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Antiapoptosis in Breast Cancer

PRINCIPAL INVESTIGATOR: Keng-hsueh Lan, M.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson
Cancer Center
Houston, Texas 77030

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Introduction

The c-erbB2 (or HER-2, *neu*) gene encodes a 185-kDa transmembrane glycoprotein (p185), which belongs to the EGF-r family. The c-erbB2 gene was found to be amplified and/or overexpressed in approximately 30% of human breast carcinomas. Our previous studies demonstrated that c-erbB2 overexpression could enhance metastatic potential and confer increased chemoresistance to breast cancer cells, thereby leading to poor clinical outcome in breast cancer patients. Also, we demonstrated that overexpression of the c-erbB2 gene can protect human breast cancer cells from apoptosis induced by the chemotherapeutic agent Taxol. One of the mechanisms is overexpression of c-erbB2 can upregulate p21^{Cip1}, which inhibits Taxol-mediated p34^{Cdc2} activation, delays cell entrance to G2/M phase, and thereby inhibits Taxol-induced apoptosis. This new finding provided an explanation for c-erbB2 mediated chemoresistance of breast cancer cells and also explored the importance of p21^{Cip1} in G2/M phase transition. Since we only use the 435 and its c-erbB2 transfectants in this study, we would like to further confirm this in other c-erbB2 overexpression breast cancer cell lines with different genetic background. Then we will find out whether other p34^{Cdc2} regulators contribute to Taxol resistance.

Body/Results

Resistance to apoptosis is a well-recognized mechanism for multidrug resistance. We first showed that induction of apoptosis is an important mechanism underlying the cytotoxic effects of Taxol on breast cancer cell lines, including MDA-MB-435 cells (Appendices Figure 1A). Then in the DNA fragmentation assays, we showed that the ErbB2 low-expressing MDA-MB-435, 435.neo and 435.neo.pool cells formed DNA ladders after 0.02 μ M Taxol treatment, whereas the appearance of the DNA ladder was inhibited in all four of the ErbB2-overexpressing 435.eB transfectants. (Figure 1B, C). DNA ladders in 435.eB transfectants appeared only when a 10-fold higher concentration of Taxol was added. The eB.1Ra and eB.2Ra revertants were derived from 435.eB1 and 435.eB2 cells after more than 15 passages that lost p185^{ErbB2} overexpression and they have lost apoptosis-resistance phenotype of the 435.eB1 and 435.eB2 cells. The results indicate that overexpression of p185^{ErbB2} in breast cancer cells can inhibit Taxol-induced apoptosis.

Our lab has previously demonstrated that Taxol-induced activation of p34^{Cdc2} kinase is required for Taxol-induced apoptosis (1). We compared the extent and kinetics of Taxol-induced activation of p34^{Cdc2} between the p185^{ErbB2}-low-expressing 435.neo cells and the p185^{ErbB2}-overexpressing 435.eB transfectants. Activation of p34^{Cdc2} kinase in 435.neo cells were detected 2 hr after addition of Taxol and it continued to increase compared to that in the control untreated cells (Figure 2A). However, activation of p34^{Cdc2} kinase in the Taxol-resistant 435.eB cells is reduced to only 47% of that in the 435.neo cells (Figure 2A). Furthermore, DNA fragmentation appeared in the 435.neo cells about 12 hr after treatment with 0.02 μ M of Taxol, and became pronounced 15 hr after treatment, while DNA fragmentation in the 435.eB cells was delayed and reduced, and this response in 435.eB1 cells correlated with their lower p34^{Cdc2} kinase activity (Figure 2B). The results suggest that inhibition of Taxol-mediated p34^{Cdc2} activation in 435.eB cells contributes to their resistance to Taxol-induced apoptosis.

Among several important mechanisms that regulate p34^{Cdc2} activity, phosphorylation on Tyr-15 of the p34^{Cdc2} protein by Wee1 kinase can negatively regulate p34^{Cdc2} activity. We compared the inhibitory Cdc2-Tyr15 phosphorylation in ErbB2-low-expressing parental MDA-MB-435

human breast cancer cells with that in 435.eB transfectants overexpressing p185^{ErbB2} (2). Western blot analysis using phosphospecific Cdc2-Tyr15 antibody showed that MDA-MB-435 cell had low levels of phosphorylation on Cdc2-Tyr15 while 435.eB transfectants exhibited much higher levels of Cdc2-Tyr15 phosphorylation (Figure 3). Expressions of Cdc25C phosphatase, which dephosphorylates p34^{Cdc2} on Tyr15, remained similar in these cell lines (1). For Wee1, the only known tyrosine kinase that phosphorylates p34^{Cdc2} on Tyr15, its expression and kinase activity are also similar in these cell lines (Figure 3). Thus, other tyrosine kinases that are activated or upregulated in 435.eB transfectants may be responsible for the increased phosphorylation of Cdc2-Tyr15.

Since p185^{ErbB2} is itself a tyrosine kinase overexpressed in 435.eB transfectants, we investigated the possibility that the ErbB2 tyrosine kinase may phosphorylate Cdc2-Tyr15 to inhibit p34^{Cdc2} activity. Cell lysates from five different human breast cancer cell lines that express p185^{ErbB2} at different levels (3) were immunoprecipitated using p34^{Cdc2} antibodies or control IgG, followed by immunoblotting for ErbB2 and Cdc2. The p185^{ErbB2} proteins were readily detected in the p34^{Cdc2} precipitates, but not the control IgG precipitates, from the 435.eB1, MDA-MB-361, and BT474 cells that overexpress p185^{ErbB2} (Figure 4). However, p185^{ErbB2} protein was not detected in the p34^{Cdc2} antibody precipitates from MDA-MB-231 and MDA-MB-435 cells that express low levels of p185^{ErbB2} (Figure 4). We also detected p185^{ErbB2} proteins in the anti-cyclin B precipitates from the BT474 cells (data not shown). These results indicated that p185^{ErbB2}, when overexpressed in human breast cancer cells, physically associates with the p34^{Cdc2}-cyclin B complex. Notably, Taxol-treated 435.eB1, MDA-MB-361, and BT474 cells showed more Cdc2-p185^{ErbB2} binding compared with untreated cells (Figure 4). This is likely due to accumulation of Taxol-treated cells at the G₂/M phase of the cell cycle, suggesting more Cdc2-p185^{ErbB2} association at the G₂/M phase. Our control experiment indicated that EGFR, another member of the ErbB RTK family, does not bind to Cdc2.

To test whether p185^{ErbB2} may phosphorylate p34^{Cdc2}, cell lysates from three cell lines were immunoprecipitated using either anti-p185^{ErbB2} antibody (Ab5) or control IgG and assayed for their ability to phosphorylate Cdc2 using GST-Cdc2N50 (amino acids 1 – 50 of Cdc2) fusion protein or GST protein (control) as substrates for *in vitro* kinase assays. The anti-p185^{ErbB2} precipitates of 435.eB1 and BT474 cells, but not of MDA-MB-435 cells, indeed contained a kinase or kinases that phosphorylated the GST-Cdc2N50 but not the GST protein (Figure 5).

To investigate the phosphorylation of p34^{Cdc2} by p185^{ErbB2}, three constructs encoding various histidine-tagged domains of the ErbB2 protein were constructed and expressed in *E. coli*. These include a wild-type tyrosine kinase domain of the ErbB2 protein (His-ErbB2KD^K), a kinase-defective mutant kinase domain of the ErbB2 protein that has a mutation at the ATP-binding site, changing Lys753 of p185^{ErbB2} to Met (His-ErbB2KD^M), and a wild-type extracellular domain of the ErbB2 protein (His-ErbB2ECD) (Figure 6). MDA-MB-435 cells transfected with these three constructs expressed mutant p185^{ErbB2} at levels comparable to those of the 435.eB transfectants (Figure 7A). Importantly, the K753M kinase-dead ErbB2 was unable to phosphorylate Cdc2N50, whereas the constitutive active ErbB2 (V659E), known to have higher kinase activity than the wt p185^{ErbB2}, phosphorylated Cdc2 to a slightly higher level compared with wt p185^{ErbB2} (Figure 7B). Thus, the ErbB2 tyrosine kinase activity is required for *in vivo* phosphorylation of Cdc2 by ErbB2.

To further explore the role of Cdc2-Tyr15 phosphorylation in ErbB2 inhibition of Taxol-induced apoptosis, these ErbB2 transfectants were compared for susceptibility to Taxol-induced apoptosis. Apoptosis was readily detected by DNA fragmentation assays in K753M

transfectants as was in parental MDA-MB-435 cells (Figure 8A). However, Taxol-induced apoptosis was inhibited in wt ErbB2 and V659E transfectants (Figure 8A). Double-label flow-cytometry analyses which simultaneously measure DNA content and DNA strand breaks showed that the majority of untreated cell lines were in G1 phase without significant DNA strand breaks (Figure 8B, top). After Taxol treatment, 47.2% of the p185^{ErbB2}-low expressing 435.neo cells progressed to G2/M phase and 28.9% of the cells had an increased dUTP labeling, indicating apoptosis (Figure 8B, bottom). In contrast, only 23.2% of the 435.eB cells progressed to G2/M phase and demonstrated minimal (0.1%) apoptosis. V659E transfectants were similar to 435.eB cells. Remarkably, 39.7% of the K753M transfectants progressed to G2/M phase and many cells died of apoptosis (18.8%). Notably, apoptosis in K753M cells is much less than that in MDA-MB-435 cells (28.9%), which is likely due to upregulation of p21^{Cip1}. On the other hand, the K753M mutant failed to block Taxol-induced apoptosis as effectively as the wt or V659E ErbB2, which is likely due to its defect in phosphorylating Cdc2-Tyr15. This indicates that the ErbB2 kinase-dependent phosphorylation of Cdc2-Tyr15 is involved in resistance to Taxol-induced apoptosis.

