Cyclin D Expression Is Controlled Post-transcriptionally via a Phosphatidylinositol 3-Kinase/Akt-dependent Pathway*

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Cyclin D expression is regulated by growth factors and is necessary for the induction of mitogenesis. Herbbimycin A, a drug that binds to Hsp90, induces the destruction of tyrosine kinases and causes the down-regulation of cyclin D and an Rb-dependent growth arrest in the G1 phase of the cell cycle. We find that the induction of D-cyclin expression by serum and its repression by herbimycin A are regulated at the level of mRNA translation. Induction of cyclin D by serum occurs prior to the induction of its mRNA and does not require transcription. Herbimycin A repression is characterized by a decrease in the synthetic rate of D-cyclins prior to changes in mRNA expression and in the absence of changes in the half-life of the protein. This effect on D-cyclin translation is mediated via a phosphatidylinositol 3-kinase (PI 3-kinase)-dependent pathway. PI 3-kinase inhibitors such as wortmannin and LY294002, and rapamycin, an inhibitor of FRAP/TOR, cause a decline in the level of D-cyclins, whereas inhibitors of mitogen-activated protein kinase kinase and farnesyltransferase do not. Cells expressing the activated, myristoylated form of Akt kinase, a target of PI 3-kinase, are refractory to the effects of herbimycin A or serum starvation on D-cyclin expression. These data suggest that serum induction of cyclin D expression results from enhanced translation of its mRNA and that this results from activation of a pathway that is dependent upon PI 3-kinase and Akt kinase.

Growth factors elicit their biological effects by activating a complex network of receptors and signaling pathways. Activation of transmembrane tyrosine kinases by serum or polypeptide growth factors results in the transit of cells through the G1 phase of the cell cycle into S-phase. Several lines of evidence suggest that the D-type cyclins and their associated kinases (Cdkks) are among the targets of these growth signals (1). The D-type cyclins, D1, D2, and D3, are closely related proteins whose expression is induced by mitogens and growth factors (2–6) and down-regulated by growth factor deprivation or by antimitogens (7, 8). The D-type cyclins associate with cyclin-dependent protein kinase Cdk4 or Cdk6 to form an active complex that phosphorylates and inactivates the retinoblastoma protein, pRb (9, 10). Inhibition of cyclin D1 expression either by antisense methodology or antibody microinjection lengthens the duration of the G1 phase and causes a reduction in proliferation (11, 12). Aberrant overexpression of D-type cyclins resulting from upstream growth factor receptor activation, gene amplification or rearrangement, or an increase in mRNA stability seems to be a common feature of a number of human cancers and may reduce the cell’s dependence on physiologic growth stimuli (13–16).

Changes in cyclin D expression integrate the proliferative signals of an array of extracellular factors, including cytokines, polypeptide growth factors, and steroid hormones (2–4, 7). Cellular stress results in the loss of cyclin D1 expression, with a concomitant arrest in the G1 phase of the cell cycle (8, 17). The networks of pathways responsible for the transduction of these signals are complex and not completely understood. There is some evidence suggesting that a Ras- and MAP kinase-dependent signaling pathway is involved. Expression of activated Ras is associated with the increased expression of cyclin D1 in both epithelial cells (12) and fibroblasts (11). Moreover, in the absence of growth factors, activation of the Raf1 → MEK → MAP kinase pathway has been shown to be sufficient to induce cyclin D1 transcription (5). Herbbimycin A is a natural product that binds to a specific site in Hsp90 and causes the degradation of transmembrane tyrosine protein kinases, Raf1, and steroid hormone receptors (18–23). We found that treatment of tumor cells with this drug causes a decrease in the expression of D-type cyclins and an Rb-dependent G1 block.2

