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The AP-1 transcription factor is a central component of many signal transduction pathways. We have shown that blocking AP-1 by over-expressing a dominant negative form of cJun (cJun-DN, Tam67) inhibits breast cancer cell growth. We hypothesize that inhibition of AP-1 blocks the cell cycle, suppression of AP-1 in vivo inhibits the development of breast tumors, the cJun-DN protein binds and inactivates important growth regulatory proteins present in breast cancer cells, these proteins include Jun and Fos family members as well as coactivators that bind cJun. In the present study, we demonstrated that TAM67 inhibits breast cancer growth both in vitro and in vivo. We determined the mechanism by which AP-1 blockade inhibits breast cancer growth. Our studies suggested that TAM67 inhibits breast cancer growth predominantly by inducing CDK inhibitors (such as P27), suppressing G1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block. We also showed that TAM67 binds all Jun and Fos family members, andit inhibits breast cancer cell also by interacting with critical cJun partners, such as Fos and ATF/CREB families. Over the next year, I will identify which coactivators are critical for breast cancer cell growth.

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INTRODUCTION

The AP-1 transcription factor is a central component of signal transduction pathways in many cells. The AP-1 family consists of multiple Jun (cJun, JunB, and JunD), Fos (cFos, FosB, Fra-1, and Fra-2), ATF/CREB (ATF1, ATF2, ATF3, CREB1, CREB2) family members and Jun dimerization partners (JDP1 and JDP2). It was shown that AP-1 is involved in controlling cellular proliferation, differentiation, apoptosis, and oncogene-induced transformation. However, most of this work has been done in fibroblasts. Relatively few studies of the function of AP-1 have been performed in epithelial cells. Thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. We and others have demonstrated that Jun and Fos family members are variably expressed in human breast tumors, and AP-1 is activated by a variety of important growth factors such as EGF, IGFs, and estrogen. Recent studies found correlations between high phospho-cJun expression and decreased overall survival in breast cancer (1); and the expression of FosB correlated with ER-positivity while expression of Fra1 showed a strong negative correlation with ER positivity (2). AP-1 complexes may be involved in regulating transcription of the ER gene as well (3). These results indicated that AP-1 proteins might play a role in the pathogenesis and growth of breast tumors. In addition, AP-1 activity has been shown to increase when human breast cancers become resistant to tamoxifen (4,5). The cJun overexpresson in MCF 7 breast cancer cells produces a tumorigenic invasive and hormone resistant phenotype (6,7). All of these studies suggest that AP-1 transcription factors may also be critical for the growth of tamoxifen-resistant or drug-resistant breast cancer cells. To investigate the role of AP-1 in regulating breast cell growth, we have developed MCF-7 breast cancer cell lines that express an inducible cJun dominant-negative mutant (cJun-DN) under the control of the Tet-off system. This cJun dominant-negative mutant lacks the transactivation domain of cJun, yet retains its DNA-binding and dimerization domains (the "TransActivation domain Mutant", TAM-67). We have shown that the TAM67 inhibits AP-1 activity and suppresses breast cancer growth [8,9]. We hypothesize that inhibition of AP-1 blocks the cell cycle, suppression of AP-1 in vivo inhibits the development of breast tumors, the cJun-DN protein binds and inactivates important growth regulatory proteins present in breast cancer cells, these proteins include Jun and Fos family members as well as coactivators that bind cJun. In the present study, we first showed that TAM67 inhibited breast cancer cell growth both *in vitro* and *in vivo*. We then investigated the mechanism by which TAM67 inhibits cell growth. We first demonstrated that TAM67 in breast cancer cells caused growth inhibition by suppressing G_1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block. We also showed that the expression of Tam67 induced apoptosis in the absence of serum. We then demonstrated that TAM67 binds all Jun and Fos family members, and it inhibits breast cancer cell also by interacting with critical cJun partners, such as Fos and ATF/CREB families. Over the next year I will identify which coactivators are critical for breast cancer cell growth. .

BODY

Statement of Work

Specific Aim 1: Determine the mechanism(s) by which AP-1 blockade inhibits breast cancer cell growth

Time Line	Task
Months 1-12	1). Determine whether AP-1 blockade affects the cell cycle and cell cycle regulators in breast cancer cells; 2). Determine whether AP-1 blockade induces apoptotic cell death and alters the expression of critical apoptosis regulators.

Specific Aim 2: Determine whether inhibition of AP-1 activity reverses tamoxifen-resistance of breast cancer cells *in vitro*

Time Line	Task
Months 1-6	Introduce the Tet-Off system into the LCC-2 cell line and screen clones expressing cJun-DN.
Months 7-12	Investigate effect of expression of cJun-DN on AP-1 activity and expression of AP-1 dependent genes in LCC2 cells
Months 13-24	Determine whether AP-1 blockade reverses TAM-resistance of LCC2 breast cancer cells.

Specific Aim 3: Determine whether inhibition of AP-1 suppresses the growth of established human breast cancer cells or reverses TAM resistance in nude mice

Time Line	Task
Months 13-24	Inject MCF-7 Tet-Off cJun-DN into nude mice to determine the effect of AP-1 blockade on the growth of human breast cancer cells *in vivo*
Months 25-36	Inject LCC2 Tet-Off cJun-DN into nude mice to determine whether AP-1 blockade reverses TAM resistance *in vivo*

PROGRESS:

Specific Aim 1. Determine the mechanism by which AP-1 blockade inhibits breast cancer cell growth.

1 a: Determine whether AP-1 blockade affects the cell cycle and cell cycle regulators in breast cancer cells. We first determined the effect of AP-1 blockade, TAM67, on cell growth by performing cell proliferation assay, we demonstrated TAM67 inhibit breast cancer cell growth. We next investigated the effect of AP-1 blockade on DNA synthesis and the cell cycle using a ^{3}H-thymidine incorporation assay and flow cytometry. The results of the ^{3}H-thymidine uptake assay showed that TAM67 dramatically inhibited ^{3}H-thymidine uptake in MCF-7 cells. Flow cytometry also showed that expression of cells of TAM67 reduced the proportion of cells in S phase, and increased the proportion in the G_0/G_1 phase. Thus, in the presence of serum, the expression of TAM67 blocked the cell cycle by causing a G_1 arrest.

1 b: Determine whether AP-1 blockade induces apoptotic cell death. We first performed TUNEL assay to measure apoptosis. We found the expression of TAM67 dramatically increased the cell apoptotic rate in serum free condition, but there is no significant difference in full serum condition. To further demonstrate the involvement of apoptosis in the inhibition of MCF-7 cell growth, we then performed western blotting to measure the cleavage of PARP, a hallmark of apoptosis. When the MCF-7 Tet-Off TAM 67 cells were cultured in serum-containing medium, we also observed no PARP cleavage neither in TAM67 induced or uninduced conditions. Under serum–free condition, we did not see obvious PARP cleavage when TAM67 was not expressed, however, when TAM67 was induced the PARP cleaved band was clearly observed. Our study showed that TAM67 induced apoptosis in serum-free condition.

1 c: We have extended these studies to determine whether AP-1 Blockade alters the expression and/or activity of cell cycle regulators. We first performed western-blotting to determine the effect of TAM67 on Rb phosphorylation, we found that TAM67 caused Rb hypophosphorylation. We next performed luciferase assays to determine whether AP-1 blockade inhibits E2F activity. The results from these experiments demonstrated that TAM67 decreased E2F activity, and thus blocked the cell cycle. Next, we performed western-blotting to determine the effects of Tam67 on the expression of cell cycle regulatory proteins. We discovered that TAM67 decreased cyclin Ds (D1, D2, D3), cyclin E, CDK4, and CDK6 expression, while increase CDK inhibitor p27 expression. Next, we used CDK2 and CDK4 kinase assays to determine whether TAM67 reduced CDK activity. We demonstrated that both CDK2 and CDK4 activity were reduced by TAM67 expression. Our study suggests that TAM67 inhibits breast cancer growth predominantly by inducing inhibitors of cyclin dependent kinases (such as p27), suppressing G1 cyclins expression and reducing CDKs activity, thus inducing a cell cycle block.

Specific aim 2. Determine whether inhibition of AP-1 activity reverses tamoxifen-resistance of breast cancer cells in vitro.

2a: Introduce the Tet-Off system into the LCC-2 cell line and screen clones expressing cJun-DN.
2b: Investigate effect of expression of cJun-DN on AP-1 activity and expression of AP-1 dependent genes in LCC2 cells.
2c: Determine whether AP-1 blockade reverses TAM-resistance of LCC2 breast cancer cells.
These studies have not yet begun, because we have not been able to generate LCC-2 expressing TAM67 cell line. Therefore we are now proposing an alternative Aim 2.

New specific aim 2. Identification of the Jun dimerization partners that are critical for the growth of breast cancer.

Time Line	Task
Months 13-16	To identify cJun-DN (TAM67)-binding proteins.
Months 17-24	To determine which of the cJun-DN-binding proteins are critical for breast cancer cells growth.

Specific aim 2a. To identify cJun-DN(TAM67)-binding proteins

I will identify the known cJun-DN-binding proteins by using immunoprecipitation-western blotting.

Experiment plan: MCF 7 Tet-Off TAM67 cells will be cultured in the presence or absence of doxycycline to induce TAM67 expression. First, western-blotting will be performed to detect the expression of each Jun and Fos family member in these cells. Then, immunoprecipitation-Western blotting (Co-IP) experiments will be performed to determine if TAM67 binds these proteins. 2-ways Co-IP will be done for this study. First, the proteins will be incubated with an anti-Flag antibody (the cJun-DN is flag-tagged), and the antibody-bound proteins will be precipitated using protein A/G sepharose beads. The precipitated proteins will be analyzed using western blotting techniques with Antibodies specific for individual Jun and Fos family members. Next, the other way of Co-IP will be performed. The proteins will be incubated with Antibodies specific for individual Jun and Fos family members, the antibody-bound proteins will be precipitated using protein A/G sepharose beads. And the precipitated proteins will be analyzed using western blotting techniques with anti-Flag antibody.

Expected results, potential pitfalls, and alternative strategies: We expect to observe cJun-DN binding of several proteins in MCF7 cells. These include Jun, and Fos family members. We will be able to detect these with specific antibodies to each of these members. However, it is also possible that some of the cJun-DN-binding proteins will not be known AP-1 transcription factors. In this case, we will in future identify the unknown proteins by using microsequencing techniques.

Specific aim 2b: To determine which of the cJun-DN-binding proteins are critical to the growth of breast cancer cells.

Because using antisense oligonucleotides to inhibit every potential candidates found in task 1 will be time-consuming, we will use an alterative strategy to narrow the list of critical Jun-DN-binding proteins. It was known that the Jun-leucine zipper domain can bind Jun, Fos, ATF/CREB, and Mafs and coactivators to form homo and heterodimerizations. However, the Fos-leucine zipper domain can not bind Fos and ATF/CREB family members. Therefore we will make specific mutant constructs of the cJun-DN that bind only Jun proteins or only coactivators, This mutant can then be used to narrow down potential candidates that bind cJun-DN.

Experiment plan: I will make new mutant constructs of the cJun-DN by mutanting its dimerization domain, so the new mutants have different partners from the cJun-DN. We will use the cJun-DN/Fos mutant, in which the cJun dimerization domain of the cJun-DN has been replaced by Fos dimerization domain. Because it has Fos dimerization domain, cJun-DN/Fos can only heterodimerize with Jun family members and coactivators and can not homodimerize with Fos and ATF/CREB family members. We have also constructed a mutant called cJun-DN/Squelcher. This mutant is similar to cJun-DN except that it lacks the dimerization domain of cJun. Because this protein does not have dimerization domain, it can not dimerize with AP-1 family members, however, it may still bind coactivators. We will transfect these cJun-DN mutants with MCF7 cells under control of a Tet-off system, select the Flag-tagged cJun-DN/Fos and cJun-DN/Squelcher inducible clones and screen them for protein expression using Western blotting. I will do luciferase assays to determine whether cJun-DN/Fos and cJun-DN/Squelcher inhibit the AP-1 activity. I will then perform proliferation assays to investigate whether cJun-DN/Fos and cJun-DN/Squelcher inhibit breast cancer cell growth.