In addition to the finding that the activity of p34^{Cdc2} is important for Taxol-induced apoptosis, we also found that p21^{Cip1} plays an important role in inhibiting p34^{Cdc2} activation in 435.eB transfectants; the results have been published in our *Molecular Cell* paper (1). One of the first goals of this study is to determine whether the activity of p34^{Cdc2} is important in other breast cancer cell lines. We use erbB2-lowexpressing control 435.neo and erbB2-overexpressing 435.eB transfectants, both derived from the MDA-MB-435 breast cancer cell line; two Taxol resistant cell lines BT 474, MDA-MB-361 are also included to compare their p34^{Cdc2} activity. Since phosphorylation of tyrosine 15 residues is an indication of inhibition of p34^{Cdc2} activity, we used immunoblotting with phospho-tyr-15 specific antibody to determine the activity of p34^{Cdc2} in these cell lines. The phosphorylation of tyrosine 15 is higher in erbB2-overexpressing (Taxol resistant) cell lines compared with erbB2-lowexpressing (Taxol sensitive) cell lines. This result indicates a positive relationship between erbB2 overexpression and increased phosphorylation on cdc2-Tyr15.

Since Wee1 and Cdc25C are two important regulators to regulate the phosphorylation status on p34^{Cdc2} tyrosine 15 residues, we screened these two factors by using immunoblotting. Expressions of Cdc25C phosphatase, which dephosphorylates p34^{Cdc2} on Tyr15, remained similar in these cell lines (1). For Wee1, the only known tyrosine kinase that phosphorylates p34^{Cdc2} on Tyr15, its expression and kinase activity are also similar in these cell lines. Thus, other tyrosine kinases that are activated or upregulated in 435.eB transfectants may be responsible for the increased phosphorylation of Cdc2-Tyr15. We also screened other p34^{Cdc2} regulators p21^{Cip1} and cyclin B1(1). As expected, p21^{Cip1} expression level is higher in BT 474 and 361 cell lines whereas cyclin B1 expression level is much lower in these two cell lines(1). From the data above, multiple regulators are involved in regulating the Taxol sensitivity.

Key Research Accomplishments

- We found that p21^{Cip1} plays an important role in inhibiting p34^{Cdc2} activation in 435.eB transfectants.
- We have produced a panel of wild-type and mutant erbB2 gene transfectants (kinase-dead His-ErbB2KD^M, wild-type extracellular domain of the ErbB2 protein His-ErbB2ECD).

These can serve as a good system for further investigation of antiapoptotic signaling of erbB2 gene.

- Validation of our hypothesis that other p34^{Cdc2} regulators contribute to Taxol resistance. Notably, we found that the ErbB2 tyrosine kinase activity is required for *in vivo* phosphorylation of Cdc2 by ErbB2.
- We demonstrated that the ErbB2 kinase-dependent phosphorylation of Cdc2-Tyr15 is involved in resistance to Taxol-induced apoptosis.

Reportable Outcomes

To date we have published one paper (Yu, D., Jing, T., Liu, B., Yao, J., Tan, M., McDonnell, T. J., and Hung, M.-C. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21^{Cip1}, which inhibits p34^{Cdc2} kinase, *Molecular Cell*. 2: 581-591, 1998), in which we acknowledged this predoctoral fellowship.

With the support of this award, the previous P.I., Tong Jing, acquired his Ph.D. degree from the Graduate School of Biomedical Sciences, University of Texas – Houston.

With the support of this award, the current P.I., Keng-hsueh Lan, is pursuing the Ph.D. degree in the Graduate School of Biomedical Sciences, University of Texas – Houston.

The majority of the figures included in this report are being prepared for manuscript submission.

Conclusions

Overexpression of p185^{erbB2} in breast cancer cells can inhibit Taxol-induced apoptosis. Our lab has demonstrated that Taxol-induced activation of p34^{Cdc2} kinase is required for Taxol-induced apoptosis. We found that p21^{Cip1} plays an important role in inhibiting p34^{Cdc2} activation in 435.eB transfectants. We also showed that multiple regulators are involved in regulating the activity of p34^{Cdc2} kinase and the Taxol sensitivity. With the panel of wild-type and mutant erbB2 gene transfectants (kinase-dead His-ErbB2KD^M, wild-type extracellular domain of the ErbB2 protein His-ErbB2ECD), we are able to demonstrate that the ErbB2 tyrosine kinase activity is required for *in vivo* phosphorylation of Cdc2 by ErbB2, rendering the breast cancer cells resistant to Taxol treatment. p34^{Cdc2} kinase may be an important target for the treatment of Taxol resistance.

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1. Yu, D., Jing, T., Liu, B., Yao, J., Tan, M., McDonnell, T. J., and Hung, M.-C. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21^{Cip1}, which inhibits p34^{Cdc2} kinase, *Molecular Cell*. 2: 581-591, 1998a.
2. Yu, D., Liu, B., Tan, M., Li, J., Wang, S.-S., and Hung, M.-C. Overexpression of c-erbB-2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms, *Oncogene* 13, 1359-1365, 1996
3. Yu, D., Liu B., Sun D., Jing T., Price J. E., Singletary S. E., Hortobagyi G. N., and Hung M.-C. *Oncogene* 16, 2087-2094, 1998b

Appendices

1. One copy of publication: Yu, D., Jing, T., Liu, B., Yao, J., Tan, M., McDonnell, T. J., and Hung, M.-C. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21^{Cip1}, which inhibits p34^{Cdc2} kinase, *Molecular Cell*. 2: 581-591, 1998
2. Figure 1-2
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Overexpression of ErbB2 Blocks Taxol-Induced Apoptosis by Upregulation of p21^{Cip1}, which Inhibits p34^{Cdc2} Kinase

Dihua Yu,*[§] Tong Jing,* Bolin Liu,* Jun Yao,*
Ming Tan,* Timothy J. McDonnell,[†]
and Mien-Chie Hung[‡]

*Department of Surgical Oncology

[†]Department of Molecular Pathology

[‡]Department of Tumor Biology

The University of Texas

M. D. Anderson Cancer Center

Houston, Texas 77030

Summary

Overexpression of the receptor tyrosine kinase p185^{ErbB2} confers Taxol resistance in breast cancers. Here, we investigated the underlying mechanisms and found that overexpression of p185^{ErbB2} inhibits Taxol-induced apoptosis. Taxol activates p34^{Cdc2} kinase in MDA-MB-435 breast cancer cells, leading to cell cycle arrest at the G2/M phase and, subsequently, apoptosis. A chemical inhibitor of p34^{Cdc2} and a dominant-negative mutant of p34^{Cdc2} blocked Taxol-induced apoptosis in these cells. Overexpression of p185^{ErbB2} in MDA-MB-435 cells by transfection transcriptionally upregulates p21^{Cip1}, which associates with p34^{Cdc2}, inhibits Taxol-mediated p34^{Cdc2} activation, delays cell entrance to G2/M phase, and thereby inhibits Taxol-induced apoptosis. In p21^{Cip1} antisense-transfected MDA-MB-435 cells or in p21^{-/-} MEF cells, p185^{ErbB2} was unable to inhibit Taxol-induced apoptosis. Therefore, p21^{Cip1} participates in the regulation of a G2/M checkpoint that contributes to resistance to Taxol-induced apoptosis in p185^{ErbB2}-overexpressing breast cancer cells.

Introduction

The *ErbB2* (or *HER-2*, *neu*) gene encodes a 185 kDa transmembrane glycoprotein (p185), and it was found to be amplified, overexpressed, or both in approximately 30% of human breast carcinomas (Slamon et al., 1987). Individuals with such carcinomas had a significantly lower overall survival rate and a shorter time to relapse than did patients whose tumors did not overexpress ErbB2. We previously demonstrated that ErbB2 overexpression can confer breast cancer cells increased resistance to paclitaxel (Taxol) (Yu et al., 1996). Our experimental findings are supported by recent reports from a phase III clinical trial that the response rate to Taxol was significantly improved in breast cancer patients when ErbB2 was downregulated using Herceptin antibodies that bind to the extracellular domain of ErbB2 (Dickman, 1998). However, the molecular mechanisms underlying ErbB2-mediated Taxol resistance in breast cancers were not defined.