We report here that the reduction in the level of D-type cyclins induced by herbbimycin A is due to inhibition of translation of cyclin D mRNAs. Furthermore, the increase in the level of D-type cyclins in cells treated with serum is due to an increase in the translation of their mRNAs. These effects are due to the regulation of a PI 3-kinase/Akt kinase-dependent, Raf1- and MAP kinase-independent pathway. This pathway is activated by serum and is blocked by the drug herbbimycin A.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Colo205, a human colon carcinoma cell line, and MCF7, a breast cancer cell line, were obtained from ATCC, and maintained in RPMI or DMEM-F12, respectively, supplemented with 8% fetal calf serum (Life Technologies, Inc.), 2 mM glutamine, and 50 units/ml each penicillin and streptomycin. MCF10A, a nontransformed human mammary epithelial cell line obtained from Dr. J. Mendelsohn (Memorial Sloan-Kettering Cancer Center, New York, NY), was maintained in DMEM-F12 containing 5% donor horse serum, glutamine, and penicillin and streptomycin as above, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin (Collaborative Biomedical Science), 20 ng/ml EGF (Coll-
laborative Biomedical Science), and 1 μg/ml amphotericin (Sigma). MCF10A were serum-starved in DMEM-F12 supplemented with penicillin and streptomycin as above plus 0.1% donor horse serum for 48 h. The cells were stimulated for the times indicated in the text with the media described above. LDL1 tetracycline-repressible cells were maintained in DMEM/F12 supplemented with 1% fetal calf serum, 40 μg/ml G418, 2.5 μg/ml puromycin, 2.0 μg/ml tetracycline, 2 mM glutamine, and 50 units/ml each penicillin and streptomycin. Herbinycin A (Life Technologies, Inc.) was used at a concentration of 500 μg/ml or as indicated in the text. Farnesyltransferase inhibitor L-744,832 was a gift from Drs. N. Kohl and A. Oliff at Merck (West Point, PA) and was used at a concentration of 20 μM. Rapamycin and Wortmannin (Sigma) were used at a concentration of 250 or 200 nM, respectively.

Western Blot Analysis—Treated cells were washed once with phosphate-buffered saline and lysed in Nonident P-40 Lysis Buffer (50 mM Tris, pH 7.5, 1% Nonident P-40, 150 mM NaCl, 2.5 mM Na3VO4, 10 mM phenylmethylsulfonyl fluoride, and 10 μM each of leupeptin, aprotinin, and soybean trypsin inhibitor) for 10 min on ice and centrifuged at 15,000 × g for 10 min. Protein concentrations were determined by a BCA protein assay reagent using the instructions provided by the manufacturer (Pierce).

Equal amounts of total protein were resolved on 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell) by electroblotting. Blots were blocked in Blotto (5% nonfat dry milk in TS (150 mM NaCl, 10 mM Tris, pH 7.4)) plus 0.1% Tween 20, and probed with rabbit polyclonal antibodies directed against cyclin D1 or D3, or Cdk2 (Santa Cruz Technologies) overnight at 4 °C. After washing twice for 10 min in TBS plus 0.1% Tween 20, a horseradish peroxidase-conjugated anti-rabbit secondary antibody was applied and incubated for 1 h at room temperature, and the blots washed twice for 10 min with TBS plus 0.1% Tween 20 and developed using ECL Reagent (Amersham Pharmacia Biotech).

32P-Pulse Labeling and Immunoprecipitation—For pulse-labeling experiments, 4 × 106 cells/100-mm plate were pre-incubated in RPMI without cysteine or methionine supplemented with 5% dialyzed fetal calf serum for 20 min and pulse-labeled for the indicated times by addition of 250 μCi of Promix (Promega). The cells were stimulated for the times (in hours) as indicated under each lane. The (0) lane indicates the amount of cyclin D1 or cyclin D3 in proliferating cells. The cell lines used are shown to the left of the figure. B. RNase protection analysis of cyclin D1 and D3. The time of herbinycin A treatment is listed above each lane. For each cell line, as shown to the left of the figure, the resultant hybridization pattern of cyclin D1 or D3 including the internal control (36B4) is shown. C. Quantification of D-cyclin protein and RNA levels. mRNA levels are standardized against the loading control. Both RNA and protein levels are expressed as relative to the level of expression in untreated proliferating cells (lane 0).

