments we noted a relatively large change in JUNB abundance following rapamycin exposure as compared with c-JUN levels (see Fig. 3D) suggesting that the absolute levels or activity of JUNB may play a role in regulating AKT-dependent cyclin D1 and c-MYC promoter activity. To test this hypothesis, we initially determined whether forced expression of JUNB or activation of a
JunB-ER conditional allele would alter the AKT-dependent cyclin D1 and c-MYC promoter activities previously observed. U87 and LAPC-4 cell line pairs, which stably overexpressed JUNB (Supplementary Fig. 2A, left) were treated with rapamycin and promoter activities determined via ChIP assays as before. As shown in Figure 4A, quiescent AKT-containing U87_{PTEN} and LAPC-4_{puro}, cells overexpressing JUNB demonstrated significantly lower levels of cyclin D1 (left) and c-MYC (right) promoter activities as compared with controls. Similar effects were observed in U87 and LAPC-4 paired lines which expressed comparable levels of a conditionally active JunB-ER protein fusion (Supplementary Fig. 2A, right; ref. 25). As shown in Figure 4B, both in control and nonactivated JunB-ER expressing cells, rapamycin exposure led to an increase in cyclin D1 (left) and c-MYC (right) promoter activity only in quiescent AKT-containing U87_{PTEN} and LAPC-4_{puro} cells. However, in U87_{PTEN} and LAPC-4_{puro} cells expressing the JunB-ER fusion protein activated by the ligand hydroxytamoxifen, rapamycin-induced cyclin D1 and c-MYC promoter activity was markedly reduced. We also attempted to determine whether JUNB was required for the inhibition of cyclin D1 and c-MYC promoter activity following rapamycin exposure lines containing elevated levels of active AKT. We employed an siRNA strategy utilizing JUNB targeting siRNAs to specifically inhibit expression (Supplementary Fig. 2B). As shown in Figure 4C. JUNB siRNA-treated cells displayed a significant derepression of both cyclin D1 and c-MYC promoter activity in active AKT-containing cell lines. We also examined the degree to which modulation of JUNB expression affected rapamycin-induced G1 arrest in these cell lines. As shown in Figure 4D, both the U87 and LAPC-4 cell line pairs that stably overexpressed JUNB demonstrated increased G1 arrest of quiescent AKT-containing cells (U87_{PTEN}, LAPC-4_{puro}) following rapamycin exposure as compared with controls. In a reciprocal fashion, JUNB siRNA-treated U87 and LAPC-4 lines which contained high levels of active AKT (U87, LAPC-4_{myrAKT}) displayed reduced G1 arrest following rapamycin treatment (Fig. 4E). These data taken together, demonstrate that modulation of JUNB profoundly effects rapamycin-induced alterations in cyclin D1 and c-MYC promoter activities in an AKT-dependent manner.
JUNB is posttranslationally regulated via AIP4/Itch ubiquitin ligase activity in an AKT-dependent manner following rapamycin exposure