Taxol is a potent and highly effective antineoplastic agent for the treatment of advanced, drug-refractory, metastatic breast cancers (Holmes et al., 1991). At the

cellular level, Taxol induces tubulin polymerization and microtubule formation (Horwitz, 1992), blocks the cell cycle in mitosis, and induces programmed cell death (apoptosis) (Wahl et al., 1996). At the biochemical level, Taxol has been shown to increase the release of tumor necrosis factor (TNF) (Ding et al., 1990), to increase lipopolysaccharide-inducible genes and protein-tyrosine phosphorylation (Manthey et al., 1992), and to activate p34^{Cdc2} inappropriately (Donaldson et al., 1994). However, the molecular pathway of Taxol-mediated cytotoxicity and apoptosis remains to be elucidated.

Apoptosis is a predominant mechanism by which cancer chemotherapeutic agents (including Taxol) kill cells (Fisher, 1994). The failure of cancer cells to detect drug-induced damage and to activate apoptosis may lead to multidrug resistance. Many intracellular signaling pathways involving growth factor receptors and their downstream signaling molecules may converge in a common apoptosis-regulatory mechanism (Collins and Rivas, 1993). Although it is known that overexpression of ErbB2 receptor confers resistance to Taxol (Yu et al., 1996), the potential impact of ErbB2 overexpression on Taxol-induced apoptosis has not been previously explored.

p34^{Cdc2}-cyclin B complexes are known to catalyze chromosomal condensation and nuclear envelope breakdown during mitosis. Growing evidence indicates that p34^{Cdc2}-cyclin B may play a role in the nuclear changes accompanying apoptosis. Activation of the p34^{Cdc2} kinase at the G2/M transition is precisely controlled by multiple regulatory factors including cyclin B, Cdc25C, Myt1, Wee1, and others (for review, see Coleman and Dunphy, 1994). Interestingly, when Cdk2 kinase activity in extracts of cycling *Xenopus* eggs was inhibited by p21^{Cip1}, mitosis was blocked and inactive p34^{Cdc2}-cyclin B accumulated (Guadagno and Newport, 1996). Whether p21^{Cip1} may directly inhibit p34^{Cdc2} requires further investigation.

p21^{Cip1} (p21^{waf1}, p21^{sd1}) encodes a 21 kDa protein and was discovered as a Cdk inhibitor (Harper et al., 1993) as well as a wild-type p53-inducible gene (El-Deiry et al., 1993). Later, p21^{Cip1} was considered critical for the coordination of the S and M phases of the cell cycle, because doxorubicin-treated p21^{-/-} cancer cells but not parental p21^{+/+} cells experienced multiple rounds of S phase without mitosis and then became apoptotic (Waldman et al., 1996). Although p21^{Cip1} has recently been shown to contribute to regulation of the G2/M transition (Dulic et al., 1998), the biological impact of such regulation when cells suffer DNA damage or disorder in mitosis (e.g., exposure to Taxol) has not been clearly demonstrated.

In an attempt to understand the molecular mechanisms underlying ErbB2-mediated Taxol resistance, we conducted the current study and found that ErbB2 blocks Taxol-induced apoptosis in breast cancer cells by upregulation of p21^{Cip1}, which participates in the negative regulation of Taxol-mediated p34^{Cdc2} activation required for Taxol-induced apoptosis. We provide experimental evidence for the role of p21^{Cip1} in the regulation of G2/M checkpoint and the impact of such regulation on Taxol-mediated apoptosis in breast cancer cells. This

[§]To whom correspondence should be addressed.

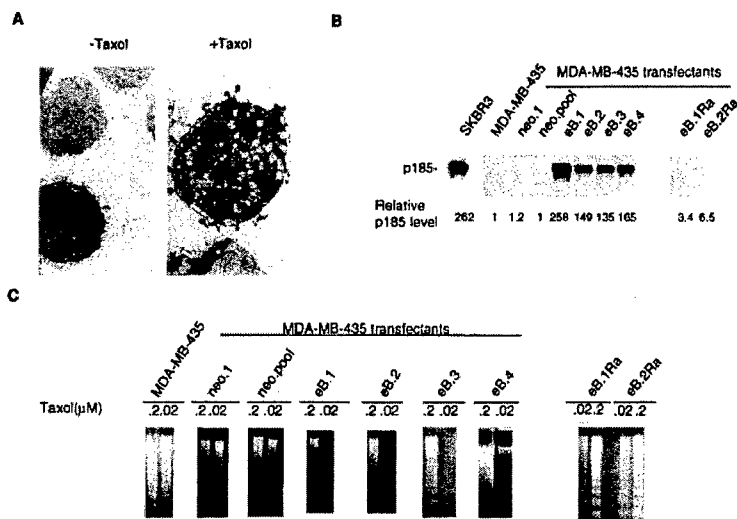


Figure 1. Overexpression of p185^{ErbB2} inhibited Taxol-Mediated Apoptosis

(A) Morphology of MDA-MB-435 cells cultured in media without or with 0.02 μM Taxol for 24 hr under transmission electron microscopy (TEM). Magnification of the originals was 2000×.

(B) Western blot analysis of p185^{ErbB2} in the indicated cell lines. The relative p185 levels represent the amount of p185 in each cell line as fold of that of the MDA-MB-435 cells and were determined by quantitation with the Personal Densitometer 50371 (Molecular Dynamics, Sunnyvale, CA).

(C) DNA fragmentation assays showing that overexpression of p185^{ErbB2} in MDA-MB-435 transfectants inhibited Taxol-induced apoptosis. The low molecular weight DNAs were isolated from the indicated cell lines cultured in media containing indicated concentrations of Taxol (0.02 or 0.2 μM) and processed for DNA fragmentation assays.

study has revealed a plausible molecular mechanism underlying the Taxol resistance phenomenon in breast cancers and potentially other cancer types that overexpress ErbB2.

Results

Breast Cancer Cells Overexpressing p185^{ErbB2} Are More Resistant to Taxol-Induced Apoptosis

The MDA-MB-435 human breast cancer cell line contains only one copy of the *ErbB2* gene per haploid, expresses low levels of p185^{ErbB2} protein, and is highly sensitive to Taxol cytotoxicity (Yu et al., 1996). To investigate whether Taxol elicited cytotoxicity by induction of apoptosis, untreated and Taxol-treated MDA-MB-435 cells were examined for apoptotic morphology using light microscopy and transmission electron microscopy (TEM). Dramatic morphological changes specific to apoptotic cells (e.g., chromatin condensation, formation of membrane-bound apoptotic bodies, etc.) were observed in Taxol-treated cells but not in the untreated cells (Figure 1A). We also detected DNA fragmentation in Taxol-treated MDA-MB-435 cells (Figure 1C). Thus, induction of apoptosis is an important mechanism underlying the cytotoxic effects of Taxol on these breast cancer cells.

To study whether p185^{ErbB2} overexpression may confer Taxol resistance by blocking Taxol-induced apoptosis, we used a panel of cell lines with the same genetic background but expressing different levels of p185^{ErbB2} (Figure 1B). The 435.eB transfectants (eB1, eB2, eB3, and eB4) were generated by transfecting MDA-MB-435 cells with the pCMV**erbB2** plasmid containing the full-length normal human *ErbB2* cDNA, and they express p185^{ErbB2} at 258-, 149-, 135-, and 165-fold, respectively, that of the parental MDA-MB-435 cells (Yu et al., 1996). The p185^{ErbB2} levels in these 435.eB transfectants are comparable to, or lower than, that in the SKBR3 breast cancer cells established from a different primary breast tumor, which express p185^{ErbB2} at 262-fold of that of the MDA-MB-435 cells (Yu et al., 1996). The control 435.neo.1 and 435.neo.pool cell lines were established

by transfecting the neomycin resistance gene pSV2-neo alone into MDA-MB-435 cells, and they express p185^{ErbB2} at levels similar to that of the MDA-MB-435 cells (Yu et al., 1996). The eB.1Ra and eB.2Ra are two spontaneous revertants derived from the 435.eB1 and 435.eB2 that lost the transfected *ErbB2* gene and do not overexpress p185^{ErbB2} anymore. These cell lines that express different levels of p185^{ErbB2} were assayed for susceptibility to Taxol-induced apoptosis by DNA fragmentation assays (Figure 1C). Taxol-treated MDA-MB-435, 435.neo, and 435.neo.pool cells (0.02 μM) formed DNA ladders, whereas DNA ladder formation was inhibited in all four of the 435.eB transfectants. DNA ladders in 435.eB transfectants appeared only when a 10-fold higher concentration of Taxol was added. The eB.1Ra and eB.2Ra revertants demonstrated DNA ladders similar to that of the parental MDA-MB-435 cells, that is, these revertants have lost the apoptosis-resistant phenotype of the 435.eB1 and 435.eB2 cells due to the loss of p185 expression. The results indicate that overexpression of p185^{ErbB2} in breast cancer cells can inhibit Taxol-induced apoptosis.