Expected results, potential pitfalls and alternate strategies: I predict that Tam/Fos will inhibit AP-1 activity and cell growth, while Tam/Squelcher will not. This result would indicate that Fos and ATF/CREB family members are not critical for growth while Jun family members are critical for breast cancer cell growth. If so, I will then identify the specific critical Jun proteins using specific antisense and/or SiRNA methods in future. It is possible that both Tam/Fos and Tam/Squelcher do not inhibit breast cancer cell growth, such a result would indicate that neither Jun family members nor coactivitors are critical for MCF 7 breast cancer cell growth, in that case, it is likely that other proteins such as Fos or ATF/CREB family members are critical. If I obtain such result I will identify the specific proteins using specific antisense and/or SiRNA methods in future. It is also possible that both Tam/Fos and Tam/Squelcher do inhibit breast cancer cell growth, such a result would indicate that coactivators and other proteins that bind Tam/Squelcher are most critical for breast cancer cell growth. If we get such a result, we will use specific antisense and/or SiRNA methods in future to identify these poteins.

Products and deliverables:

a. New MCF 7 Tet-off cell lines expressing Jun-DN mutants will be established.

b. A manuscript describing the cJun-DN-binding proteins critical for breast cancer cells growth will be prepared.
c. Second year annual report will be submitted to funding agency.

Progress on new specific aim 2:

Specific Aim 2: Identification of the Jun dimerization partners that are critical for the growth of breast cancer.

2a. To identify the known cJun-DN (TAM67)-binding proteins

First, using Western-blotting assay, I detected the expression of individual AP-1 family members in MCF7 Tet off TAM67 cells. I found that all Jun and Fos proteins were expressed in these cells. I then identified if TAM67 binds these AP-1 family members by immunoprecipitation-Western blotting assay. First, immunoprecipitation was done with an anti-Flag antibody (TAM67 is flag-tagged), and the precipitated proteins was analyzed using western blotting techniques with antibodies specific for individual Jun and Fos family members. Next, immunoprecipitation was done with antibodies specific for individual Jun and Fos family members, and the precipitated proteins was analyzed using western blotting with anti-Flag antibody. The data demonstrates that TAM67 binds all Jun and Fos family members.

2b. To investigate which of the cJun-DN-binding proteins are critical for breast cancer cell growth by using more selective cJun-DN mutants experiments.

It is known that the Jun-leucine zipper domain can bind Jun, Fos, ATF/CREB, Mafs, and coactivators to form homo- and heterodimerizations. However, the Fos-leucine zipper domain cannot bind Fos and ATF/CREB family members. Therefore we made specific mutant constructs of the cJun-DN that bind only Jun proteins or only coactivators, This mutant was used to narrow down potential candidates that bind cJun-DN.

2b.1. Make new mutant constructs of the cJun-DN by mutating its dimerization domain

I made cJun-DN/Fos mutant by replacing the cJun dimerization domain of the cJun-DN with Fos dimerization domain. Because it has Fos dimerization domain, cJun-DN/Fos can only heterodimerize with Jun family members and coactivators and cannot homodimerize with Fos and ATF/CREB family members. I have also constructed a mutant called cJun-DN/Squelcher by deleting the dimerization domain of cJun. Because this protein does not have dimerization domain, it cannot dimerize with AP-1 family members, it may still bind coactivators.

2b.2. Introduce the Tet-off system into MCF7 cell line and screen clones expressing cJun-DN/Fos and cJun-DN/Squelcher mutants.

I transfected cJun-DN/Fos and cJun-DN/Squelcher mutants with MCF7 cells under control of a Tet-off system, selected the Flag-tagged cJun-DN/Fos and cJun-DN/Squelcher inducible clones and screen them for protein expression using Western blotting. The MCF 7 Tet-off cJun-DN/Fos and MCF 7 Tet-off cJun-DN/squelcher cell lines has been established.

2b.3. Investigate the effect of expression of cJun-DN/Fos and cJun-DN/Squelcher on AP-1 activity and the growth in MCF7 cells.

I did luciferase assays to determine whether cJun-DN/Fos and cJun-DN/Squelcher inhibit the AP-1 activity. I found that cJun-DN/Fos inhibited AP-1 activity while cJun-DN/Squelcher did not. I then performed proliferation assays to investigate whether cJun-DN/Fos and cJun-

DN/Squelcher inhibit breast cancer cell growth. The data showed that both cJun-DN/Fos and cJun-DN/Squelcher did not affect the growth of MCF 7 cells.
These results indicate that proteins that bind cJun-DN/Fos are not critical for breast cancer cell growth. While proteins that bind TAM67 but not bind cJun-DN/Fos are critical for the growth of breast cancer cells. Thus, we hypothesized that Fos and ATF/CREB family members may be required for breast cancer cell growth.

Specific aim 3. Determine whether AP-1 blockade suppresses breast cancer growth and reverses tamoxifen resistance in vivo.
3a: Inject MCF-7 Tet-Off cJun-DN into nude mice to determine the effect of AP-1 blockade on the growth of human breast cancer cells *in vivo.* To investigate the effect of AP-1 blockade on *in vivo* tumor growth, we injected the MCF-7 Tet-Off TAM67 cells into nude mice receiving doxycycline to suppress the expression of the AP-1 inhibitor. After the mice developed tumors, they were randomized to either continue to receive Dox or not. In mice not receiving Dox, the expression of TAM67 was induced, and tumor growth was inhibited, while the tumors in mice receiving Dox continued to grow. We have completely this task and reported last year.
3b: Inject LCC2 Tet-Off cJun-DN into nude mice to determine whether AP-1 blockade reverses TAM resistance *in vivo.* This task has not yet begun, because we have not been able to generate LCC-2 expressing TAM67 cell line. Therefore we are now proposing an alternative Aim 3.

New specific aim 3. Determine whether differential recruitment of coactivators accounts for the ability of the TAM67 to inhibit AP-1 transcriptional activation .

Time Line	Task
Months 25-31	To compare the ability of cJun and TAM67 to bind coactivators, such as Jab1, AIB1, CBP, P300, and CAPER in MCF7 cells by using immunoprecipitation-Western blotting.
Months 32-36	To determine whether TAM67 alters recruitment of these coactivators by performing ChIP assays using primers specific for the collagenase(MMP1) promoters.

Specific aim 3a. To compare the ability of cJun and TAM67 to bind coactivators, such as Jab1, AIB1, CBP, P300, and CAPER in MCF7 cells.
I will perform immnoprecipitation-Western blotting to compare the ability of cJun and TAM67 to bind coactivators such as Jab-1, AIB1, CBP, P300 and CAPER. MCF 7 clones that stable express either cJun or TAM67 (Tet off cJun and MCF 7 Tet off TAM67 cell lines) under control of a Tet-off system will be used in the experiments.
Experiment plan: MCF 7 Tet-Off cJun and MCF 7 Tet off TAM67 cells will be cultured in the presence or absence of doxycycline to induce cJun or TAM67 expression. First, western-blotting will be performed to detect the expression of cJun and TAM67, as well as coactivators Jab-1, AIB1, CBP, P300 and CAPER, in these cells. Then, immunoprecipitation-Western blotting (Co-IP) experiments will be performed to determine if cJun and TAM67 bind these coactivators, and to compare the ability of cJun and TAM67 to bind coactivators. 2-ways Co-IP will be done for this study. First, the proteins will be incubated with an anti-Flag antibody (the cJun and TAM67 are flag-tagged), and the antibody-bound proteins will be precipitated using protein A/G sepharose beads. The precipitated proteins will be analyzed using western blotting techniques.

Antibodies specific for individual coactivators will be used. Next, the other way of Co-IP will be performed. The proteins will be incubated with Antibodies specific for individual coactivators, the antibody-bound proteins will be precipitated using protein A/G sepharose beads. And then the precipitated proteins will be analyzed using western blotting techniques with anti-Flag antibody.

Expected results, potential pitfalls, and alternative strategies: I expect to observe the different coactivator binding ability of cJun and TAM67 in MCF 7 cells, for example, cJun binds some of these coactivators while TAM67 does not. However, it is also possible that there is no difference of coactivator binding ability of cJun and TAM67 will be observed in this experiment. In this case, I will perform GST-Pull down or yeast-2-hybrid Assays to investigate the different ability of cJun and TAM67 to bind coactivators.

Specific aim 3b. To determine whether TAM67 alters recruitment of these coactivators.

I will perform ChIP assays using primers specific for the collagenase(MMP1) promoters to determine whether TAM67 alters recruitment of coactivators such as Jab-1, AIB1, CBP, P300 and CAPER. MCF 7 clones that stable express either cJun or TAM67 (Tet off cJun and MCF 7 Tet off TAM67 cell lines) under control of a Tet-off system will be used in the experiments.

Experiment plan: MCF 7 Tet-Off cJun and MCF 7 Tet off TAM67 cells will be cultured in the presence or absence of doxycycline to induce cJun or TAM67 expression. First, western-blotting will be performed to detect the expression of cJun, TAM67, Jab-1, AIB1, CBP, P300 and CAPER in these cells. Then, ChIP Assay will be performed to determine whether TAM67 alters recruitment of these coactivators compare to cJun. Cells will be fixed, lysed after cross-link, the cell lysate will be chopped to small pieces and incubated with antibodies specific for individual coactivator, the antibody-bound protein complex will be precipitated using protein A/G sepharose beads, and The precipitated complex will be heated to reverse the cross-link and followed by DNA purification. Next, PCR will be performed using primers specific for the collagenase (MMP1) promoters.

Expected results, potential pitfalls and alternate strategies: I expect to observe these coactivators present in MMP1 promoters in MCF 7 cells when cJun is over-expressed, while some coactivators do not show up when TAM67 is overexpressed. It is also possible that the same coactivators present in MMP1 promoter under either cJun or TAM67 over expressed condition. In this case, I will first perform GST-Pull down or yeast-2-hybrid Assays to investigate the different ability of cJun and TAM67 to bind coactivators, then use ChIP assays to determine whether TAM67 alters recruitment of these coactivators.

Products and deliverables:

a. A manuscript describing whether differential recruitment of coactivators accounts for the ability of the TAM67 to inhibit AP-1 transcriptional activation will be prepared.
b. A abstract will be submitted to AACR annual meeting.
c. Third year annual report will be submitted to funding agency.

Progress on new specific aim 3:

Specific aim 3. Determine whether differential recruitment of coactivators accounts for the ability of the TAM67 to inhibit AP-1 transcriptional activation .

3a. Compare the ability of cJun and TAM67 to bind coactivators, such as Jab1, AIB1, CBP, P300, and CAPER in MCF7 cells.

First, using Western-blotting assay, I detected that Jab-1, AIB1, CBP, P300 were expressed in both MCF 7 Tet-off cJun and MCF 7 Tet-off TAM67 cells under doxycycline present and absent conditions. I then compared the ability of cJun and TAM67 to bind these coactivators. Immunoprecipitation was done with antibodies specific for Jab-1, AIB1, CBP, and P300, and the precipitated proteins was analyzed using Western-blotting with an anti-Flag antibody (both cJun and TAM67 are flag-tagged). I found that cJun binds all of these coactivators, while TAM67 only binds AIB1. These data suggest that TAM67 appears to inhibit breast cancer cell growth by preventing recruitment of co-activators. I have not been able to detect CAPER expression in MCF 7 cells because I have not got the antibody yet. I am working on confirming these results by using the second way of immunoprecipitation-Western assay which is to precipitate proteins by anti-Flag antibody and to analyze the proteins with antibodies specific for these coactivators.

3b. To determine whether TAM67 alters recruitment of these coactivators. These studies are ongoing.

KEY RESEARCH ACCOMPLISHMENTS

We studied the mechanism by which AP-1 blockade inhibits breast cancer cell growth. .Our data demonstrated that the AP-1 blockade, TAM67, inhibited breast cancer growth both *in vitro* and *in vivo*. TAM67 suppressed cyclin D and E expression, increased p27 expression, decreased CDk2 and CDk4 kinase activity, caused Rb hypophosphorylation and reduced E2F activity, thus results a G1 cell cycle block leading to cell growth inhibition. We also observed that TAM67 induced MCF7 breast cancer cell apoptosis in serum free condition. Our recent data suggests that Jun and Fos family members are expressed in MCF 7 cells at TAM67 over-expressed condition, TAM67 binds all of these Jun and Fos family members. Our studies also showed that Jun families is not critical while Fos, or ATF families are required for breast cancer cell growth. Therefore, TAM67 appears to inhibit breast cancer cell growth by interacting with Fos and ATF family members, and preventing recruitment of co-activators.