Rnase Protection Analysis—Total RNA was isolated using a RNA-sol B RNA isolation kit by procedures supplied by the manufacturer (Biozym). A 440-bp EcoRI/PstI fragment encompassing sequences +1 to +440 of the human cyclin D1 cDNA (24), a 546-bp PstI fragment encompassing sequences +248 to +794 of the human cyclin D3 cDNA (25) both provided by Dr. Y. Xiong (Chapel Hill, NC), as well as a 139-bp PstIEcoRV fragment of 36B4, a ribosomal protein subunit whose expression does not vary with the cell cycle (26), were subcloned into Bluescript KS+ (Stratagene) by standard procedures (27). The D1 and D3 subclones were linearized with EcoRI, and 36B4 subclone was linearized with PvuII. The linearized glyceraldehyde-3-phosphate dehydrogenase probe was a gift from Dr. D. Hochhauser (Memorial Sloan-Kettering Cancer Center). 32P-Labeled antisense probes were synthesized according to the manufacturer (Promega). The specific activity of the 36B4 internal control was adjusted by increasing the amount of cold nucleotide and was empirically determined to be within the linear range of the assay. Rnase protection analysis was performed as described previously (16). Briefly, 5 × 106 cpm of each probe was hybridized to 30 μg of total RNA overnight at 50 °C. Hybridization reactions were digested with a combination of Rnase A (U. S. Biochemical Corp.) and Rnase T1 (Life Technologies, Inc.). After ethanol precipitation, the RNase protection analysis of cyclin D1 or D3 in proliferating cells. The cell lines used are shown to the left of the figure. The result of hybridization pattern of cyclin D1 or D3 including the internal control (36B4) is shown. C. Quantification of D-cyclin protein and RNA levels. mRNA levels are standardized against the loading control. Both RNA and protein levels are expressed as relative to the level of expression in untreated proliferating cells (lane 0).

Viral Infection and Akt Kinase Assays—Hemagglutinin (HA)-tagged c-Akt and 32P-Akt were cloned into the retroviral vector pMV12-SRα. Retroviral vectors were propagated by transient co-transfection into COS cells with a packaging plasmid. Retroviral infections were performed by treating MCF7 cell monolayers with 40 μg/ml DEAE-dextran for 1 h and then incubating with viral stocks overnight. G418 was added to 500 μg/ml, and resistant colonies were pooled.

Akt kinase assays were performed as described previously (28). Briefly, total cellular lysate, treated as described in the text, was immunoprecipitated with an anti-HA antibody (Babco) and used in a kinase assay with histone H2B as the exogenous substrate.

The tetracycline system plasmids pUHD10–3–neo and pUHD10–3 as described (29) were used. HA-tagged Akt was cloned into the pUHD10–3 vector at the EcoRI site. LDL1 cells expressing the tTA gene (pUHD15–1 neo) were obtained from T. Papas (30) and were transfected with pUHD10–3/Akt plasmids and MSCPac (31) using LipofectAMINE reagent (Life Technologies, Inc.). Single colony clones of tet-inducible Akt were isolated following selection with 2.5 μg/ml puromycin (Sigma).

RESULTS

Effect of Herbinycin A on D-type Cyclin Steady State Protein and mRNA Levels—Herbinycin A caused a G1 arrest in mul-

FIG. 1. Effect of herbinycin A on D-type cyclin protein and RNA levels. A, representative Western blot analyses of cyclin D1 or D3 or PI 3-kinase (p85) protein expression in cells treated with drug for the times (in hours) as indicated under each lane. The (0) lane indicates the amount of cyclin D1 or cyclin D3 in proliferating cells. The cell lines used are shown to the left of the figure. B, RNase protection analysis of cyclin D1 and D3. The time of herbinycin A treatment is listed above each lane. For each cell line, as shown to the left of the figure, the resultant hybridization pattern of cyclin D1 or D3 including the internal control (36B4) is shown. C, quantification of D-cyclin protein and RNA levels. mRNA levels are standardized against the loading control. Both RNA and protein levels are expressed as relative to the level of expression in untreated proliferating cells (lane 0).
multiple cell lines, which was preceded by a decrease in the expression of cyclins D1 and D3 (data not shown and Fig. 1). Fig. 1A shows the effects of herbimycin A on D-cyclin and PI 3-kinase (p85) expression in two Rb-positive cell lines. Neither cell line expressed cyclin D2 protein or mRNA (data not shown). In MCF7, a breast carcinoma cell line, cyclin D1 and D3 levels were reduced to 30% and 50% of control levels, respectively, by 24 h of drug treatment. In the nontransformed mammary epithelial cell line, MCF10A, the decline in D-type cyclins occurred more rapidly: 50% reduction by 3 h of H A treatment. The p85 subunit of PI 3-kinase was unaffected by herbimycin A treatment in both cell lines.