Taxol Induces Apoptosis at the G2/M Phase of the Cell Cycle, which Is Impeded by Overexpression of p185^{ErbB2}

Taxol was previously shown to induce phosphorylation of Bcl-2, thereby inactivating Bcl-2 and inducing apoptosis in several cancer types (Blagosklonny et al., 1996). Phosphorylated Bcl-2 proteins can be detected as slower migrating bands in Western blots. No significant difference in Bcl-2 protein levels or Bcl-2 phosphorylation was observed between Taxol-treated, apoptosis-sensitive MDA-MB-435 cells and apoptosis-resistant 435.eB transfectants by Western blot analysis using Bcl-2 antibody (Figure 2A). Therefore, inhibition of Taxol-induced apoptosis by p185^{ErbB2} was unlikely to be the result of increased Bcl-2 activity.

It has been recognized that the position of cells in the cell cycle may play a role in determining susceptibility to apoptosis in many cell types (Meikrantz and Schlegel, 1996). To explore the mechanisms of Taxol-induced

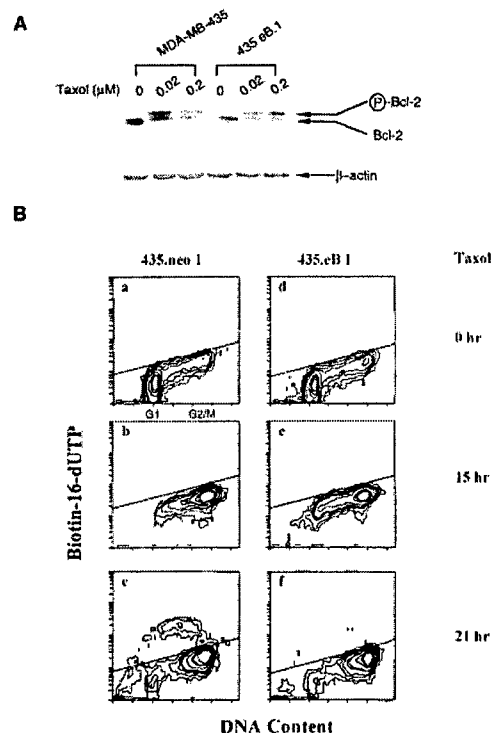


Figure 2. p185^{ErbB2} Inhibits Taxol-Induced Apoptosis by Delaying Cell Entrance to G2/M Phase of the Cell Cycle but Not by Increasing Bcl-2

(A) Bcl-2 protein levels and induction of Bcl-2 phosphorylation are similar between MDA-MB-435 and 435.eB cells. Immunoblot analysis of the Bcl-2 proteins in the cell lysates of the MDA-MB-435 cells and 435.eB1 transfectants treated with Taxol-free media (0) or with media containing the indicated concentrations of Taxol (0.02, 0.2) was performed using an antibody against human Bcl-2. Positions of the phosphorylated [(p)-Bcl-2] and nonphosphorylated Bcl-2 (Bcl-2) are indicated on the right. Immunoblotting of β -actin was used as the protein loading control.

(B) Taxol induces apoptosis at the G2/M phase of the cell cycle, which is inhibited by p185^{ErbB2}. The 435.neo and 435.eB1 transfectants were treated with 0.02 μ M Taxol for 0, 15, or 21 hr and were harvested for double-label flow cytometry analyses. Apoptotic cells with DNA strand breaks are shown to have higher levels of biotin-16-dUTP labeling above the sloped lines, which were placed based on the biotin-16-dUTP signal levels in the control cells without Taxol.

apoptosis and p185^{ErbB2}-mediated antiapoptosis, we examined the cell cycle stage or stages where Taxol induced apoptosis by multiparameter flow cytometry. Cell cycle stage and apoptosis were simultaneously determined by measuring DNA content and by labeling DNA strand breaks with biotinylated dUTP (Darzynkiewicz et al., 1992). Flow cytometric analysis demonstrated that the majority of the untreated 435.neo control cells were at the G1 phase of the cell cycle without significant DNA strand breaks (Figure 2Ba). After 15 hr of 0.02 μ M Taxol treatment, 72% of these p185-low-expressing cells progressed to the G2/M phase (Figure 2Bb). After 21 hr of Taxol treatment, more of these cells were arrested at the G2/M phase, and some of these cells were at the sub-G1 phase indicative of apoptosis (Figure 2Bc). That the 435.neo cells were arrested at G2/M by Taxol seems

to be an abortive event, since 21% of cells died at the G2/M phase as demonstrated by a high-level dUTP labeling indicating apoptotic DNA strand breaks (Figure 2Bc, over the sloped line). In contrast, the Taxol-resistant 435.eB1 cells progressed less effectively toward the G2/M phase after 15 hr of Taxol treatment (55%, Figure 2Be) and demonstrated minimal apoptotic DNA strand breaks (5%) after 21 hr of Taxol treatment (Figure 2Bf), despite untreated 435.eB cells showing a similar cell cycle distribution profile to that of the untreated 435.neo cells (Figure 2Bd). These results indicate that Taxol-induced apoptosis of the 435.neo cells occurs preferentially at the G2/M phase of the cell cycle, whereas overexpression of p185^{ErbB2} in 435.eB cells delays Taxol-mediated cell entrance to the G2/M phase, thereby inhibiting Taxol-induced apoptosis.

Taxol Induces G2/M Arrest and Apoptosis by Activation of p34^{Cdc2}-Cyclin B Kinase

Entry to mitosis is delicately controlled by the Cdk-cyclin complex consisting of the p34^{Cdc2} protein kinase and cyclin B (Draetta et al., 1989). Here, we show that Taxol can activate the p34^{Cdc2}-cyclin B1 kinase in MDA-MB-435 breast cancer cells (Figures 3A and 3C, lane 1 versus lane 4), which correlated with the induction of apoptosis at the G2/M phase (Figure 3Db). To determine whether the activation of p34^{Cdc2}-cyclin B1 kinase was required for Taxol-induced apoptosis, we investigated the effects of inhibiting p34^{Cdc2} kinase on Taxol-induced apoptosis by two approaches. First, we examined the effects of olomoucine, a potent chemical inhibitor of p34^{Cdc2}, on Taxol-induced apoptosis. MDA-MB-435 cells were cultured for 24 hr in Taxol-free media or Taxol-containing media with the addition of isoolomoucine (a control chemical) or olomoucine, respectively. The differently treated cells were then assayed for the p34^{Cdc2} kinase activities and apoptotic cell death. Olomoucine, but not isoolomoucine, reduced (30%) Taxol-mediated activation of p34^{Cdc2} (Figure 3A). Parallel to the inhibition of p34^{Cdc2} kinase activity, olomoucine effectively inhibited Taxol-induced apoptosis, since flow cytometry demonstrated that MDA-MB-435 cells treated with olomoucine plus Taxol had dramatically reduced sub-G1 apoptotic cells compared with cells treated with Taxol alone or with isoolomoucine plus Taxol (Figure 3B).

Since olomoucine functions by competing for the ATP binding domain of the kinase and may have weak inhibitory effects on other Cdk (Vesely et al., 1994), our second approach was to specifically inhibit p34^{Cdc2} using the dominant-negative mutant of p34^{Cdc2}, pCMVcdc2-dn (van den Heuvel and Harlow, 1993). MDA-MB-435 cells were transfected with the pCMVcdc2-dn, or pCMVneo for 36 hr, then cultured with or without Taxol for an additional 21 hr and harvested. RNA expression from the transfected Cdc2-dn was detected by RT-PCR using PCR primers that specifically amplify Cdc2-dn (Figure 3G). Taxol-mediated activation of p34^{Cdc2} was reduced in the pCMVcdc2-dn-transfected cells (76% reduction), whereas no discernible inhibitory effect was observed in the pCMVneo-transfected cells (Figure 3C). Meanwhile, Cdc2-dn retarded Taxol-induced cell progression to