REPORTABLE OUTCOMES

Specific aim 1:

1. The paper untitled "Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth" was published in Oncogene 21, 2002, Pages 7680-7689.
2. The manuscript "AP-1 Blockade in Breast Cancer Cells Causes Cell Cycle Arrest by Suppressing G1 Cyclin Expression and reducing cyclin dependent kinase activity" was submitted to Oncogene, now under review.
3. Poster untitled "AP-1 inhibitor Causes a Cell Cycle Block by Inducing the Expression of CDK Inhibitors and by Suppressing the Expression of Cyclins D and E" was presented at *Era of Hope DOD Breast Cancer Research meeting, 2002.*
4. First year annual report had been submitted to funding agency.

Specific aim 2:

1. New MCF 7 Tet-Off inducible cell lines include MCF 7 Tet-Off TAM/Fos and MCF 7 Tet-Off TAM/Squelcher were established.
2. The manuscript "cJun-Dominant-Negative Mutant Inhibits Breast Cancer Cell Growth by interacting with critical cJun partner" is under preparation.
3. Second year annual report is submitted to funding agency .

Specific aim 3:

1. Poster untitled "AP-1 Blockade Inhibits Breast Cancer Cell Growth by Preventing the Recruitment of Coactivators" was presentated at AACR annual meeting 2003.

CONCLUSIONS

The cJun-dominant negative mutant, TAM67, inhibits breast cancer growth both *in vitro* and *in vivo*. Studies supported by this grant have shown that TAM67 inhibits breast cancer growth predominantly by inducing the expression of inhibitors of cyclin dependent kinases (such as p27), suppressing G1 cyclins expression and reducing CDKs activity, thus causing a cell cycle block. TAM67 also induces apoptosis in cells grown in serum free condition. We have also demonstrated that TAM67 binds all Jun and Fos family members, and that Fos and ATF family members are critical for growth of breast cancer cells. Our studies on coactivators also suggest that TAM67 inhibits breast cancer cell growth by preventing recruitment of coactivators.

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AP-1 Blockade in Breast Cancer Cells Causes Cell Cycle Arrest by Suppressing G1 Cyclin Expression and Reducing Cyclin Dependent Kinase Activity

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Running Title: AP-1 blockade causes a cell cycle block in breast cancer cells

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Abbreviations: AP-1: Activating Protein-1; CDK: Cyclin Dependent Kinase; Dox: Doxycycline.

Abstract

The AP-1 transcription factor is a central component of signal transduction pathways in many cells, though the exact role of AP-1 in controlling cell growth and malignant transformation is unknown. We have previously shown that AP-1 complexes are activated by peptide and steroid growth factors in both normal and malignant breast cells, and that blocking AP-1 by over-expressing a dominant negative form of cJun (cJun-DN, Tam67) inhibits breast cancer cell growth both in *vivo* and *in vitro*. We hypothesized that Tam67 inhibits cell growth by altering the expression of cell cycle regulatory proteins, thus causing a cell cycle block. In the present study, we used clones of MCF7 breast cancer cells that express Tam67 under the control of an inducible promoter. First, we determined the effect of AP-1 blockade on cell growth, then we performed ^{3}H-thymidine incorporation and flow cytometry assays to investigate whether Tam67 inhibits the cell cycle. We observed that in the presence of serum Tam67 inhibited cell growth and caused a block in the G1 phase of the cell cycle. Next, we performed western-blotting and CDK kinase assays to determine the effects of Tam67 on Rb phosphorylation, the expression of cell cycle regulatory proteins, and CDK activity. We discovered that Tam67 inhibited Rb phosphorylation and reduced E2F activity. We also found that Tam67 decreased the expression of the D and E cyclins, reduced CDK2 and CDK4 activity, and increased the CDK inhibitor p27. Finally we performed RPA to determine the cyclin D1 mRNA expression, we found that TAM67 decreased cyclin D1 mRNA expression. Our study suggests that in the presence of serum Tam67 inhibits breast cancer growth predominantly by inducing inhibitors of cyclin dependent kinases (such as p27) and by reducing the expression of the cyclins involved in transitioning from G1 into S phase of the cell cycle. This effectively blocks breast cell proliferation. These studies lay the foundation for future attempt to develop new agents for the treatment and prevention of breast cancer.

Introduction

The AP-1 transcription factor is a complex of proteins composed of basic region-leucine zipper proteins that belong to the Jun, Fos, Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factors (ATF/CREB) subfamilies. These proteins bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 is a central component of many signal transduction pathways in a variety of cell types, and is critical for mitogenesis, apoptosis, and carcinogenesis depending on the cell type. We and others have shown that AP-1 is involved in controlling cellular proliferation, differentiation, apoptosis, and oncogene-induced transformation (Brown *et al.*, 1993; Brown *et al.*, 1994; Ham *et al.*, 1995; Holt *et al.*, 1986; Rodgers *et al.*, 1994; Szabo *et al.*, 1991). However, the exact molecular mechanism by which AP-1 transcription factors control cell proliferation, survival and death is still being elucidated.

In breast cells, the previous studies have suggested that growth factors and hormones, such as IGF, EGF, estrogens and retinoids, can modulate AP-1 transcriptional activity (Chen *et al.*, 1996b; Lin *et al.*, 2000; Schule & Evans, 1991; Webb *et al.*, 1999). Other studies demonstrate that ER and AP-1 interact to regulate the expression of certain estrogen and tamoxifen-regulated genes (Paech *et al.*, 1997). Activation of AP-1 may also contribute to tumor cell invasive capacity and to tamoxifen resistance (Johnston *et al.*, 1999; Schiff *et al.*, 2000; Smith & Prochownik, 1992; Yang *et al.*, 1997). In previous studies we have used a specific inhibitor of AP-1, a dominant negative cJun mutant, TAM67, to block AP-1 activity in breast cancer cells. Results from these studies demonstrated that Tam67 blocks AP-1 activation in normal, immortal, and malignant breast cells (Ludes-Meyers *et al.*, 2001). We have also demonstrated that Tam67 inhibited breast cancer growth both in *vivo* and *in vitro* (Liu *et al.*, 2002). These studies suggest that AP-1 transcription factor is an important regulator of breast cancer cell growth, invasion, and resistance to anti-estrogens.

In the present study, we studied the effect of Tam67 on the expression and activity of cell cycle regulators and on markers of apoptosis. We found that Tam67 inhibited MCF7 cell growth and also caused apoptosis in serum-free conditions. Tam67 decreased the expression of cyclin D proteins (cyclin D1, D2, D3) and cylcin E, the main cyclins in G1 phase of cell cycle, and their associated CDK2 and CDK4 activities, which in turn caused hypophosphorylation of Rb that blocked the release of E2F from Rb/E2F complex. TAM67 also decreased cyclin D1 mRNA expression. Thus, AP-1 blockade causes growth inhibition by suppressing the expression of G1 cyclins, inducing p27, and ultimately inhibiting E2F activity. These studies provide a strong rationale for the foundation for future attempts to develop specific signal transduction inhibitors to effectively treat or prevent breast cancer.

Materials and Methods:

Cell Culture and Transfection

The generation of the MCF-7 Tet-off TAM67 Clones #62, #67 and vector clones #1, #3 has been previously described (Ludes-Meyers *et al.*, 2001). The cells were maintained in Improved MEM (high zinc option, Life Technologies, Grand Island, New York) with 100 ug/ml of geneticin and 100 ug/ml hygromycin. The cells were transfected using Fugene 6 reagent (Roche, Indianapolis, Indiana) according to the manufacturer's recommendations.

Cell Growth Assays

The CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. Approximately 12,000 cells were seeded in a 24 well plate and doxycycline was added or removed to block or induce the expression of TAM67 of MCF-7 Tet-Off

TAM67 cells. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37° C and absorption at 550 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

^{3}H-Thymidine Incorporation assay

MCF-7 Tet-Off TAM67 Clone #62 cells were kept in –Dox and +Dox media to induce or block the expression of TAM67 for a total of 7 days. 48 hours before harvest (thus after 5 days in the absence or presence of Dox), the medium was changed to serum-free medium. Two days later the cells were stimulated with serum for 0, 6, 12, 24, or 48 hours, and then labeled with ^{3}H-thymidine (2 μ Ci/ml) for 3 hours. The cells were then incubated with 5% TCA at 4°C for 30 minutes, and were then lysed by addition of 0.1 N NaOH. The level of protein in the lysates was determined using a BCA assay (Pierce, Rockford, IL, USA). ^{3}H-Thymidine uptake was measured by counting ^{3}H c.p.m. in a scintillation counter. Each data point was performed in triplet, and the results were reported as mean c.p.m. + / - standard error. All results were normalized to protein content.

Flow Cytometry

MCF-7 Tet-Off TAM67 Clone #62 cells were kept in –Dox or +Dox media to induce or block the expression of TAM67 for a total of 7 days. 48 hours before harvest (thus, after 5 days in the absence or presence of Dox), the medium was changed to serum-free medium. 48 hours later the cells were stimulated with serum, harvested at 0, 6, 12, 24, 48 hours after serum stimulation, and fixed in 95% ethanol for 30 minutes in room temperature. The cells were then stained with propidium iodide in PBS. Stained cells were analyzed with EPICS XL-MCL flow cytometer (Coulter Co.).

Luciferase Assay

E2F1 transcriptional activity in cells was measured using the Dual-Luciferase™ Reporter Assay (Promega, Medison, Wisconsin) as previously described (Ludes-Meyers *et al.*, 2001). The cells were co-transfected with the E2F1-luc reporter gene and pRL-TK, a Ranilla construct for normalizing of transfection efficiency. Transfected cells were lysed 36 hours after transfection and luciferase activity was measured with equal amounts of cell extract using microplate luminometer (Labsystems, Helsinki, Finland) and normalized with the Renilla activity.

Western Blot Analysis

For cell cycle study, the cells were cultured in the medium for 7 days with or without Dox to block or induce the expression of TAM67, then synchronized in 50 ng/ml of nocodazole for 18 hours. After the synchronization, the floating cells (in M phase) were collected and washed in PBS for 3 times. The cells were then cultured in full medium. At time points 6, 12, 18, 24, and 36 hours, the cells were harvested and cells lysates were prepared. 20 μg of total cellular protein extract were electrophoresed on SDS-PAGE gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad, Hercules, California). The following antibodies were used: Ab-1 (Oncogene Science, Cambridge, MA, 1:200) for cJun; 14001A for Rb (Pharmingen, 1:1000); 14561A for cyclin D1 (Pharmingen, 1:300); C17 for cyclin D2 (Santa Cruz, sc-181, 1:300), C16 for cyclin D3 (Santa Cruz, sc-182, 1:200), HE-12 for cyclin E (Santa Cruz, sc-247, 1:200); F-12 for p16 (Santa Cruz, sc-1661, 1:200); H-164 for p21 (Santa Cruz, sc-756, 1:1000); F-8 for p27 (Santa Cruz, sc-1641, 1:200); MAB1501 for actin (Chemicon, 1: 2000). Anti-rabbit or anti-mouse antibody (1:4,000, Amersham, Piscataway, New Jersey) was used as secondary antibody. Blots were developed using the enhanced chemiluminescence (ECL) system (Amersham, Piscataway, New Jersey).

Kinase Assay

The methods for the kinase assay have been previously described (Yang *et al.*, 2001). For the CDK2 kinase assay, cells were lysed in a buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EGTA, 1mM DTT, 1 % Triton X-100, 10 % glycerol, 10 mM β-glycerophosphate, 100 mM NaF, 0.2 mM $NaVO_3$, 1.5 mM $MgCl_2$, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM PMSF. For the CDK4 kinase assay, the cells were lysed in a buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 2.5 mM EGTA, 1mM DTT, 0.1 % Tween 20, 10 % glycerol, 10 mM β-glycerophosphate, 1mM NaF, 0.1 mM NaVO3, 1.5 mM $MgCl_2$, 2 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.1 mM PMSF. Protein G agarose (Life Technologies, Gaithersburg, MD) was incubated with CDK2 or CDK4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4^0C for an hour followed by incubating with 500-800 μg of protein extracts at 4°C overnight. The agarose mixture was pelleted and washed in lysis buffer for four times. For immunoprecipitation–western blotting, the agarose was resuspended in 40 μl 1x sample buffer (125 mM Tris pH 6.8, 4% SDS, 0.005% bromophenol blue, 20% glycerol, 0.7M β-mercaptoethenol) and 20μl was loaded on 12% SDS-PAGE. Western blotting was performed as described above. For the CDK2 kinase assay, the agarose mixture was washed in 1x cold kinase buffer (20 mM Tris pH 7.5, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, and 1mM DTT) and resuspended in final volume of 25 μl containing 5 μl 5x kinase buffer, 1 μl γ-^{32}P ATP and 20 μg of histone H1 (Roche, Indianapolis, IN). For the CDK4 kinase assay, the agarose mixture was washed in 1x cold kinase buffer (50 mM Hepes pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 mM β-glycerophosphate, 1mM NaF and 0.1 mM $NaVO_3$) and resuspended in final volume of 25 μl containing 5 μl 5x kinase buffer, 1 μl γ-^{32}P ATP and 1.5 μg GST-Rb (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The kinase reaction was performed at 30°C for 30 minutes and was stopped by adding 25 μl of 2x sample buffer. The samples were heated at 90°C for 5 minutes and 25 μl of reaction mixture was loaded on 10

% SDS-PAGE gel. The gel was dried and exposed to X-ray film. The intensity of the bands was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RNAase Protection

RNA was isolated from MCF 7 Tet off Tam67 cells under DOX presence or absence conditions using Qiagen MidiEasy Kit. RNA yield was determined by UV absorption at 260nm after dissolving in sterile H2O. RNAase protection was done follow Phamingen protocol using BD RiboQuant ™ RPA kit.