To determine whether the decrease in cyclin D protein levels results from a decrease in mRNA levels, RNase protection analyses were performed. These results are shown in Fig. 1B. Neither cyclin D1 nor D3 mRNA levels were affected by herbimycin A treatment of MCF7 cells. In MCF10A cells, cyclin D1 mRNA levels were reduced to 50% of the control at 9–12 h of drug treatment; cyclin D3 mRNA levels were unchanged. Fig. 1C shows a summary of the quantitation of these results. In both cell lines tested, a reduction in D-type cyclin protein levels preceded the effect, if any, on the corresponding transcript. These results suggest that the primary effect of herbimycin A on cyclin D levels is at the post-transcriptional level.

**Herbimycin A Causes a Decrease in the Rate of Cyclin D3 Protein Synthesis**—Changes in steady state protein levels can be accomplished by alterations in either the rate of synthesis or in protein half-life. As shown in Fig. 2, treatment of MCF7 cells with herbimycin A specifically decreased the incorporation of labeled amino acids into cyclin D3. The drug reduced the rate of incorporation into cyclin D3 by approximately 50%, while isotope incorporation into total protein synthesis remained unaffected. Chase experiments were performed in MCF7 cells, following a 45-min pulse labeling (Fig. 3A, Control) and showed that the half-life of cyclin D3 was approximately 2 h (Fig. 3B). This half-life was unaffected when the chase media contained herbimycin A, or when the cells were pretreated with herbimycin A for 6 h and chased with herbimycin A-containing media (Fig. 3A). The pulse-chase experiment was repeated using a 10-min pulse, and the half-life was identical (data not shown). These results indicate that herbimycin A affects cyclin D3 expression by reducing its rate of synthesis. Given that the commercially available antibodies against cyclin D1 were inadequate for immunoprecipitation, we were unable to confirm that herbimycin A also affected the synthesis of cyclin D1.

**Serum Induction of D-type Cyclins Is Controlled by an Increase in the Rate of Protein Synthesis**—These data suggest that herbimycin A affects D-cyclin levels by inhibiting the translation of their mRNAs. This could reflect a direct effect of herbimycin A on the translational machinery or result from...
inhibition of signaling pathways such as those initiated by tyrosine kinase activation. Herbimycin A inhibits tyrosine kinase signaling by inducing the degradation of TKs and of Raf1 (18, 20, 21). We therefore investigated the mechanism of serum induction of cyclin D expression. MCF10A is an immortalized, untransformed human mammary epithelial cell line, which requires growth factors and hormone supplements for proliferation and arrests in G1 when deprived of growth factors (32). As shown in Fig. 4A, addition of complete media to serum- and growth factor-starved MCF10A cells caused a 7-fold increase in the amount of cyclin D1 and a 3-fold increase in cyclin D3 proteins within 12 h. RNase protection analyses shown in Fig. 4B indicate that although the cyclin D1 message was increased almost 2-fold, this increase occurred at approximately 12 h, much later than the increase in protein level. The background hybridization due to the internal control is indicated by the asterisks. When compared with the internal control, cyclin D3 mRNA levels remained unchanged during the 12-h time period tested. Quantitation of these results is shown in Fig. 4C. To confirm the contribution of a post-transcriptional component to the induction of cyclin D protein levels, additional serum induction experiments were performed. The pulse-labeling experiment shown in Fig. 5A indicates that the synthesis of cyclin D3 was increased 13-fold over the control 6 h after addition of fresh media to serum-starved MCF10A cells. Treatment of these cells with actinomycin D had little effect on the induction of cyclin
D1 or D3 by serum, whereas the induction of c-Fos, which is transcriptionally controlled, was completely inhibited (Fig. 5B). Early induction of cyclin D1 was not affected by actinomycin D treatment. However, the level of expression was slightly reduced after 6 h of treatment as compared with the control. The magnitude of cyclin D3 induction was also slightly reduced in the presence of actinomycin D. Although these results do not preclude a transcriptional component, they confirm that the induction of cyclin D by serum results from a post-transcriptional mechanism.