To gain further insight as to how AKT-dependent JUNB expression might be regulated following rapamycin exposure we examined whether JUNB may be subject to differential ubiquitin mediated proteosomal degradation. JUNB expression is known to be regulated posttranslationally via this mechanism (28). The U87 and LAPC-4 cell line pairs were transfected with a HA-tagged ubiquitin construct and treated with rapamycin as indicated in Figure 5A. Extracts were prepared, immunoprecipitated with JUNB antibodies and subjected to immunoblot analysis using anti-HA sera. As can be seen, increased endogenous JUNB ubiquitination was observed in quiescent AKT-containing U87PTEN and LAPC-4puro cells following rapamycin exposure in these ubiquination assays relative to their active AKT-containing isogenic counterpart lines. We also assessed the affects of the proteasome inhibitor MG132 in both paired lines following rapamycin exposure. MG132 blocked the rapamycin-induced decrease in JUNB levels in quiescent AKT-containing U87PTEN and LAPC-4puro cells (see Supplementary Fig. 3). JUNB ubiquitin (Ub)-mediated turnover has been demonstrated to be induced via the JNK-dependent E3 ligase AIP4/Itch (29). This prompted us to examine whether AIP4 activity was involved in this regulation. We initially examined the effects of rapamycin exposure on AIP4 expression in the paired cell lines. As shown in Figure 5B, a time-dependent increase in AIP4 protein levels was observed following rapamycin treatment irrespective of AKT activity. JUNB levels increased in active AKT-containing U87 and LAPC-4myrAKT cells, whereas displayed reductions in protein amounts in quiescent AKT-containing U87PTEN and LAPC-4puro lines, consistent with the large alterations in JUNB levels previously observed in the in vitro DNA-pull down assays following rapamycin exposure (see Fig. 3D). Increases in phosphorylated c-JUN were observed in all lines correlating with increased JNK activity following rapamycin treatment (see Supplementary Fig. 1B) whereas total c-JUN levels did not appreciably change. We then examined whether rapamycin exposure resulted in alterations of AIP4 activity in extracts prepared from the cell line pairs. AIP4 immune
complexes were used in *in vitro* ubiquitination assays from the indicated cells lines and rapamycin treatments using GST-JUNB as a substrate. As shown in Figure 5C, AIP4 activity was significantly increased in both quiescent AKT-containing lines, U87<sub>PTEN</sub> and LAPC-4<sub>puro</sub> relative to their active AKT-containing counterpart lines following rapamycin treatment. These results demonstrate a differential AKT-dependent increase in ubiquitin-conjugation of JUNB and AIP4 activity in response to rapamycin.

AIP4/Itch knockdown abrogates rapamycin-induced AKT-dependent cyclin D1 and c-MYC promoter regulation

Our results demonstrating AKT-dependent differential AIP4 activity and JUNB ubiquitination motivated us to determine whether knockdown of AIP4 would have effects on cyclin D1 and c-MYC promoter activity in the paired cell lines upon mTOR inhibition. As shown in Figure 6C, treatment of lines with siRNAs targeting AIP4 resulted in inhibition of

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Knockdown of AIP4 abrogates AKT-dependent cyclin D1 and c-MYC promoter activity following mTOR inhibition. A, U87, U87<sub>PTEN</sub>, LAPC-4<sub>myrAKT</sub>, or LAPC-4<sub>puro</sub> cells were treated with siRNA targeting AIP4 or a scrambled (scr) nontargeting control siRNA in the presence of rapamycin and extracts immunoblotted for the indicated proteins. B, cyclin D1 (left) and c-MYC (right) promoter Pol II occupancy in the paired cell lines (U87 and U87<sub>PTEN</sub>, shaded bars; LAPC-4<sub>myrAKT</sub> and LAPC-4<sub>puro</sub>, open bars) following treatment with the indicated siRNAs and rapamycin. Mean ± SD are shown; n = 3. C, S-phase cell-cycle analysis of AIP4 siRNA-treated treated cell lines in response to rapamycin exposure. Mean ± SD; n = 3.
AIP4 expression to below detectable levels whereas actin levels remained unchanged. Control treatments or treating cells with a nontargeting scrambled siRNA did not effect AIP4 or actin expression. We then treated the lines with the indicated siRNAs, as shown in Figure 6B, and determined the effects of AIP4 knockdown on cyclin D1 (left) and c-MYC (right) PolII association following rapamycin exposure as before (see Figs. 1 and 4). In control and nontargeting scrambled siRNA–treated cultures, both the U87 and LAPC-4 cell line pairs displayed the AKT-dependent alterations in cyclin D1 and c-MYC promoter activity previously observed however, in cells in which AIP4 expression was silenced, rapamycin induced cyclin D1 and c-MYC PolII association was markedly reduced in quiescent AKT-containing cells (U87PTEN, LAPC-4puro). In addition, knockdown of AIP4 also resulted in a marked increase in G1 arrest of quiescent AKT-containing lines (U87PTEN and LAPC-4puro) as compared with control or nontargeting siRNA–treated cells in response to rapamycin.