Results:

AP-1 blockade induced by TAM67 inhibits MCF-7 cell growth in the presence of serum

Previously, using MCF-7 clones that stably express a dominant-negative cJun mutant TAM67, under the control of an inducible promoter, we showed that TAM67 suppresses AP-1 activity, inhibits cell growth induced by several different growth factors, (EGF, IGF-1 heregulin-β, bFGF, and estrogen), and also inhibits MCF-7 xenograft tumor growth (Liu *et al.*, 2002; Ludes-Meyers *et al.*, 2001). In this study, we investigated whether AP-1 blockade inhibited cell proliferation or induced apoptosis. The MCF-7 Tet-Off TAM67 clone cells (#62) and vector transfected cells (clone #1) were cultured in the presence or absence of Dox to inhibit or stimulate the expression of TAM67. As shown in Figure 1A, in complete medium without Dox, the expression of TAM67 totally inhibited MCF-7 cell growth. These cells proliferated normally in the presence of Dox. Vector clone cells grew well both in the presence and absence of Dox.

We also investigated the effect of Tam67 on cell growth in serum-free conditions. In serum-free medium, the TAM67 clone cells still grew, although slowly, in the presence of Dox. But in the absence of Dox, the expression of TAM67 not only inhibited cell growth but also appeared to cause cell death. In the absence of serum, the vector clones grow slowly but equally in the presence or absence of Dox (Fig. 1B).

TAM67 blocks the cell cycle

When MCF 7 cells were grown in the presence of serum, TAM67 inhibited MCF-7 cell growth without inducing cell death. Therefore, we next investigated the effect of AP-1 blockade on DNA synthesis and the cell cycle using a ^{3}H-thymidine incorporation assay and flow cytometry. The results of the ^{3}H-thymidine uptake assay showed that TAM67 dramatically inhibited ^{3}H-thymidine uptake in MCF-7 cells (Fig. 3). Flow cytometry also showed that expression of cells of TAM67 reduced the

proportion of cells in S phase, and increased the proportion in the G_0/G_1 phase (Fig. 4). Thus, in the presence of serum, the expression of TAM67 blocks the cell cycle by causing a G_1 arrest.

Effects of TAM67 on Rb phosphorylation and E2F activity

The above results show that TAM67 blocks the cell cycle by inducing a G1 cell cycle arrest. Molecules that regulate G1 to S transition include Rb, cyclins (Ds, and E), CDKs (2, 4, and 6), and CDK inhibitors. We first investigated the effects of TAM67 on the phosphorylation of Rb. As seen in Figure 6A, Rb is highly phosphorylated in the presence of Dox. When the MCF-7 Tet-Off TAM67 cells were cultured in the absence of Dox, Rb phosphorylation was reduced (Fig. 5). Given this hypophosphorylation of Rb, we predicted that E2F activity would be reduced. We therefore measured E2F transactivation activity by transfecting an E2F responsive luciferase plasmid into the MCF 7 cells in the presence and absence of Dox. These results showed that in the absence of Dox, E2F transcription factor activity was inhibited (Fig. 6).

Effects of TAM67 on expression of cell cycle regulatory proteins

Rb is phosphorylated by cyclin E-CDK2 or cyclin D-CDK4/6 complexes, and this phosphorylation is suppressed by CDK inhibitors. Therefore, we next determined the effects of TAM67 on protein expression levels of these cell cycle regulators. The cells were first synchronized in M phase by the addition of nocodazole, and then released from cell cycle block and cells harvested at different time points for western blot analysis. As shown in Fig. 7, we found that TAM67 decreased the expression of cyclin E and cyclin Ds, including D1, D2, and D3, while increased p27 expression. TAM67 did not affect p16 expression. Interestingly, Tam67 also decreased p21 expression.

TAM67 inhibits CDK2 and CDK4 kinases activity

The observed hypophosphorylation of Rb induced by TAM67 suggested a defect in activation of essential G1 CDKs. Accordingly, the activity of the CDK2 and CDK4 kinases was measured using immunoprecipitates from MCF-7 Tet-Off TAM67 cells grown in the presence or absence of Dox. The cells were then synchronized in M phase and restimulated to enter the cell cycle. A significant reduction of CDK2 activity was observed when cells were cultured in the absence of Dox at different time points (Fig. 8A). CDK4 activity was also decreased in cells expressing TAM67 (Fig. 8B). These immunoprecipation-western blot experiments also showed that CDK2-associated cyclin E and CDK4-associated cyclin D1 were also reduced in cells expressing TAM67 (cultured in the absence of DOX, Fig. 8A and B).

TAM67 inhibits the expression of cyclin D1 mRNA

Our data showed TAM67 decreased the cyclin D1 protein expression. Next we performed RPA to determine the cyclin D1 mRNA expression. The cells were first synchronized in M phase by the addition of nocodazole, and then released from cell cycle block and cells harvested at different time points for RPA. As shown in Fig. 9, we found that TAM67 decreased the expression of cyclin D1 mRNA expression.

Discussion:

The above results demonstrate that blockade of the AP-1 transcription factor in MCF 7 breast cancer cells leads to inhibition of cell growth in the presence of serum and programmed cell death in serum-free conditions. The effects of AP-1 blockade are shown as a model in Fig. 9. AP-1 blockade causes decreased expression of D and E cyclins, the main cyclins in G1 phase of cell cycle, and increases the expression of the CDK inhibitor p27. These changes in the expression of cell cycle regulators leads to reduced CDK2 and CDK4 activity, which in turn causes hypophosphorylation of Rb and inhibition of E2F activity, ultimately inducing a G1 cell cycle block. In combination with our previous results showing that expression of Tam67 blocks signal transduction by multiple growth factors (Liu *et al.*, 2002), these studies demonstrate that AP-1 blockade can effectively block signal transduction and inhibit the growth of breast cancer cells.

We and others have previously shown that the AP-1 transcription factor is critical for cell proliferation and transformation of several cell types (Brown *et al.*, 1994; Chen *et al.*, 1996a; Holt *et al.*, 1986; Liu *et al.*, 2002). Studies of *jun* and *fos*-null cells and animals indicate that c-Fos and c-Jun are critical growth promoting components of AP-1 (Brown *et al.*, 1998; Johnson *et al.*, 1993; Schreiber *et al.*, 1999; Wisdom *et al.*, 1999), whereas JunB and JunD are negative regulators of cell proliferation (Potapova *et al.*, 2001; Weitzman *et al.*, 2000). In fibroblasts, c-Jun is required for transit beyond the G1/S interphase (Schreiber *et al.*, 1999; Smith & Prochownik, 1992). The most severe defects are exhibited by c-Jun-/- fibroblasts, which can be passed only once or twice in culture before they exhibit a pseudo-senescent phenotype and their cell cycle transit time increases dramatically (Johnson *et al.*, 1993; Schreiber *et al.*, 1999; Wisdom *et al.*, 1999).

In breast cells, AP-1 is important for regulating cell growth (Liu *et al.*, 2002; Ludes-Meyers *et al.*, 2001), invasion (Smith *et al.*, 1999), chemotherapy resistance (Potapova *et al.*, 2001), and tamoxifen resistance (Schiff *et al.*, 2000). We have also shown that AP-1 blockade induced by

TAM67 suppresses AP-1 activity induced by different peptide growth factors, including EGF, IGF-1, heregulin-β, b-FGF and estrogen. The present results showing that TAM67 inhibits MCF-7 cell growth by inducing a cell cycle block are consistent with these previous results showing that AP-1 blockade causes general inhibition of growth factor signal transduction.

The results presented here show that blockade of AP-1 leads to reduction in critical G1 cell cycle regulators (cyclins D and E), and an increase in the CDK inhibitor p27. The results of Hennigan *et al* (Hennigan & Stambrook, 2001) are consistent with our present study. These investigators used GFP-TAM67 to study the role of AP-1 in human fibrosarcoma cells. They demonstrated that GFP-TAM67 caused pRB hypophosphorylation and arrested cells in the G1 phase of the cell cycle, findings similar to those presented here. However, unlike our present findings, this group found that GFP-TAM67 did not inhibit the expression of cyclin D1, cyclin E in fibrosarcoma cells. These differences may be due to the different types of cell used for these two studies (fibrosarcoma vs. breast cancer cells).

The human cyclin D1 gene regulatory sequences contain two AP-1 binding sites (Albanese *et al.*, 1999; Herber *et al.*, 1994). Results from previous studies have suggested that c-Jun induces while JunB inhibits cyclin D1 transcription (Bakiri *et al.*, 2000). It has been suggested that AP-1 family members regulate cell cycle by inducing the expression of cyclin D1 via AP-1 sites in its promoter region (Brown *et al.*, 1998). Our data showed that expression of TAM67 reduced the expression of cyclin D1 suggesting that down-regulation of cyclin D is responsible, at least partially, for TAM67's inhibition effects. Because the cyclin D1 promoter contains typical AP-1 binding sites, this inhibitory effect of TAM67 could be due to direct binding of TAM67 to these sites. Cyclins D2 and D3, show considerable structural and functional homologies with cyclin D1, and in certain instances, they may complement each other functionally. Thus, the downregulation of cyclins D1, D2 and D3 may also contribute to the inhibitory effect of TAM67.

Cyclin E is thought to act as a rate limiting factor after cyclin D1 at the G1-S transition. In breast cancer, cyclin E also drives proliferation (Yu *et al.*, 2001), its overexpression is a negative prognostic factor (Nielsen *et al.*, 1996) and an independent risk factor of visceral relapse in breast cancer (Kim *et al.*, 2001). In multivariate analysis, a high level of cyclin E is significantly correlated with poor outcome (Keyomarsi *et al.*, 2002). Our studies demonstrated that TAM67 inhibits cyclin E protein expression and cyclin E-CDK2 kinase activity. Thus, cyclin E down regulation is also likely to contribute to the cell cycle arrest induced by TAM67. How TAM67 inhibits cyclin E expression and reduces cyclin E-CDK2 activity is currently under investigation. However, in the future, agents that inhibit the expression of both cyclin D and cyclin E (as does TAM67) may be particularly effective drugs for the treatment of breast cancer.

AP-1 transcription factors have also been implicated in the control of cell death and survival. Increased AP-1 activity may promote apoptosis in some cell types, while promoting survival in other cell types. Ectopic expression of c-Jun or c-Fos can induce apoptosis in sympathetic neurons as well as in mouse fibroblasts, Syrian hamster embryo cells and a human colorectal carcinoma cell line (Bossy-Wetzel *et al.*, 1997; Ham *et al.*, 1995; Preston *et al.*, 1996). In breast cancer cells, AP-1 sensitizes cells to Vitamin E succinate-induced apoptosis (Zhao *et al.*, 1997). On the other hand, in some circumstances inhibition of AP-1 activity may also promote apoptosis. The present results show that AP-1 inhibition induced by TAM67 sensitizes MCF-7 breast cancer cells to apoptosis, when these cells are starved of serum. Previous data are consistent with our results showing that reducing AP-1 activity in breast cancer cells caused increased cell death after treatment with UV light or cisplatin chemotherapy are consistent with our current results (Potapova *et al.*, 2001; Sauter *et al.*, 1999; Smith *et al.*, 1999).