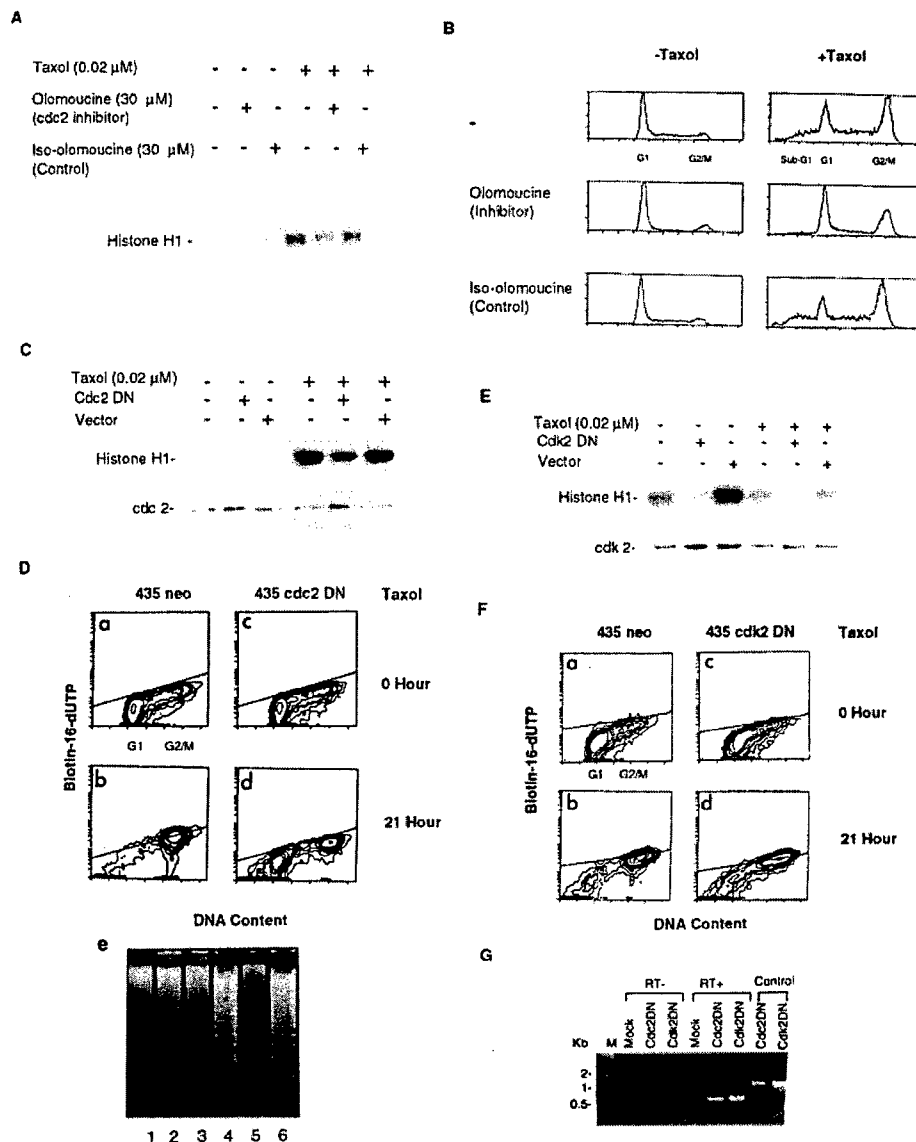


Figure 3. Activation of p34^{Cdc2} Kinase Is Required for Taxol-Mediated Apoptosis

(A) Inhibition of p34^{Cdc2} kinase activity by olomoucine. MDA-MB-435 cells were cultured for 24 hr in Taxol-free media, or Taxol-containing media (0.02 μ M), alone, with addition of iso-olomoucine, or with olomoucine. These differently treated cells were then assayed for p34^{Cdc2} kinase activities by determining their ability to phosphorylate histone H1 substrate.

(B) Olomoucine inhibited Taxol-induced apoptosis. MDA-MB-435 cells treated in a similar manner as in Figure 3A were collected and stained with propidium iodide for flow cytometric analysis. Apoptosis was represented by the appearance of cells at the sub-G1 phase.

(C) Inhibition of p34^{Cdc2} kinase activity by the dominant-negative mutant of p34^{Cdc2} (Cdc2-dn). MDA-MB-435 cells were mock transfected or transfected with pCMVneo vector, or with pCMVcdc2-dn using cationic liposome, DC-Chol:DOPE. Thirty-six hours later, cells were grown in fresh media with (0.02 μ M) or without Taxol for another 21 hr. These differently treated cells were then harvested and assayed for p34^{Cdc2} kinase activities as in (A). The p34^{Cdc2} protein levels were determined by Western blot analysis using anti-Cdc2 antibodies (bottom). Activation of p34^{Cdc2} kinase activities was reduced (76.2% reduction after normalized with Cdc2 protein level) in the Cdc2-dn-transfected cells compared to that in mock-transfected cells.

(D) Inhibition of Taxol-induced apoptosis by Cdc2-dn. Cells treated in a similar manner as in (C) were collected and detected for Taxol-induced apoptosis by double-label flow cytometry analysis (a-d) and by DNA fragmentation assay (e, lanes 1-6 correspond to lanes 1-6 in [C]).

(E) Inhibition of Cdk2 kinase activity by the dominant-negative mutant of Cdk2 (Cdk2-dn). MDA-MB-435 cells were treated and assayed in a similar manner as in (C) except pCMVcdk2-dn was used here.

(F) Cdk2-dn did not inhibit Taxol-induced apoptosis. MDA-MB-435 cells were treated and assayed in a similar manner as described in (D) except pCMVcdk2-dn was used here.

(G) RNA expression of the transfected pCMVcdc2-dn and pCMVcdk2-dn.

MDA-MB-435 cells were mock transfected, transfected with pCMVcdc2-dn or pCMVcdk2-dn for 36 hr, and processed for RT-PCR experiments. RT-, PCR without reverse transcription; RT+, reverse transcription followed by PCR; control, direct PCR using pCMVcdc2-dn and pCMVcdk2-dn plasmids as PCR template, which yielded signals ~600 bp larger than that from RNA transcripts. This was due to the β -globin intron 2 sequence existing in both plasmids.

G2/M phase and dramatically reduced apoptosis compared with pCMVneo-transfected cells that were effectively blocked at the G2/M phase by Taxol and underwent apoptosis (Figure 3Dd versus 3Db). Inhibition of Taxol-induced apoptosis by Cdc2-dn in MDA-MB-435 cells was also observed using DNA fragmentation assays (Figure 3De, lane 5 versus 4). Blocking of Taxol-induced apoptosis by inhibition of p34^{Cdc2} with olomoucine and Cdc2-dn indicated that activation of p34^{Cdc2} was required for Taxol-induced apoptosis.

Cdk2 was previously shown to be required for entry into mitosis as a positive regulator of p34^{Cdc2} kinase in extracts of cycling *Xenopus* eggs (Guadagno and Newport, 1996). We next investigated whether Cdk2 is required for Taxol-mediated entrance of MDA-MB-435 cells to G2/M phase and apoptosis using the dominant-negative mutant of Cdk2, pCMVcdk2-dn (van den Heuvel and Harlow, 1993). MDA-MB-435 cells were transfected with the pCMVcdk2-dn, or CMV vector for 36 hr, then cultured in the presence or absence of Taxol for an additional 21 hr and harvested. RNA expression from the transfected Cdk2-dn was also detected by RT-PCR (Figure 3G). Interestingly, Taxol did not activate Cdk2 kinase activity in MDA-MB-435 cells (Figure 3E, lane 1 versus lane 4), suggesting that Taxol did not induce apoptosis by activating Cdk2. Additionally, Cdk2-dn did not block Taxol-induced apoptosis in these cells (Figure 3Fd), although Cdk2-dn effectively inhibited the kinase activities of Cdk2 (Figure 3E), indicating that Cdk2 is not required for Taxol-induced apoptosis. Similarly, Cdk4 was found not necessary for Taxol-induced apoptosis in MDA-MB-435 cells (data not shown) using dominant-negative Cdk4 mutant pCMVcdk4-dn (van den Heuvel and Harlow, 1993). Taken together, activated p34^{Cdc2}, but not Cdk2 or Cdk4, is one of the mediators for Taxol-induced apoptosis.

Taxol-Induced Activation of p34^{Cdc2} Is Inhibited in 435.eB Cells

To examine whether overexpression of p185^{ErbB2} may inhibit Taxol-mediated activation of p34^{Cdc2} and thereby confer resistance to Taxol-induced apoptosis, we compared the extent and kinetics of Taxol-induced activation of p34^{Cdc2} kinase between the p185^{ErbB2}-low-expressing 435.neo cells and the p185^{ErbB2}-overexpressing 435.eB transfectants. Activation of p34^{Cdc2} kinase in 435.neo cells was detected 2 hr after addition of Taxol, and it continued to increase 8 hr after Taxol treatment compared to that in the untreated cells (Figure 4A). However, activation of p34^{Cdc2} kinase in the Taxol-resistant 435.eB cells is reduced to only 47% of that in the 435.neo cells (Figure 4A), whereas the p34^{Cdc2} protein levels are similar between the 435.neo cells and 435.eB cells (Figure 4A, bottom). To investigate whether the inhibition of p34^{Cdc2} activation by p185^{ErbB2} may contribute to resistance to Taxol-induced apoptosis, 435.neo cells and 435.eB cells were compared for the kinetics of Taxol-induced apoptosis using DNA fragmentation assays (Figure 4B). DNA ladders appeared in the 435.neo cells 12 hr after 0.02 μ M Taxol treatment and became pronounced 15 hr after Taxol treatment. However, DNA ladders in the 435.eB cells did not appear until 18 hr after Taxol treatment, and

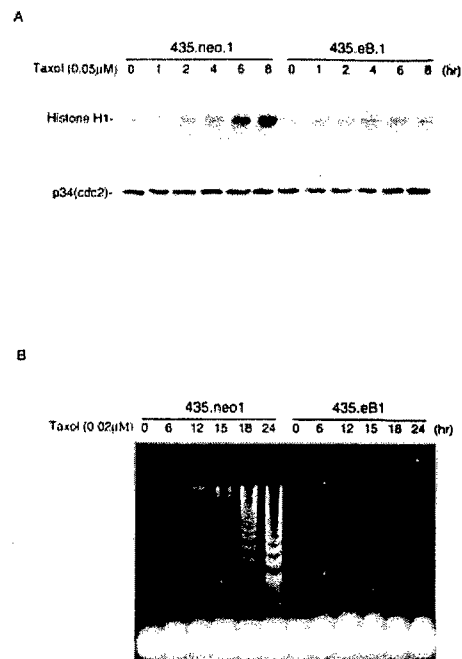


Figure 4. Taxol-Induced Activation of p34^{Cdc2} and Apoptosis Are Inhibited in p185-Overexpressing 435.eB1 Cells

(A) Taxol-mediated p34^{Cdc2} activation is inhibited in 435.eB1 cells. Protein lysates from either 435.neo1 or 435.eB1 cells were collected at the indicated time points after addition of 0.05 μ M Taxol. Equal amounts of protein lysates were immunoprecipitated using the anti-Cdc2 antibodies, and immunocomplexes were subjected to kinase assays. Cdc2 protein levels were determined by Western blotting using anti-Cdc2 antibodies.