Figure 7. JNK activity is required for differential AKT-dependent AIP4 activity, cyclin D1 and c-MYC transcriptional responses, and G1 arrest in response to rapamycin. A, effects of JNK inhibitor (JNKi VIII) on in vitro AIP4 activity. The paired cell lines were treated with rapamycin and JNKi VIII (4 μmol/L) as indicated and AIP4 immune complexes used in in vitro ubiquitination reactions as in Figure 5 (C). B, cyclin D1 (left) and c-MYC (right) PolII association in the cell lines (U87 and U87PTEN, shaded bars; LAPC-4myrAKT and LAPC-4puro, open bars) following treatments with JNKi VIII (4 μmol/L) and rapamycin as indicated. Mean ± SD are shown; n = 3. C, cyclin D1 and c-MYC PolII occupancy in U87, U87PTEN, LAPC-4myrAKT, and LAPC-4puro cells treated with nontargeting scrambled (scr) or JNK siRNA as indicated in the presence of rapamycin (U87 and U87PTEN, shaded bars; LAPC-4myrAKT and LAPC-4puro, open bars). Mean ± SD are shown; n = 3.
These data demonstrate that AIP4 is required for the AKT-dependent effects on cyclin D1 and c-MYC promoter activity and G1 arrest following rapamycin exposure.

JNK activity is required for AIP4-mediated JUNB ubiquination and stimulation of cyclin D1 and c-MYC promoter activity in cells with quiescent AKT activity following rapamycin exposure.

As AIP4/JUNB signaling is known to be regulated by JNK (29), we were curious as to whether pharmacological inhibition of JNK activity would lead to dramatic effects on AKT-dependent cyclin D1 and c-MYC transcriptional responses following mTOR inhibition. To inhibit JNK activity we utilized the selective pyridinylamide inhibitor (referred to as JNKi VIII; ref. 30), which effectively inhibited activity in both cell line pairs (see Supplementary Fig. 1B). Moreover, exposure to JNKi VIII did not affect AKT activity in either cell line pair as determined by phospho-serine473 levels (see Supplementary Fig. 4A). We subsequently assessed in vitro AIP4 activity in extracts of cells treated without or with JNKi VIII following exposure to rapamycin as before (see Fig. 5C). As shown in Figure 7A,

![Figure 7. (Continued) D, S-phase cell-cycle analysis of paired cell lines treated with nontargeting (scr) or JNK siRNAs in the presence of rapamycin. Mean ± SD; n = 3. E, regulation of cyclin D1 and c-MYC transcription by AP-1 in response to rapamycin.](image)

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In this model, rapamycin-FKBP12 inactivation of TORC1 results in sustained activation of the JNK cascade via increased ASK1 activity (27, 52). This leads to AIP4-mediated JUNB ubiquitination and degradation in quiescent AKT-containing cells and is inhibited in tumor cells whose AKT activity is elevated. As a result, accumulated JUNB and c-JUN-JUNB heterodimers bind to AP-1 elements within the cyclin D1 and c-MYC promoters and inhibit transcription. In contrast, in cells with relatively quiescent AKT, rapamycin exposure results in activation of JNK/AIP4 signaling, degradation of JUNB and stimulation of cyclin D1 and c-MYC promoters via c-JUN.
JNK inhibition resulted in no significant increase in the levels of detectable ubiquinated JUNB in both the quiescent AKT-containing lines U87PTEN and LAPC-4_act relative to their active AKT-containing matched lines. We then pre-treated both cell line pairs with JNKi VIII and the determined the effects on cyclin D1 and c-MYC promoter Pol II association following rapamycin exposure as before (see Figs. 1 and 6). As shown in Figure 7B, exposure of both paired lines to rapamycin resulted in reduced Pol II occupancy of cyclin D1 (left) and c-MYC (right) promoters in U87 and LAPC-4(myrAKT) cells while increasing Pol II occupancy of these promoters in U87PTEN and LAPC-4(puro) cells as previously observed. However, when the cell line pairs were treated in combination with both JNKi VIII and rapamycin, both the cyclin D1 and c-MYC promoters displayed reduced Pol II association irrespective of AKT activity. We also examined the effects of JNK knockdown on the cell-cycle distributions of the paired cells following rapamycin exposure. As shown in Figure 7D, siRNAs targeting JNK effectively inhibited its expression (see Supplementary Fig. 4B) and resulted in significant increases in G1 arrest following rapamycin treatment in quiescent AKT-containing cell lines (U87PTEN and LAPC-4(myrAKT)) as compared with controls. These data suggest that JNK activity is required for the differential AKT-dependent effects on cyclin D1 and c-MYC promoter activity and relative cell resistance following rapamycin exposure.