The present results demonstrate that in the presence of serum Tam67 inhibits breast cancer growth predominantly by inducing the expression of cyclin dependent kinase inhibitors (such as p27),

by reducing the expression of the G1 cyclins, and by reducing CDK activity, thus leading to Rb hypophosphorylation, inhibition of E2F activity, and a G1 cell cycle block. This effectively blocks breast cell proliferation. These studies lay the foundation for future attempts to inhibit the activation of the AP-1 transcription factor for the prevention or treatment of cancer.

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Figure Legends:

Fig. 1. TAM67 inhibition of MCF 7 cell growth both in (a) serum present and (b) serum absent conditions. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 5 days, starved of fetal bovine serum for 2 more days. The cells were then stimulated with serum or without serum, then the cell proliferation was determined by MTS assay.

Fig. 2. TAM67 causes apoptosis in serum-free conditions. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, in the last 2 days cells were cultured in medium with serum or without serum. A. TUNEL assay was used to measure the apoptotic cells in different conditions. a, DOX (+), serum (+); b, DOX (+), serum (-); c, DOX (-), serum (+); d, DOX (-), serum (-). B. The percentage of apoptotic cells was determined using TUNEL assay data. C. Western-blotting assay was performed to measure the cleavage of PARP, a hallmark of apoptosis.

Fig. 3. TAM67 inhibition of ^{3}H-thymidine uptake in MCF 7 cells. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 5 days, starved of fetal bovine serum for 2 more days. The cells were then stimulated with serum or without serum, and ^{3}H-Thymidine incorporation assay was performed at time points.

Fig. 4. TAM67 inhibits normal cell cycle by causing G1 arrest. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, 48 hours before harvest the medium is changed to serum-free to synchronize cells. Then Flow Cytometry Assay was performed. a: TAM67 increases cell numbers in G0/G1 phase. b: TAM67 caused reduces cell numbers in S phase. c: TAM67 does not dramatically affect the cell distribution in G2/M phase.

Fig. 5. TAM67 cause Rb hypophosphorylation. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, and synchronized using nocodazole, then Rb phosphorylation status was determined by western-blotting assay. Rb hypophosphorylation was observed in the absence of DOX condition.

Fig. 6. TAM67 decreased E2F1 activity. MCF 7 Tet off TAM67 cells and MCF 7 Tet off vector cells were cultured in the presence or absence of DOX for 7 days, then the cells were cotransfected with the E2F1-luc reporter gene and pRL-TK, luciferase activity was measured and normalized with the Renilla activity. E2F1 activity was decreased in DOX absence condition in Tam67 cells, while there is no difference in vector cells between DOX present and absent conditions.

Fig. 7. Effect of TAM67 on the expression of cell cycle regulatory protein. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, synchronized using nocodazole, then

cells in M phase were replated in full medium and harvested at several time points. The cell cycle regulatory proteins expression were determined by Western-Blotting. TAM67 decreased cyclin E, Ds, CDK4, CDK6, and P21 expression, and increased p27 expression.

Fig. 8. Effect of TAM67 on CDK 2 and CDK 4 kinase activity. MCF 7 Tet off TAM67 cells were cultured in the presence or absence of DOX for 7 days, synchronized using nocodazole for 18 hours, then cells in M phase were replated in full medium and harvested at several time points. CDK 2 and CDK4 kinase assay were performed as described in materials and methods. CDK2 and Cyclin E protein expression in CDk2/Cyclin E complex, CDK4 and Cyclin D1 proteins expression in CDK 4/Cyclin D1 complex were determined by immunoprecipitation-western blotting. a: TAM67 suppressed Cyclin E expression and CDK2 kinase activity, while did affect CDK 2 protein expression. b: TAM67 inhibited Cyclin D1 and CDk4 expression, and suppress CDk4 activity in some time points.

Fig. 9. Mechanism of AP-1 blockade in breast cancer cells causes cell cycle arrest. AP-1 blockade causes decreased expression of D and E cyclins, and increases the expression of the CDK inhibitor p27, leads to reduced CDK2 and CDK4 activity, which in turn causes hypophosphorylation of Rb and inhibition of E2F activity, ultimately inducing a G1 cell cycle block.

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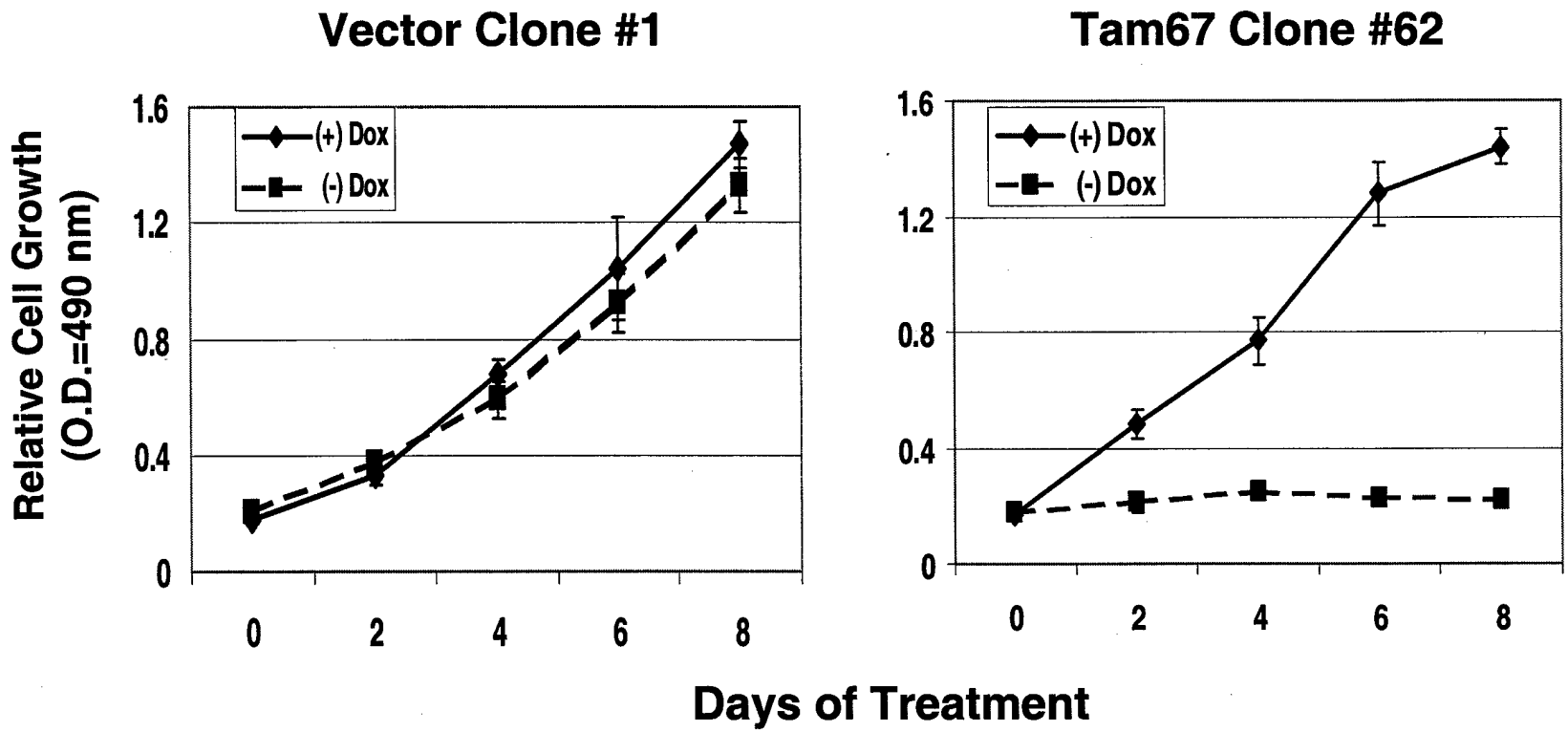
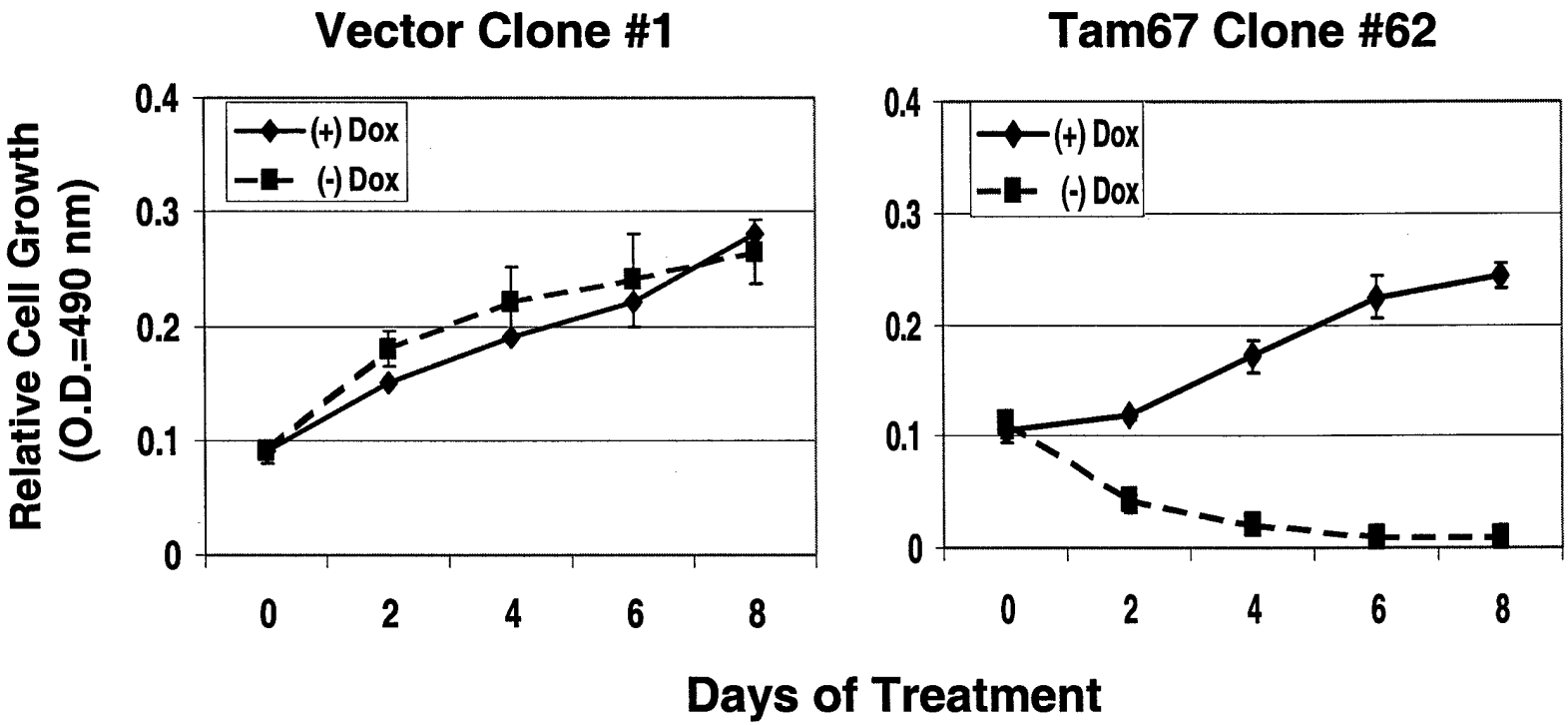