**D-type Cyclin Levels Are Down-regulated in Response to Inhibition of the PI 3-Kinase Pathway**—Several pharmacological inhibitors were used to investigate the pathways responsible for serum regulation of cyclin D expression. Treatment of MCF7 cells with the MAP kinase kinase (MEK) inhibitor PD98059 for increasing amounts of time (33, 34) had no effect on cyclin D1 or D3 levels in proliferating cells (Fig. 6A), while under similar conditions, treatment of MCF7 cells with PD98059 for 12 h inhibited the activation of Erk1 and Erk2 by EGF (Fig. 6A2). Growth arrest induced by treatment with a farnesyltransferase inhibitor was also not associated with a down-regulation of D-cyclins (data not shown). This was true in multiple cell lines, including those with wild-type Ras such as MCF7 and Caco205 and in v-Ha-ras transfectants of MCF10A and NIH3T3 cells.

The effects of wortmannin and LY294002, inhibitors of PI 3-kinase, and rapamycin, an inhibitor of the PI 3-kinase-related protein TOR/FRAP, were also assessed (35–38). Growth factor activation of PI 3-kinase results in the activation of p70S6 kinase, a protein involved in the enhanced translation of a number of mRNAs (39, 40). As shown in Fig. 6B, treatment of MCF7 cells with either 250 nM rapamycin or 200 nM wortmannin, a concentration that specifically inhibits PI 3-kinase, or 50 nM LY294002 caused the down-regulation of both cyclin D1 and cyclin D3. Since rapamycin is immunosuppressive, we also tested the effect of rapamycin on a lymphoid cell line. Treatment of Jurkat cells with rapamycin caused the down-regulation of cyclin D3; however, cyclin D2 levels were unaffected (data not shown). Quantitation of these results is shown in Fig. 6C. Rapamycin, wortmannin, and LY294002 treatment down-regulated D-type cyclins to approximately 50% of the control by 5–8 h of treatment while the MEK inhibitor had no effect.

An Activated Form of the Akt Kinase Abrogates the Effects of Herbinycin A and Serum on D-type Cyclin Levels—The Akt kinase is a downstream target of PI 3-kinase and is activated by platelet-derived growth factor, EGF, insulin, and insulin-like growth factor (28, 41, 42). Activation of PI 3-kinase leads to the formation of phosphatidylinositol 3′-phosphates that bind to proteins containing pleckstrin homology domains, such as Akt, causing them to partition to the plasma membrane (43, 44). When c-Akt is engineered to contain an N-terminal Src myristoylation motif that confers targeting to the plasma membrane, it is constitutively activated (45). Viral expression constructs containing a HA-derived tagged wild-type (c-Akt) or an activated form of c-Akt, which carries the Src myristoylation signal (m-myAkt), were used to infect MCF7 cells. A Western blot analysis showing the expression of the HA-tagged proteins in the MCF7 infectants is shown in Fig. 7A. In cells expressing the neomycin control and those expressing c-Akt, both cyclin D1 and cyclin D3 were down-regulated in response to herbinycin A treatment. However, in the cells expressing the activated form of Akt (m-myAkt), the D-type cyclins were no longer affected by herbinycin A (Fig. 7B). The expression of Raf1, one of the direct targets of herbinycin A, was down-regulated by the drug in each of the cells, as was Akt. Akt kinase activity was elevated in cells expressing m-myAkt, and, although the level of Akt expression was reduced by approximately 50% after a 24-h treatment with herbinycin A (Fig. 7B), the kinase activity remained markedly elevated over the level in the neomycin- or c-Akt-expressing cells (Fig. 7C). These results indicate that expression of an activated form of Akt abrogates the effect of herbinycin A on D-type cyclins, but not on direct targets of the drug such as Raf1.

Wortmannin caused a down-regulation of the D-type cyclins in the cells expressing the empty vector and those expressing c-Akt, but not in the cells expressing m-myAkt (Fig. 7D). Thus, the activation of Akt by membrane targeting resulted in PI 3-kinase independence of cyclin D expression. In contrast, rapamycin, which is downstream of Akt, caused a down-regulation of the D-type cyclins in all three infectants.

To confirm that an activated form of Akt abrogates the effect of herbinycin A on cyclin D, colon carcinoma cells (DLD1) expressing a tetracycline-repressible m-myAkt were treated with herbinycin A in the presence and absence of tetracycline. As shown in the top of Fig. 7E, in the absence of tetracycline, HA-tagged m-myAkt was induced approximately 2-fold. Under
these conditions, herbimycin A had no effect on the level of cyclin D1. However, in the presence of tetracycline, herbimycin A still down-regulated cyclin D1 50% of the control level by 12 h of drug treatment. In these cells, herbimycin A did not have as pronounced an effect on the level of Akt as in MCF7 cells (data not shown). These results confirm that an ectopically expressed activated form of Akt, whether induced or constitutive, abrogates the effect of herbimycin A on cyclin D levels.