(B) Taxol-induced apoptosis is delayed and reduced in 435.eB1 cells compared with that in 435.neo1 cells. The low molecular weight DNA was isolated from either 435.neo1 or 435.eB1 cells at the indicated time points after addition of 0.02 μ M Taxol and processed for DNA fragmentation assays.

the degree of DNA fragmentation was less pronounced than that in 435.neo cells. The delayed and reduced apoptotic response in the Taxol-treated 435.eB cells correlated with their lower p34^{Cdc2} kinase activity, suggesting that inhibition of Taxol-mediated p34^{Cdc2} activation in 435.eB cells may contribute to their resistance to Taxol-induced apoptosis.

Elevated p21^{Cip1} Expression in 435.eB Transfectants

It is known that activation of p34^{Cdc2} is precisely regulated by the accumulation of cyclin B and by three phosphorylation sites on the p34^{Cdc2} subunit that are modulated by Cdc25 and Wee1. To determine how p185^{ErbB2} inhibited the activation of p34^{Cdc2}, we compared the expression levels of these p34^{Cdc2} regulators in the p185-low-expressing 435.neo cells and p185-overexpressing 435.eB cells by immunoblot analysis, and no significant difference was detected among these cells (data not shown). This suggests that overexpression of p185^{ErbB2} did not inhibit activation of p34^{Cdc2} by regulating cyclin B, Cdc25, and Wee1 expression.

In addition to these regulators of p34^{Cdc2}, p21^{Cip1} was suggested to regulate a G2/M checkpoint, blocking the entrance of cells into M phase (Jacks and Weinberg,

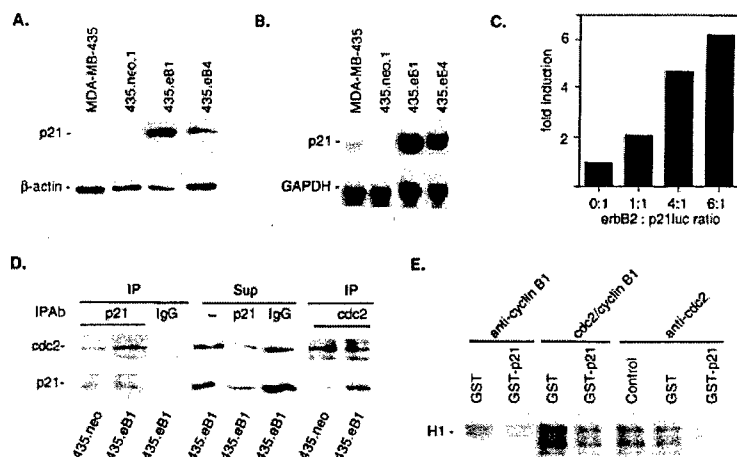


Figure 5. p185^{ErbB2} Upregulates p21^{Cip1}, which Inhibits p34^{Cdc2}

(A) p21^{Cip1} protein levels are higher in the p185^{ErbB2}-overexpressing 435.eB transfectants than in the parental MDA-MB-435 and control 435.neo.1 cells. Immunoblot analyses were performed using the p21^{Cip1} antibody (top) or the β -actin antibody as a control (bottom).

(B) Northern blot analysis demonstrated higher steady-state p21^{Cip1} mRNA levels in the p185^{ErbB2}-overexpressing 435.eB transfectants than in the control cells. RNA blot was hybridized with a ³²P-labeled cDNA probe of p21^{Cip1} (top) or GAPDH as a control (bottom).

(C) ErbB2 transcriptionally upregulated p21^{Cip1}. MDA-MB-435 cells (1×10^6) in 60 mm dishes were cotransfected using cationic liposome with 1.5 μ g pCMV-lacZ, 1 μ g pWWP-Luc, and different concentrations of pCMVVerbB2 resulting in the pCMVVerbB2 to pWWP-Luc

(ErbB2: p21luc) ratios of 0:1, 1:1, 4:1, and 6:1. The total amount of transfected DNA was brought up to 9 μ g by adding pCMVneo. Luciferase activities were measured 40 hr after transfection and were standardized by transfection efficiency.

(D) Association of p21^{Cip1} with p34^{Cdc2}. Cell lysates were prepared from 435.neo and 435.eB1 cell lines. Total protein (500 μ g) was immunoprecipitated with antibodies against p21^{Cip1} or p34^{Cdc2}. Immunoprecipitates (IP) and 80 μ g of the supernatants (Sup) were resolved on 12% SDS-PAGE, and associated p21^{Cip1} and p34^{Cdc2} proteins were analyzed by immunoblotting. Normal mouse and rabbit IgG were used as controls.

(E) Recombinant p21^{Cip1} protein directly inhibited p34^{Cdc2}-cyclin B1 kinase activity. Protein lysates from MDA-MB-435 cells were collected and immunoprecipitated using the anti-cyclin B1 antibody. Equal amounts of the immunocomplexes or equal amounts of the recombinant p34^{Cdc2}-cyclin B1 proteins were then subjected to kinase assays with 10 μ g of recombinant GST or GST-p21^{Cip1} proteins. As controls, protein lysates from MDA-MB-435 cells were also immunoprecipitated using the anti-Cdk2 antibody and subjected to kinase assays without (control) or with 10 μ g of recombinant GST or GST-p21^{Cip1} proteins.

1996). To investigate whether p185^{ErbB2} may inhibit p34^{Cdc2} kinase activity by upregulation of p21^{Cip1}, we examined the p21^{Cip1} expression levels in MDA-MB-435 cells and 435.neo cells that express low levels of p185^{ErbB2} and in 435.eB transfectants that express high levels of p185^{ErbB2}. Higher levels of p21^{Cip1} protein were detected in the 435.eB transfectants compared with MDA-MB-435 and 435.neo control cells (Figure 5A), which was due to upregulation of p21^{Cip1} mRNA expression (Figure 5B). To further investigate whether p185^{ErbB2} upregulated p21^{Cip1} by transcriptional activation, we cotransfected into MDA-MB-435 cells the pWWP-Luc plasmid, containing 2.4 kb wild-type p21^{Cip1} promoter and upstream sequences fused with the luciferase reporter gene (El-Deiry et al., 1993), with either the pCMVneo control vector or with increasing amounts of the pCMVVerbB2, an ErbB2 expression vector (Yu et al., 1996). Luciferase activities driven by the p21^{Cip1} promoter increased in the presence of ErbB2 expression vector in an ErbB2 concentration-dependent manner (Figure 5C). These data demonstrated that p185^{ErbB2} can upregulate p21^{Cip1} in 435.eB transfectants at the transcriptional level.

p21^{Cip1} Associates with p34^{Cdc2} and Inhibits p34^{Cdc2} Kinase Activity

Although p185^{ErbB2} upregulated p21^{Cip1} expression in 435.eB cells and p21^{Cip1} was shown to regulate the G2/M transition (Dulic et al., 1998; Niculescu et al., 1998), it is not clear whether the upregulated p21^{Cip1} in 435.eB cells may inhibit p34^{Cdc2} and mediate the antiapoptotic function of p185^{ErbB2}. We therefore examined whether the upregulated p21^{Cip1} in 435.eB cells may associate with p34^{Cdc2} in vivo. The 435.neo and 435.eB1 cell lysates were immunoprecipitated using p21^{Cip1} antibodies or

control IgG. Immunoprecipitates (IP) and supernatants (Sup) were separated on SDS-PAGE and immunoblotted with antibodies against p34^{Cdc2} and p21^{Cip1}. Abundant p34^{Cdc2} protein was detected in the 435.eB1 cell precipitates of p21^{Cip1} antibodies (Figure 5D, left panel), whereas little p34^{Cdc2} protein remained in the supernatant due to depletion of p21^{Cip1} (Figure 5D, middle panel). In addition, a stronger p34^{Cdc2} signal can be detected in the 435.eB1 cells expressing high levels of p21^{Cip1} compared with the 435.neo cells expressing low levels of p21^{Cip1} (Figure 5D, left panel). Reverse experiments detected p21^{Cip1} in the p34^{Cdc2} antibody immunoprecipitates (Figure 5D, right panel). These results indicate that the upregulated p21^{Cip1} protein in 435.eB cells physically associates with p34^{Cdc2}, suggesting that upregulation of p21^{Cip1} may contribute to inactivation of p34^{Cdc2} kinase in 435.eB cells.