Vector Clone #1
Tam67 Clone #62
Relative Cell Growth
(O.D.=490 nm)
(+) Dox
(-) Dox
1.6
1.2
0.8
0.4
0
0
2
4
6
8
Days of Treatment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(O.D.=490 nm)
(+) Dox
(-) Dox
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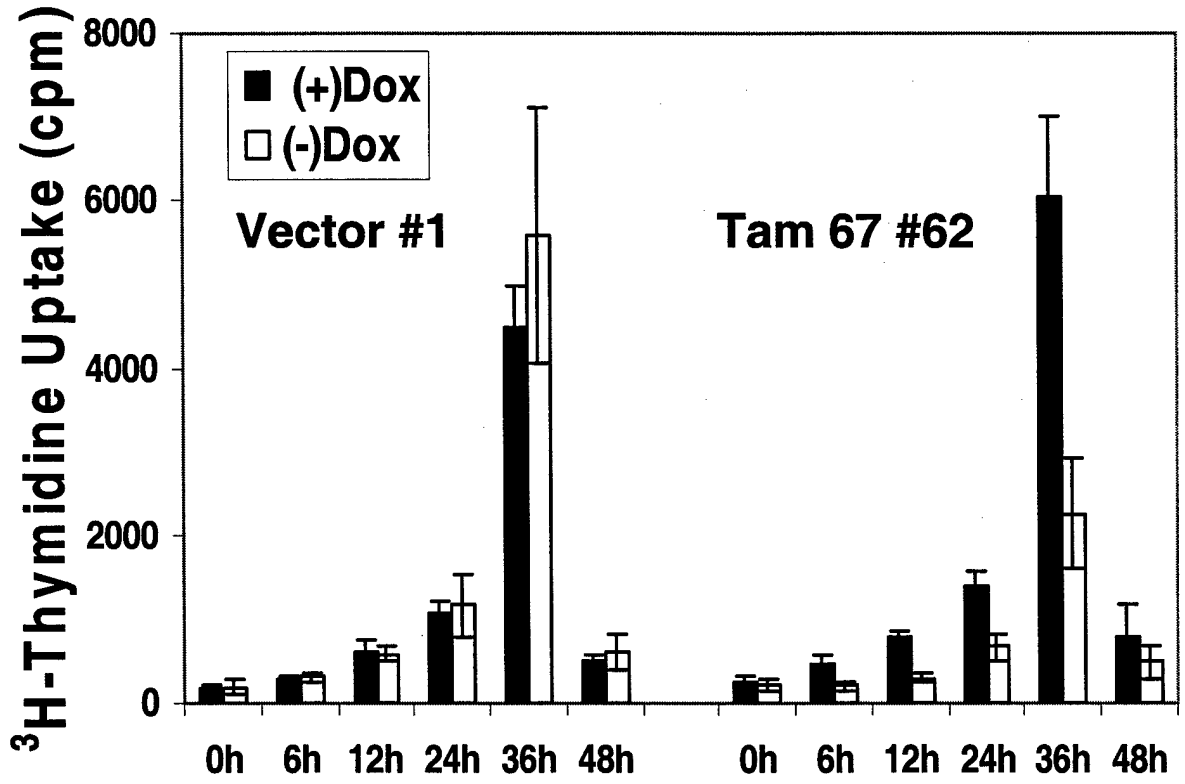
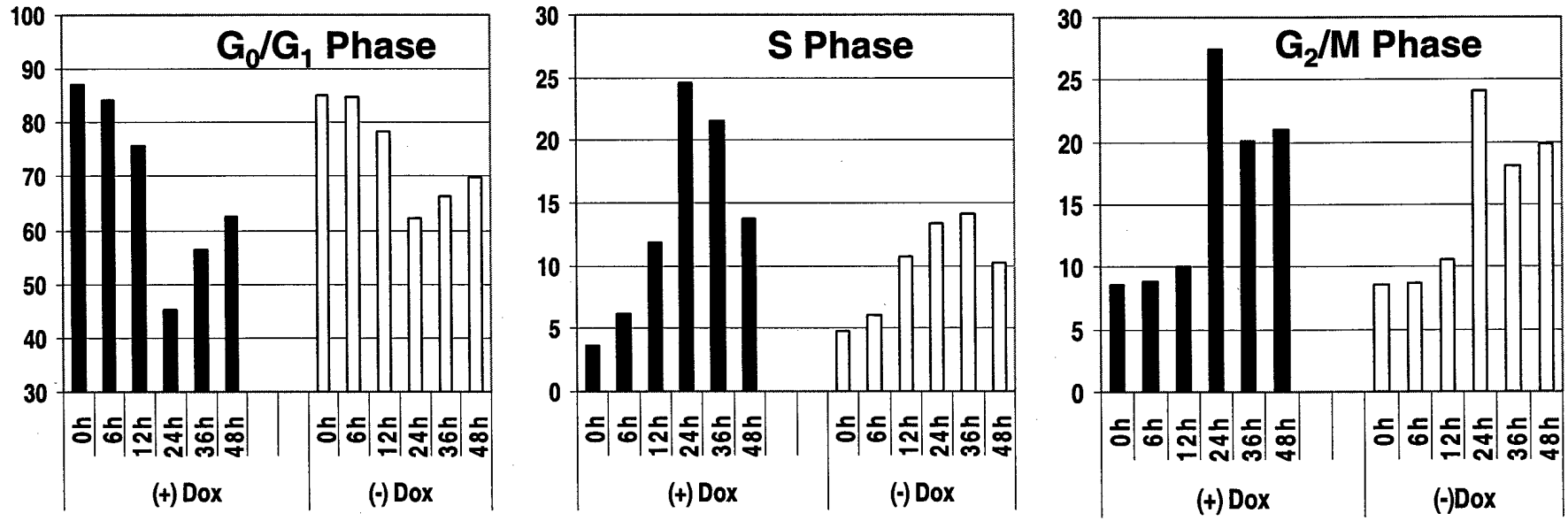
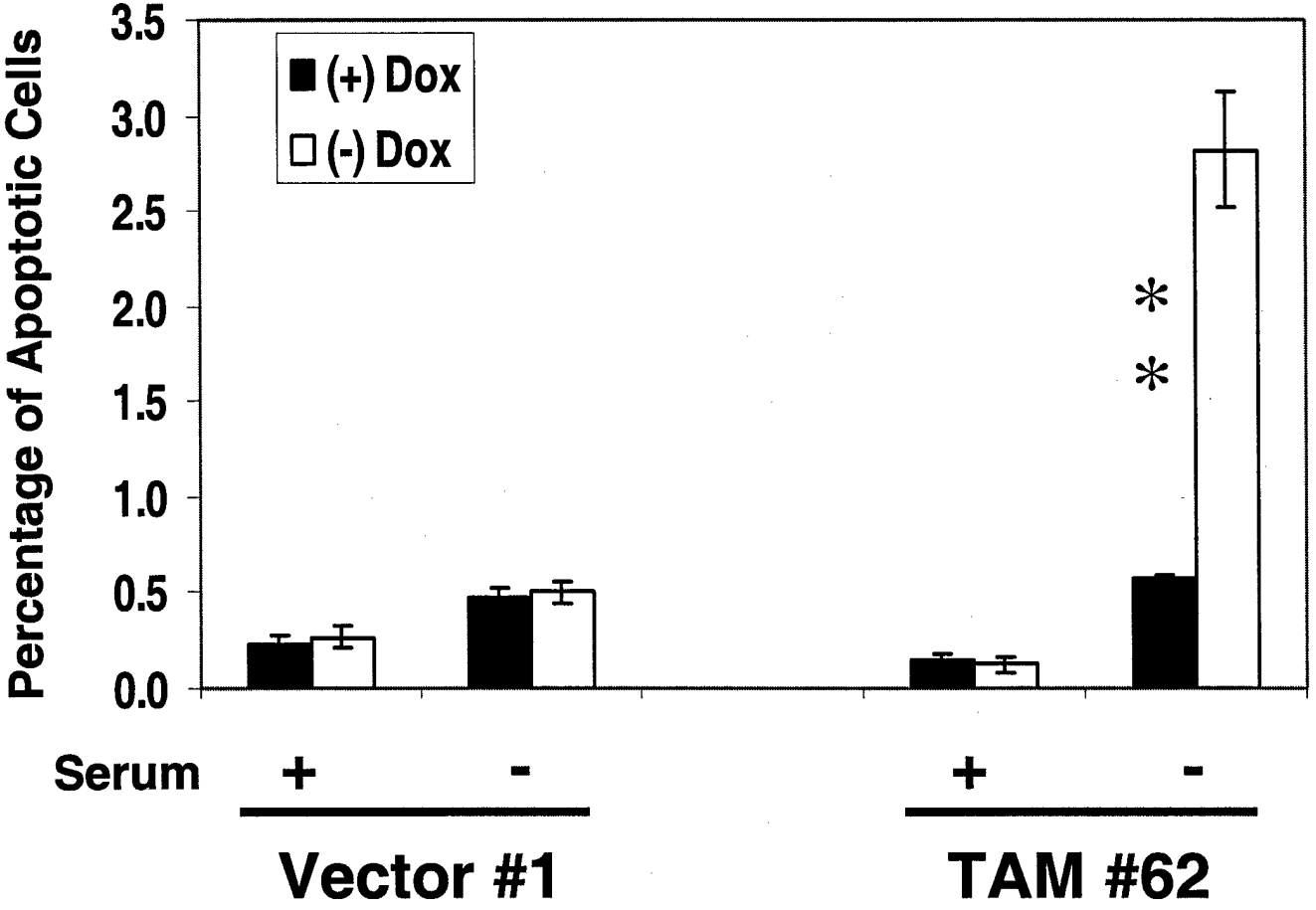
0.3
0.2
0.1
0
0
2
4
6
8
Days of Treatment

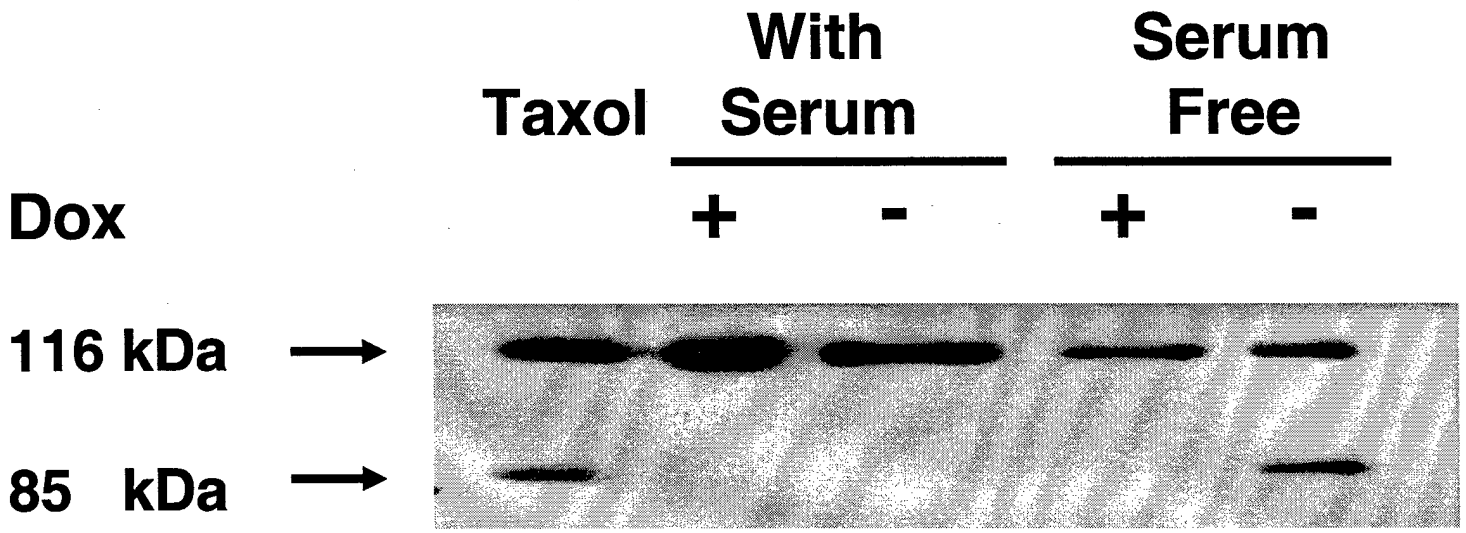

8000
6000
4000
2000
0
^{3}H-Thymidine Uptake (cpm)
(+)Dox
(-)Dox
Vector #1
Tam 67 #62
0h
6h
12h
24h
36h
48h
0h
6h
12h
24h
36h
48h