As shown in Fig. 8A, addition of serum to serum-deprived MCF7 cells expressing the empty vector caused a 2.5-fold increase in cyclin D levels but had no effect on the MCF7 cells expressing mmyAkt. Quantitation of these data is shown in Fig. 8B. Therefore, an activated form of Akt kinase caused cyclin D expression to become insensitive to serum stimulation and may overcome the need for serum factors for the maintenance of D-type cyclin levels.

**DISCUSSION**

The signals induced by extracellular growth factors regulate levels of expression of D-cyclins (2–6). We have shown that the ansamycin antibiotic herbimycin A induces a specific Rb-dependent G1 block, which is characterized by an early decline in the levels of D-cyclins in epithelial tumor cells. We sought to determine whether this decline is due to a direct effect of ansamycins on the half-life of D-cyclin proteins or to the degradation of a protein that transduces an upstream signal necessary for D-cyclin expression. We show that down-regulation of cyclin D1 and D3 proteins is neither secondary to changes in the levels of the mRNAs that encode the D cyclins nor to an increase in the rate of D-cyclin turnover (Figs. 1 and 3). We conclude that herbimycin A inhibits the translation of D-cyclin mRNA and that this drug may be affecting a member of an upstream signaling pathway.

Ansamycin antibiotics, which include herbimycin A and geldanamycin, were discovered on the basis of their ability to revert the malignant phenotype of v-src-transformed cells (46, 47). The direct target of these drugs seems to be the chaperone Hsp90. This chaperone contains a conserved, deep pocket that binds tightly to ansamycins (19). Occupancy of the pocket by...
FRAP/TOR activates the translation machinery in a discrete pathway or -independent manner, which affects the translation of cyclin D.

Signals transduced by activation of tyrosine kinases are direct targets of herbimycin A action, it seemed that the drug inhibits Hsp90-mediated refolding of proteins but not their translation or initial folding. Apparently, this phenomenon is a consequence of inhibition of the ATP-dependent release of the refolded protein from Hsp90 (23). The stabilized complex is then degraded. However, ansamycins induce the degradation of only a selected subset of cellular proteins. These include steroid receptors, the Raf1 kinase, v-Src, and certain transmembrane tyrosine kinases, notably members of the HER and IGF receptor families (18, 20–22).

Ansamycins could affect D-cyclin translation via a direct effect on the translational machinery or by interrupting an upstream regulatory pathway. In this regard, we showed that serum induction of D-cyclin protein expression precedes any effect on D-cyclin mRNA and, in addition, is actinomycin D-resistant (Figs. 4 and 5). Thus, it seems that serum activates and herbimycin A suppresses a pathway(s) required for D-cyclin translation. Activation of receptor tyrosine kinases has been shown to up-regulate D-cyclin expression, and this effect is thought to involve engagement of the Ras → Raf1 → MAP kinase cascade (5). As both receptor tyrosine kinases and Raf1 kinase are direct targets of herbimycin A action, it seemed likely that degradation of these targets was responsible for the action of the herbimycin A. However, whereas PI 3-kinase inhibitors and rapamycin were found to decrease D-cyclin expression, MEK inhibitors do not (Fig. 6). Furthermore, neither reversion of Ha-ras-transformed cells by farnesyltransferase inhibitors nor destruction of Raf1 by herbimycin A in cells expressing constitutively active Akt kinase is associated with a decrease in D-cyclin levels. These data suggest that, in these cells, D-cyclin expression is under the control of a PI 3-kinase-dependent pathway and is regulated independently of Raf1 and MAP kinase.