To examine whether p21^{Cip1} can indeed inhibit p34^{Cdc2}, we tested the inhibitory effects of purified recombinant GST-p21^{Cip1} fusion protein (El-Deiry et al., 1993) on p34^{Cdc2}-cyclin B1 kinase activity. p34^{Cdc2}-cyclin B1 complexes were immunoprecipitated by anti-cyclin B1 antibodies, and Cdk2-cyclin complexes were immunoprecipitated by anti-Cdk2 antibodies (as a positive control) from MDA-MB-435 total cell lysates. The immunoprecipitates were incubated with GST-p21^{Cip1} or control GST protein and then assayed for kinase activity. GST-p21^{Cip1} effectively inhibited the G2/M phase p34^{Cdc2}-cyclin B1 kinase activity (Figure 5E, left panel). Since GST-p21^{Cip1} could inhibit activities of p34^{Cdc2}-cyclin B1 complexes precipitated by anti-cyclin B1 antibodies, the data indicated that p21^{Cip1} inhibited the p34^{Cdc2} kinases that were active in the mitosis-promoting p34^{Cdc2}-cyclin B1 heterodimer. In the control experiment, GST-p21^{Cip1} inhibited Cdk2 activity as expected (Figure 5E, right panel). Furthermore, the purified GST-p21^{Cip1} fusion protein also

inhibited the activities of the purified recombinant p34^{Cdc2}-cyclin B1 kinases (Figure 5E, middle panel). Therefore, p21^{Cip1} can directly inhibit p34^{Cdc2}-cyclin B1 kinase activity in vitro.

Sensitization of 435.eB Cells to Taxol-Induced Activation of p34^{Cdc2} and Apoptosis by Antisense p21^{Cip1}

To investigate whether the upregulated p21^{Cip1} in p185^{ErbB2}-overexpressing 435.eB cells may inhibit Taxol-mediated activation of p34^{Cdc2} kinase, thereby blocking Taxol-induced apoptosis in these cells, we blocked p21^{Cip1} mRNA expression in 435.eB cells by transient transfection of p21^{Cip1} antisense oligonucleotides (P21AS) that hybridize to the transcription initiation site. In the control experiment, the 435.eB cells were transfected with scrambled p21^{Cip1} antisense oligonucleotide (P21S). Thirty-six hours after transfection, the 435.eB cells were treated with or without Taxol for an additional 21 hr and cell lysates harvested. Western blot analysis using p21^{Cip1} antibodies demonstrated that the p21^{Cip1} scrambled oligonucleotide-treated 435.eB cells expressed readily detectable p21^{Cip1} protein, and the p21^{Cip1} protein was slightly upregulated by Taxol (Figure 6A), which is consistent with previous reports that Taxol can induce p21^{Cip1} in certain cell types (Barboule et al., 1997). However, p21^{Cip1} antisense-treated 435.eB cells had significantly reduced p21^{Cip1} protein levels, and Taxol did not upregulate p21^{Cip1} in these cells. Higher p34^{Cdc2} kinase activity was detected in Taxol-treated 435.eB cells transfected with p21^{Cip1} antisense compared with those transfected with the scrambled oligonucleotides (Figure 6B), whereas the p34^{Cdc2} protein levels remained similar. These experiments suggest that p21^{Cip1} contributes to inhibition of Taxol-induced p34^{Cdc2} activation in 435.eB cells. We next compared the induction of apoptosis by Taxol in the antisense or scrambled oligonucleotide-transfected 435.eB cells using flow cytometry (Figure 6C). The majority of the untreated 435.eB cells with scrambled oligonucleotide were at the G1 phase of the cell cycle without significant apoptotic DNA strand breaks (Figure 6C, top panel, 0 hr). After Taxol treatment for 15 hr and 21 hr, these cells cycled toward the G2/M phase of the cell cycle with no significant DNA strand breaks (Figure 6C). Although the untreated 435.eB cells with antisense p21^{Cip1} had similar cell cycle profiles (Figure 6C, bottom panel, 0 hr) as the control cells, these cells showed a high level of apoptotic DNA strand breaks after 15 hr of Taxol treatment that became more pronounced after 21 hr of Taxol treatment (Figure 6C). Since blocking of p21^{Cip1} by antisense in 435.eB cells relieved the inhibition of Taxol-mediated p34^{Cdc2} activation and sensitized the 435.eB cells to Taxol-induced apoptosis, the data indicate that upregulated p21^{Cip1} in 435.eB transfectants participated in the inhibition of Taxol-mediated p34^{Cdc2} activation, which contributes to resistance to Taxol-induced apoptosis.

Overexpression of ErbB2 Does Not Inhibit Taxol-Induced Apoptosis in p21^{-/-} Mouse Embryonic Fibroblasts

To further test the requirement of p21^{Cip1} for p185^{ErbB2} antiapoptotic function, we examined whether p185^{ErbB2}

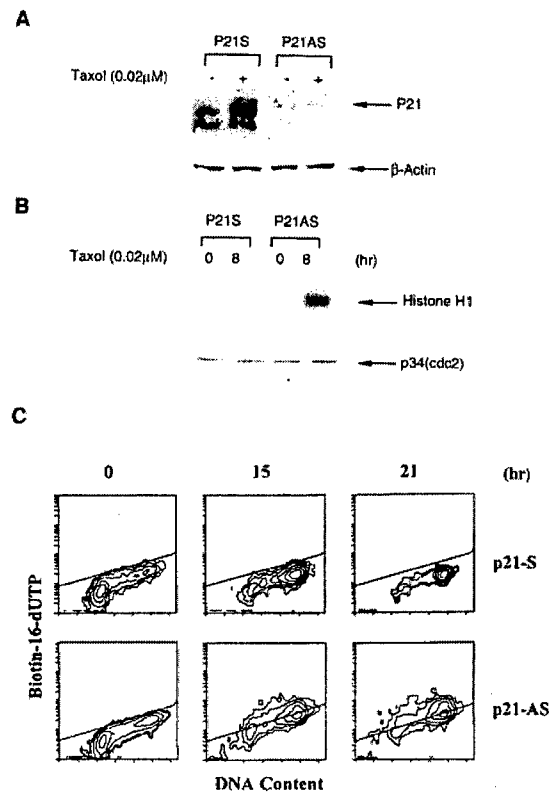


Figure 6. Antisense p21^{Cip1} Sensitized 435.eB Cells to Taxol-Induced Activation of p34^{Cdc2} and Apoptosis

(A) Transfection of p21^{Cip1} antisense oligonucleotide reduced p21^{Cip1} protein levels. The 435.eB cells were transfected with p21^{Cip1} antisense oligonucleotide (P21AS) or scrambled p21^{Cip1} antisense oligonucleotide (P21S) as a control. Thirty-six hours later, the cells were cultured in the absence (–) or presence (+) of 0.02 μ M Taxol for another 21 hr. Cell lysates were collected for immunoblot analysis using p21^{Cip1} antibody.

(B) P21AS relieved inhibition of Taxol-mediated p34^{Cdc2} activation by p185^{ErbB2}. The 435.eB cells were treated with oligonucleotide in a similar way as in (A), and the cells were cultured in media containing 0.02 μ M Taxol for 0 or 8 hr. Activation of p34^{Cdc2} by Taxol and the p34^{Cdc2} protein levels were assayed as in (A).

(C) P21AS sensitized the 435.eB cells to Taxol-induced apoptosis. The 435.eB cells were treated in a similar way as in (A), and the cells were cultured in Taxol-containing (0.02 μ M) media for 0, 15, and 21 hr and harvested. Flow cytometry analysis was performed as in Figure 2B.

could block Taxol-induced apoptosis in p21^{Cip1} null cells. The ErbB2 expression vector (pCMVerbB2) or control vector (pCMVneo) was transfected into mouse embryonic fibroblasts (MEF) from a p21^{Cip1} knockout mouse (p21^{-/-} MEF) or from a wild-type mouse (wt MEF) (Montes de Oca Luna et al., 1997). Western blot analysis using p185^{ErbB2} antibodies demonstrated overexpression of p185^{ErbB2} in pCMVerbB2-transfected p21^{-/-} MEF and wt MEF compared to controls (Figure 7A). In addition, the overexpressed p185^{ErbB2} in wt MEF has led to a dramatic increase of p21^{Cip1} expression (Figure 7A). However, although overexpression of p185^{ErbB2} in wt MEF conferred resistance to Taxol-induced apoptosis, overexpression of p185^{ErbB2} in p21^{-/-} MEF did not demonstrate a similar antiapoptotic response (Figure 7B). Similar results were

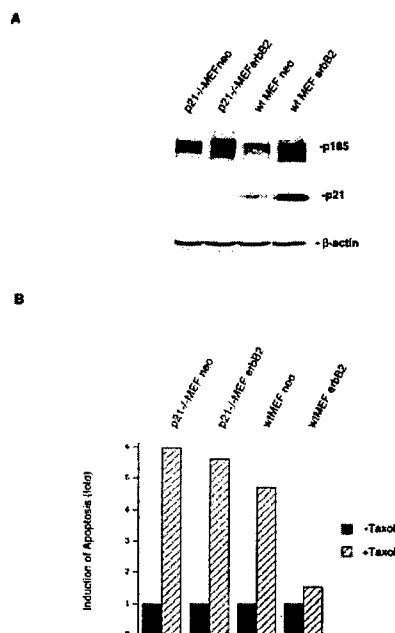


Figure 7. Overexpression of p185^{ErbB2} Cannot Protect p21^{-/-} Mouse Embryonic Fibroblasts from Taxol-Induced Apoptosis

(A) Transfection of pCMVVerb2 led to enhanced expression of p185^{ErbB2} in p21^{-/-} MEF and wt MEF (top), and p185^{ErbB2} upregulated p21^{Cip1} in wt MEF (middle). The p21^{-/-} MEF and wt MEF were transfected with pCMVneo or pCMVVerb2. Forty hours later, protein lysates were extracted for Western blot analyses using anti-p185^{ErbB2}, anti-p21^{Cip1}, or anti- β -actin antibodies as controls (bottom).