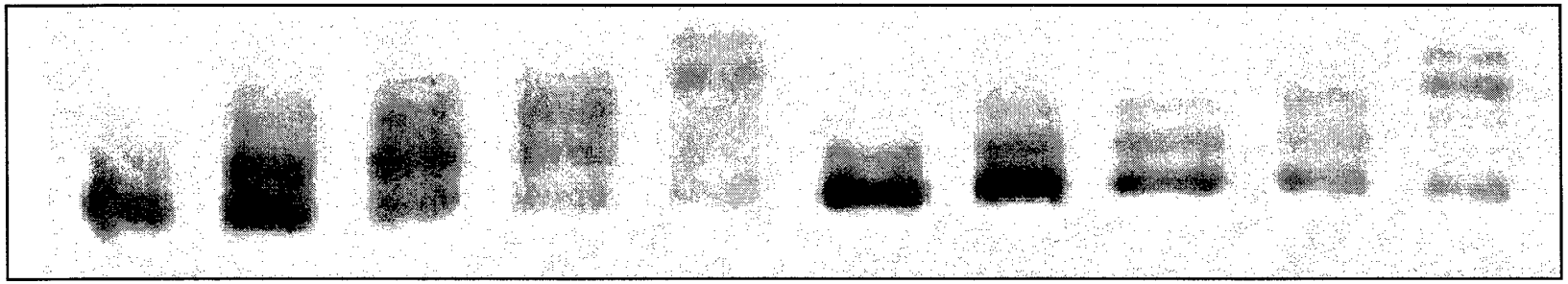

G_0/G_1 Phase
100
90
80
70
60
50
40
30
0h
6h
12h
24h
36h
48h
(+) Dox
(-) Dox
S Phase
30
25
20
15
10
5
0
G_2/M Phase
(-)Dox

a b c

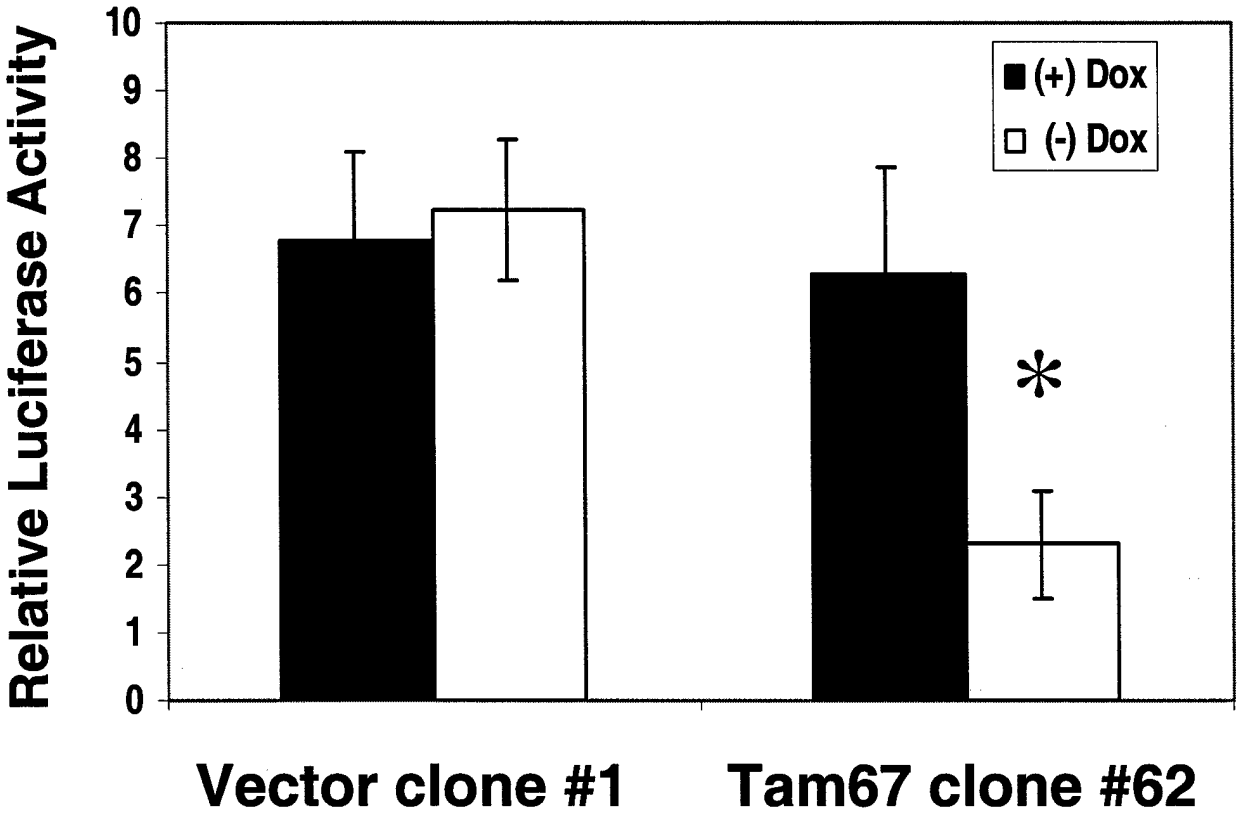
a
b
c
d

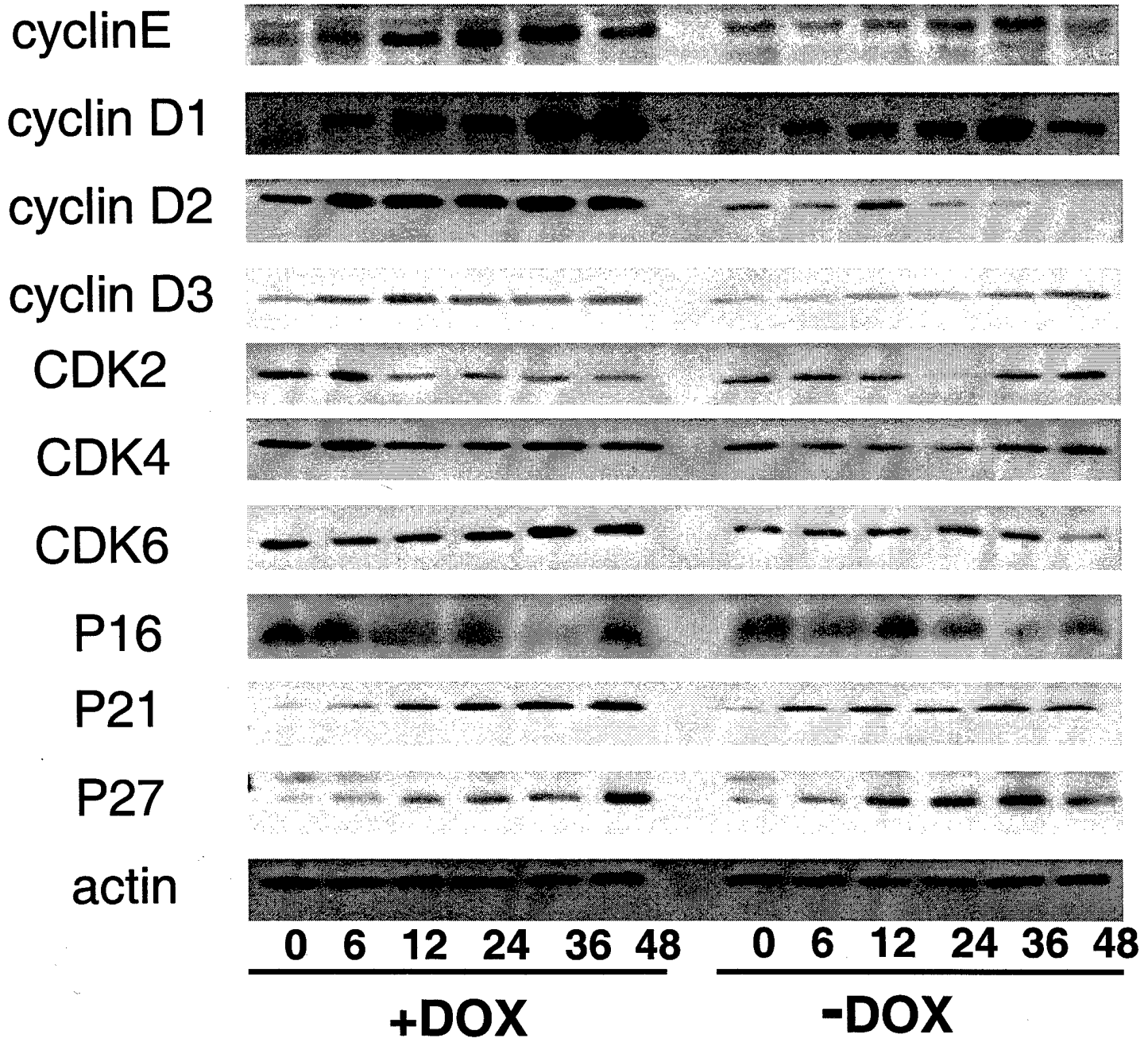

Percentage of Apoptotic Cells
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(+) Dox
(-) Dox
* *
Serum
+
-
+
-
Vector #1
TAM #62


Taxol
With Serum
Serum Free
Dox
+
-
+
-
116 kDa
85 kDa

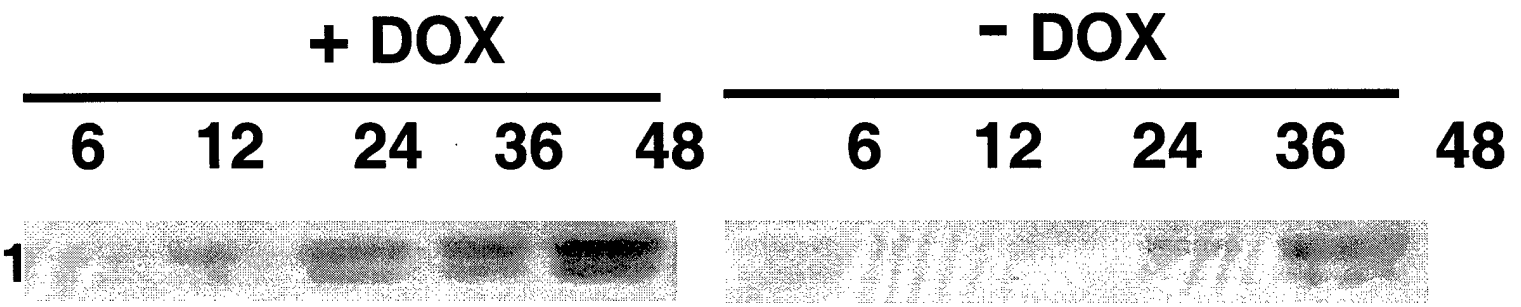


p-Rb/ total Rb	.16	.41	.66	.82	.84	.10	.28	.37	.42	.58
Hours After Nocodazole	6	12	24	36	48	6	12	24	36	48
	(+) Dox					(-) Dox				

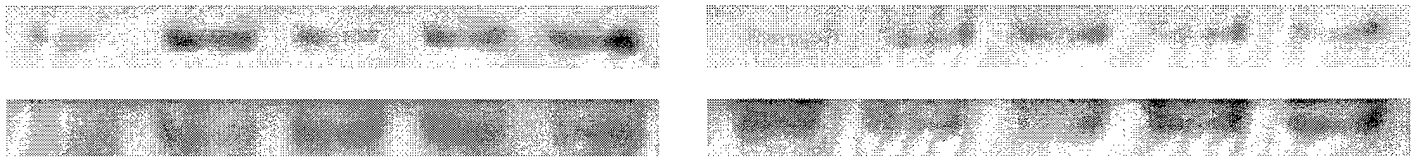

Relative Luciferase Activity
10
9
8
7
6
5
4
3
2
1
0
(+) Dox
(-) Dox
*
Vector clone #1
Tam67 clone #62


cyclinE
cyclin D1
cyclin D2
cyclin D3
CDK2
CDK4
CDK6
P16
P21
P27
actin
0 6 12 24 36 48
0 6 12 24 36 48
+DOX
-DOX