Discussion

We previously demonstrated that the AKT-dependent control of cyclin D1 and c-MYC mRNA translational efficiency and stability regulate the responses of these 2 critical cell-cycle determinants to rapamycin (13, 14, 31). Here, we have expanded on these studies and demonstrated that AKT-dependent transcriptional regulation contributes to the coordinate responses of these genes to rapamycin. The differential promoter activities appear to be regulated by distinct AP-1 family member binding within the specific contexts of the cyclin D1 and c-MYC promoters. Moreover, our data demonstrate that posttranslational regulation of JUNB plays a major role in this response and suggests that AKT activity may directly or indirectly regulate AIP4-mediated JUNB degradation. Our data are consistent with a model in which rapamycin-induced stress leads to activation of JNK-mediated phosphorylation of c-JUN (27), while concomitantly JNK phosphorylation of AIP4 and subsequent ubiquination of JUNB is inhibited via AKT function in cells containing elevated levels of active AKT. However, in cells with relatively quiescent AKT activity, following rapamycin-induced activation of JNK, AIP4 activity leads to rapid degradation of JUNB. The relative ratio of phosphorylated c-JUN to JUNB bound to either the cyclin D1 or c-MYC promoters functions as a rheostat leading to either transcriptional repression or activation resulting in G1 arrest or progression, respectively (see Fig. 7E).

Many studies have reported on the ability of AKT signaling to positively influence promoter activities (32). PI3K/AKT signaling is known to regulate MYC-mediated transcription via posttranslational effects on the negative regulator MAD1 by promoting its degradation (33). AKT activity also regulates basic-loop-helix transcription factor–cofactor complex formation to promote transcriptional activity (34). In addition, AKT activity stimulates CREB activity (35) and contributes to IkB degradation resulting in NFkB induction (36). However, there are also examples wherein AKT signaling has been reported to negatively regulate transcription. For example, Forkhead transcription factors are negatively regulated by phosphorylation via AKT (37) and Mdm2 phosphorylation by AKT promotes increases ubiquitination of p53 leading to reductions in p53-mediated transcriptional responses (38). In addition, following myostatin treatment of skeletal muscle cells, cyclin D1 expression is silenced via effects on p300 through AKT signaling (39). Our data demonstrates the ability of AKT to regulate transcription of the cyclin D1 and c-MYC promoters following rapamycin exposure and illustrates how the same, or similar stimuli, can lead to differential transcriptional responses. To our knowledge, our work is also the first to demonstrate that JUNB binds to the c-MYC promoter and results in inhibition of its promoter activity and collectively provides another example of a physiologically relevant antagonism between c-JUN and JUNB (40).

The observation that AKT regulates E3 ligase activity is supported by several studies (41–43), yet how it may control AIP4-mediated JUNB degradation is unclear. It is possible that AKT indirectly controls an effector of AIP4 to govern overall ligase function. Alternatively, AKT may phosphorylate AIP4 to influence activity or regulate ubiquitin-dependent AIP4 stability (44). It is interesting to note that an AKT consensus phosphorylation site is predicted in AIP4 under conditions of limited stringency using ScanSite (45), and experiments to test whether a physiologically relevant phosphorylation event in this regard may regulate AIP4 activity are underway. It is of interest that Panner et al. (42), reported a PTEN/AKT/AIP4 signaling cascade which regulates FLIP protein stability in glioblastomas. Under conditions of quiescent AKT activity, AIP4, although found in a highly ubiquinated form, correlated with high levels of ubiquinated FLIP, and decreased overall protein stability. However, in cells with elevated levels of active AKT, FLIP, ubiquination was reduced and increased steady-state expression was observed. Our data support these findings in that increased AKT activity was found to correlate with decreased JUNB ubiquitination (see Fig. 5A) and suggests that AKT negatively regulates AIP4. AKT may also regulate a deubiquitination process so as to exert its effects on JUNB stability. More recently, Panner et al. (46) have reported that the deubiquinase USP8 may link AKT activity to downstream AIP4 function in the regulation of FLIP, protein stability.