To confirm these results, we tested the effects of herbimycin A in cells in which an activated form of the Akt kinase is either induced or expressed constitutively. Akt kinase is a serine kinase that lies downstream of PI 3-kinase and is activated by phosphatidylinositol 3-phosphates (28, 41, 48, 49). Herbimycin A does not affect D-cyclins but still causes Raf1 degradation in cells expressing actively activated Akt kinase (Fig. 7). These results confirm that cyclin D translation is regulated by a PI 3-kinase/Akt-dependent pathway. Serum and growth factors stimulate this pathway, in part by engag-

The Ras → MAP kinase pathway has been shown in several systems to be responsible for the induction of D-cyclin transcription (5, 11, 12) and also for assembly of the active cyclin D-Cdk4 protein kinase complex (56). In the epithelial cell lines we examined, inhibition of Ras processing, MEK kinase activity, or Raf1 expression did not affect D-cyclin levels. In these systems, the Raf1 → MEK → MAP kinase pathway is not required for cyclin D-expression (Fig. 6). Whether this is generally true in epithelial cells or reflects activation of an alternative pathway in these carcinoma cells is unknown. However, a potential link between cyclin D expression and PI 3-kinase has been demonstrated in epithelial cells, as inhibition of PI 3-kinase by LY294002 or by inostamycin, a phosphatidylinositol synthesis inhibitor, leads to a decrease in cyclin D levels (57–59). We show a direct link between PI 3-kinase/Akt kinase activation and cyclin D expression, with a combination of inhibitors and by expression of an activated form of Akt (Figs. 6 and 7). The role of Ras is less clear. Farnesyltransferase inhibitors efficiently prevent the processing of Ha-Ras but not N- or Ki-Ras. Even so, reversion of Ha-ras-transformed cells by the drug is not associated with repression of D-cyclin expression.

The mechanism whereby Akt stimulates D-cyclin translation is not completely understood. It could directly regulate the translational apparatus, resulting in an increase in the efficiency of translation of D-cyclin message, or cause changes in the compartmentalization of D-cyclin mRNA. Mitogenic stimulation is associated with a generalized 2–3-fold increase in translation, while a group of transcripts are translated at much higher rates. The explanation of how Akt affects D-cyclin translation is likely to be more complex since G1 progression is also associated with decreased translation of certain inhibitors such as p27kip1 (60). The PI 3-kinase/Akt kinase pathway has been implicated in the growth factor-dependent phosphorylation of a number of key regulators of translation such as p70S6 kinase

3 J. Chow and J. Mendelsohn, personal communication.
Phosphorylation of PHAS-1 and p70S6 kinase are also activities that correlate with the increase in protein synthesis caused by growth factors such as insulin (42, 61, 62, 64). Phosphorylation of PHAS-1 and p70S6 kinase are also rapamycin-sensitive, implicating an additional need for the TOR/FRAP pathway in the activation of these two regulators of translation (65–67). Importantly, forced overexpression of eIF4E has been shown to cause an increase in cyclin D1 levels by increasing the cap binding protein eIF4E (64). Both eIF4E and p70S6 kinase are activities that correlate with the increase in protein synthesis caused by growth factors such as insulin (42, 61, 62, 64).

Akt kinase has been shown to be a potent regulator of cellular proliferation. v-akt encodes a mutant activated kinase and is an oncogene (70). Expression of activated Akt rescues G1 arrest, stimulating cell cycle progression in the absence of growth factors, in part by affecting the expression of c-Myc and Bcl-2 (43). It has also been reported that Akt activation leads to apoptosis via stimulation of PI 3-kinase and Akt kinase (43, 45, 72) possibly through the phosphatidylinositol-3 kinase sensitive, implicating an additional need for the TOR/FRAP pathway in the activation of these two regulators of translation (65–67). Importantly, forced overexpression of eIF4E has been shown to cause an increase in cyclin D1 mRNA (68, 69).

Activation of PT3 kinase and Akt by tyrosine kinases in tumor cells can therefore lead to both activation of cell cycle progression and a decrease in apoptosis via stimulation of PI 3-kinase and Akt kinase. Activation of PI 3-kinase by mutated Ras would also feed into this pathway. This is perhaps one explanation for the high frequency of mutational activation of Ras and tyrosine kinases, but not RafI and MAP kinase in human cancers. A variety of environmental stresses also lead to a specific down-regulation of D-type cyclins and G1 block (17). It is tempting to speculate that this results from engagement of an Hsp90-regulated pathway and is mimicked by hermaphroditic Akt binding to Hsp90. In many tumor cell lines, the ansamycins cause a striking reversion of transformation associated with G1 arrest and apoptosis. This phenomenon may reflect down-regulation of a key pathway for the maintenance of the malignant phenotype and could be the basis of a new set of therapeutic strategies.

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