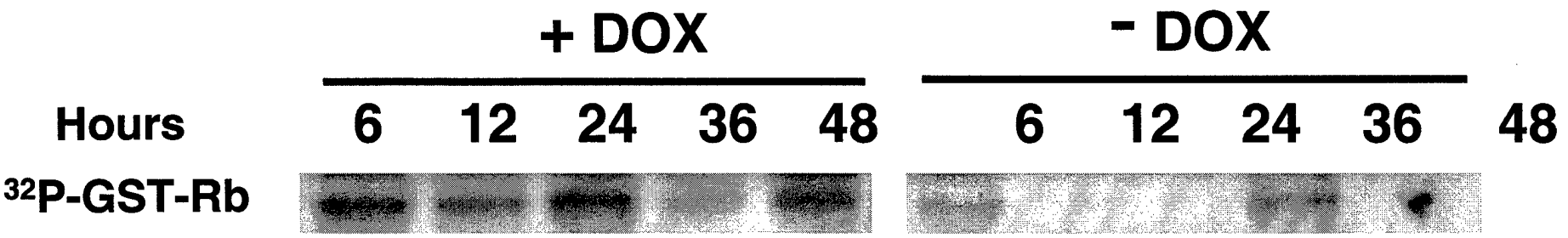
a. CDK2 Kinase Assay



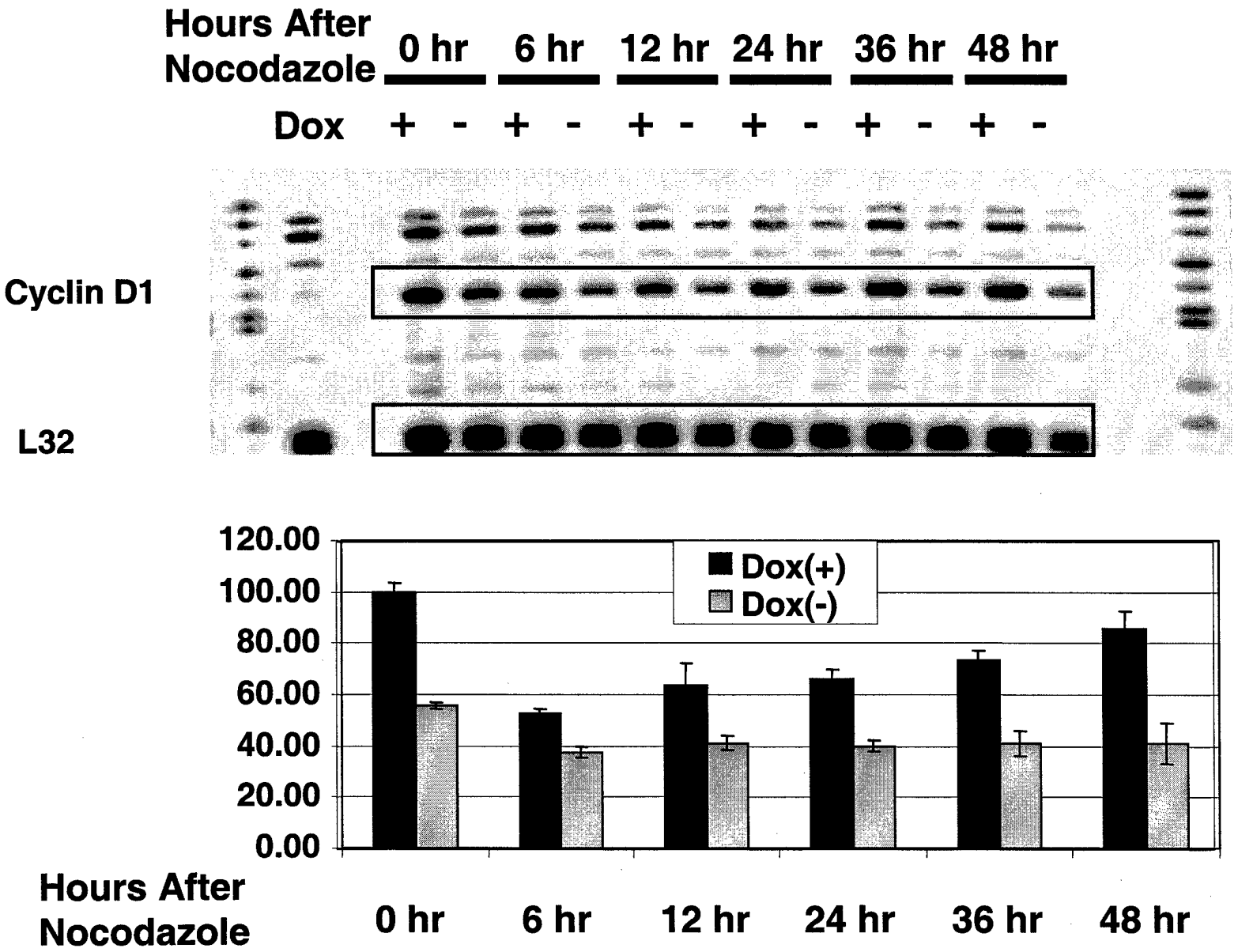
b. IP-western: IP with CDK2, Western with Cyclin E or CDK2

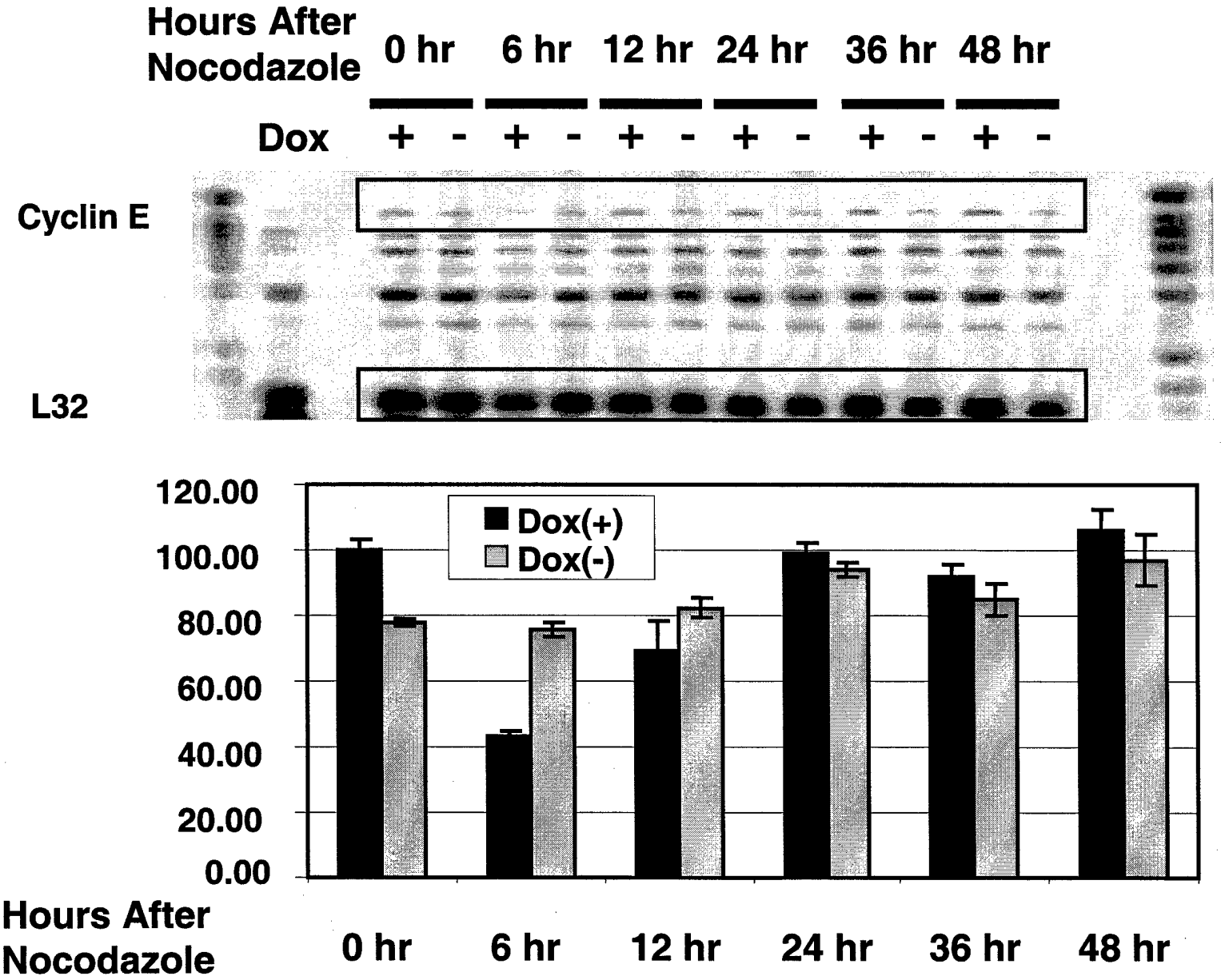


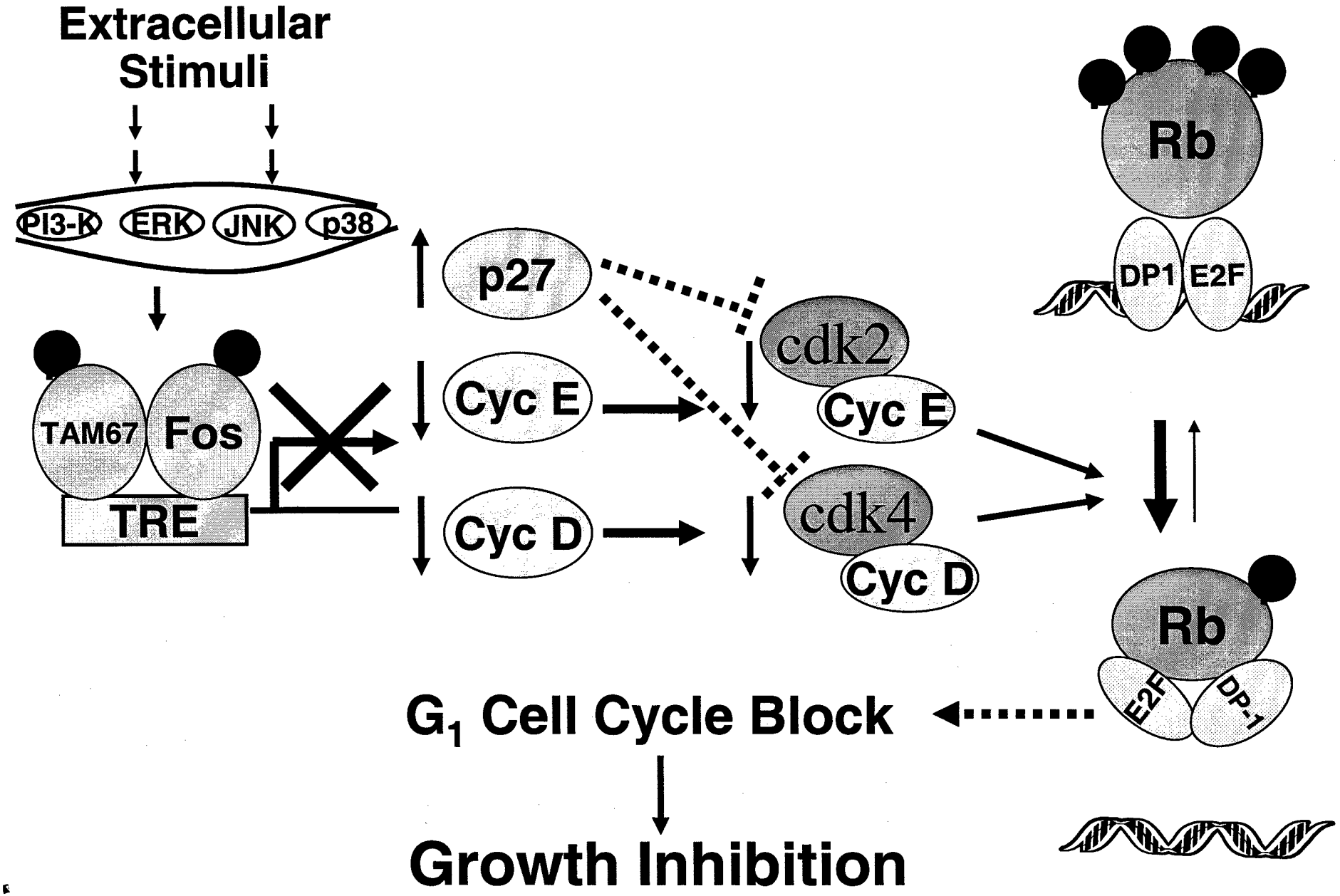
a. CDK4 Kinase Assay



b. IP-western: IP with CDK4, Western with Cyclin D1 or CDK4


Hours After Nocodazole
0 hr
6 hr
12 hr
24 hr
36 hr
48 hr
Dox + - + - + - + - + - + -
Cyclin D1
L32
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Dox(+)
Dox(-)
Hours After Nocodazole
0 hr
6 hr
12 hr
24 hr
36 hr
48 hr


Hours After Nocodazole
0 hr
6 hr
12 hr
24 hr
36 hr
48 hr
Dox
+ - + - + - + - + - + -
Cyclin E
L32
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Dox(+)
Dox(-)
Hours After Nocodazole
0 hr
6 hr
12 hr
24 hr
36 hr
48 hr


Extracellular Stimuli
PI3-K
ERK
JNK
p38
TAM67
Fos
TRE
p27
Cyc E
Cyc D
cdk2
Cyc E
cdk4
Cyc D
Rb
DP1
E2F
Rb
E2F
DP-1
G1 Cell Cycle Block
Growth Inhibition