We observed that AIP4 expression was induced following rapamycin exposure irrespective of PTEN or AKT status, yet AIP4 activity was blunted in cells containing active AKT (see Fig. 5B and C). These data suggest AIP4 activity is regulated posttranslationally. MTOR is known to have
effects on the control of autophagy and rapamycin exposure induces this process in yeast, as well as in a variety of mammalian cell types (47). Recent data also provides evidence for a role in ubiquitin in the selective degradation of proteins in response to starvation (48). The increase in AIP4 expression would be consistent with the global upregulation of E3 ligase activity following rapamycin exposure and the induction of selective autophagy. We speculate that the induction of selective autophagy, as a consequence of mTOR inhibition, may be a result of rapamycin mimicking a starvation-like response in cells. It is possible that enzymes from both major classes of E3 ligases may play a role in such a response including AIP4.

Because c-JUN–JUNB heterodimer formation also contributes to AP-1–mediated transcriptional regulation (49), we examined whether heterodimers were present following rapamycin exposure. The observation that rapamycin significantly increased heterodimer formation in active AKT-containing cells, where cyclin D1 and c-MYC promoter activity is inhibited, is consistent with previous data demonstrating that JUNB can attenuate trans-activation by c-JUN. Thus, it is likely that heterodimer formation of these transcriptional regulators contributes to the differential regulation of cyclin D1 and c-MYC transcription in an AKT-dependent manner in response to rapamycin. We also observed that rapamycin-induced significant accumulation of phosphorylated c-JUN independent of AKT activity, which via a positive autoregulatory mechanism acting on its own promoter (50), would be predicted to result in elevated levels total c-JUN. However, c-JUN levels remained relatively constant following rapamycin exposure (Fig. 5B). This suggests that additional regulatory mechanisms independent of the autoregulatory circuit result in the overall unchanged levels of total c-JUN.

Our data support the notion that tumors cells coordinately regulate cyclin D1 and c-MYC expression in an AKT-dependent manner, at both the transcriptional and posttranscriptional levels, as an intrinsic mechanism of resistance to mTOR inhibitors. Several genes are known to exhibit similar coordinately regulated gene expression programs in response to various stimuli with alterations occurring in transcription, mRNA stability and translation (51). To our knowledge, this is the first report showing that mTOR inhibitors have marked effects on AIP4.

An increased understanding of the mechanistic details regulating the AKT-dependent hypersensitivity of tumor cells to mTOR inhibitors may lead to the identification of additional therapeutically amenable targets whose modulation may have synergistic antitumor effects with these inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Anil Ruangli, Linda Penn, Latifa Bakirti, Mohos Yaniv, Ted Dawson, and Edward Yeh for the reagents. We also thank Drs. Robert Nishimura and Alan Lichtenstein for comments on the manuscript and Johnathan Lee for technical assistance. We are grateful to Ardella Sherwood for excellent administrative assistance.

Grant Support

This work was supported, in whole or in part, by VA MERIT and NIH R01CA109312 grants. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 12, 2010; revised September 10, 2010; accepted December 01, 2010; published OnlineFirst December 6, 2010.

References


Molecular Cancer Research

AP-1 Regulates Cyclin D1 and c-MYC Transcription in an AKT-Dependent Manner in Response to mTOR Inhibition: Role of AIP4/Itch-Mediated JUNB Degradation

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