(B) p185^{ErbB2} did not protect p21^{-/-} MEF from Taxol-induced apoptosis. The p21^{-/-} MEF and wt MEF were cultured with (+Taxol) or without (-Taxol) Taxol for an additional 24 hr after transfection with pCMVneo or pCMVVerb2 for 36 hr. Cells were then harvested for double-label flow cytometry analyses. The folds of induction of apoptosis in Taxol-treated samples were calculated by standardization of the percent of apoptotic cells (including cells with high levels of biotin-16-dUTP labeling in G2/M phase as well as sub-G1 apoptotic cells) in these samples over that in the corresponding untreated cells.

obtained when apoptotic cells were quantitated by staining with annexin-specific antibodies (data not shown). These findings provided consistent evidence that p21^{Cip1} participates in the p185-mediated resistance to Taxol-induced apoptosis.

Discussion

Activation of p34^{Cdc2} Is Required for Taxol-Induced Apoptosis

Activation of p34^{Cdc2} kinase is the biochemical step required for mitosis and has been implicated as an important event during chemotherapy-induced apoptosis in certain cancer types (Meikrantz and Schlegel, 1996). Although it was suggested that chromatin condensation and lamina disassembly during apoptosis involve different processes from those operating in mitosis, similar transient activations of p34^{Cdc2} kinase were observed in Taxol-treated apoptotic HeLa cells (Donaldson et al., 1994). In this study, we demonstrated that Taxol induced apoptosis of MDA-MB-435 breast cancer cells at the

G2/M phase of the cell cycle through activation of p34^{Cdc2}. Activation of p34^{Cdc2}-cyclin B1 kinase is required for Taxol-induced apoptosis in breast cancer cells because Taxol-mediated activation of p34^{Cdc2} kinase occurred prior to Taxol-induced apoptosis and because inhibition of Taxol-mediated activation of p34^{Cdc2} kinase by a chemical inhibitor and by the dominant-negative mutant of p34^{Cdc2} diminished Taxol-induced apoptosis.

p185^{ErbB2} Confers Resistance to Taxol-Induced Apoptosis by Inhibiting p34^{Cdc2} Activation

Several growth factors and growth factor receptors have been shown to modulate apoptosis (Rodeck et al., 1997). Here, we have provided direct evidence that overexpression of p185^{ErbB2} in breast cancer cells can confer resistance to Taxol-induced apoptosis. The antiapoptotic effect of p185^{ErbB2} was also observed in p185^{ErbB2}-overexpressing MDA-MB-361 and BT-474 breast cancer cell lines (Yu et al., unpublished data) that were established from breast tumors of other patients, indicating that resistance to Taxol-induced apoptosis in p185^{ErbB2}-overexpressing breast cancer cells is not limited to a single cell line but is of general importance. Our data indicated that overexpression of p185^{ErbB2} in breast cancer cells conferred resistance to Taxol-induced apoptosis via Bcl-2 independent pathways. Interestingly, overexpression of p185^{ErbB2} in 435.eB transfectants resulted in a reduced activation of p34^{Cdc2} kinase compared to p185-low-expressing 435.neo control cells. Moreover, inhibition of Taxol-mediated activation of p34^{Cdc2} kinase by p185^{ErbB2} corresponded to the delayed cell entrance to G2/M phase after Taxol treatment and paralleled the inhibition of Taxol-induced apoptosis. Therefore, at least one of the mechanisms for p185^{ErbB2} antiapoptotic function is inhibition of Taxol-mediated activation of p34^{Cdc2} kinase.

Upregulation of p21^{Cip1} Contributes to Inhibition of Taxol-Mediated p34^{Cdc2} Activation by p185^{ErbB2}

In this study, we found that overexpression of p185^{ErbB2} transcriptionally upregulates the Cdk inhibitor p21^{Cip1} in MDA-MB-435 cells. The p185^{ErbB2}-mediated upregulation of p21^{Cip1} is p53 independent, because the MDA-MB-435 cells do not contain the wt p53 gene (Lesoon-Wood et al., 1995).

p21^{Cip1} was originally believed not to be a universal inhibitor of Cdks but to display selectivity for G1/S phase Cdk-cyclin complexes (e.g., Cdk2) (Harper et al., 1993). Recently, p21^{Cip1} was also demonstrated to contribute to regulation of G2/M transition (Dulic et al., 1998), and Taxol was shown to increase p21^{Cip1} expression, which associated with p34^{Cdc2} (Barboule et al., 1997). We report here that in 435.eB cells, p185^{ErbB2} upregulated p21^{Cip1}, which also complexed with p34^{Cdc2}. Moreover, we demonstrated that p21^{Cip1} directly inhibited the G2/M phase p34^{Cdc2}-cyclin B1 in vitro, although less effectively than it inhibits the G1/S phase Cdk2. The association of p21^{Cip1} with p34^{Cdc2} in 435.eB cells and the ability of p21^{Cip1} to inhibit p34^{Cdc2} activity in vitro suggest that p21^{Cip1} may inhibit p34^{Cdc2} activity in 435.eB cells. This notion is also

supported by our data that p21^{Cip1} antisense oligonucleotide sensitized 435.eB cells to Taxol-mediated activation of p34^{Cdc2}. The p21^{Cip1}-mediated dual inhibition at G1/S and G2/M phases may protect p185-overexpressing breast cancer cells from Taxol-induced apoptosis: p21^{Cip1} directly inhibits Taxol-mediated activation of p34^{Cdc2}, which delays or prevents cell entrance to G2/M phase, resulting in inhibition of apoptosis; or p21^{Cip1} arrests those cells progressing through aberrant mitosis in G1, which indirectly prevents or delays the cells from entering G2/M phase and undergoing apoptosis. Although multiple mechanisms may be involved, inhibition of p34^{Cdc2} by p21^{Cip1} is likely one of the major mechanisms of apoptosis resistance in p185-overexpressing cells, since activation of p34^{Cdc2}, but not of Cdk2 or Cdk4, is required for Taxol-induced apoptosis.

Molecular Basis of Taxol-Induced Apoptosis and p185^{ErbB2} Antiapoptosis

Based on previous studies and data presented in this study, we propose a model for Taxol-induced apoptosis and p185^{ErbB2}-mediated antiapoptosis in breast cancer cells. Taxol can induce activation of p34^{Cdc2} kinase in MDA-MB-435 breast cancer cells that contributes to the induction of apoptosis at the G2/M phase, whereas overexpression of p185^{ErbB2} in MDA-MB-435 cells (435.eB transfectants) impedes Taxol-induced apoptosis by upregulation of the Cdk inhibitor p21^{Cip1}, which in turn inhibits Taxol-mediated activation of p34^{Cdc2}. This model synthesized the critical role of the G2/M Cdk p34^{Cdc2} in apoptosis induction, the function of p21^{Cip1} as a mammalian (human breast cancer) cell G2/M Cdk inhibitor, and the effects of overexpression of the receptor tyrosine kinase p185^{ErbB2} on p21^{Cip1} expression into our understanding of the molecular basis of Taxol-induced apoptosis and p185^{ErbB2} antiapoptosis. Furthermore, the model provides a molecular mechanism, although it may not be the only mechanism, that underlies the Taxol-resistance phenomenon in ErbB2-overexpressing breast cancers.

Experimental Procedures

Cell Lines and Culture

The human breast cancer cell line MDA-MB-435, the 435.eB transfectants, their revertants, and control 435.neo cells were obtained, established, and cultured as previously reported (Yu et al., 1996). The p21^{-/-} MEF and wt MEF were from Dr. Guillermina Lozano (The University of Texas, M. D. Anderson Cancer Center).

Antibodies and Reagents

Antibodies were purchased from commercial sources: human p34^{Cdc2}, cyclin B1 monoclonal antibodies, and Cdk2 polyclonal antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Bcl-2 antibody from DAKO Corporation (Carpinteria, CA); human p21^{Cip1} polyclonal and p185^{ErbB2} monoclonal antibodies from Oncogene Science, Inc. (Cambridge, MA); and β -actin monoclonal antibody from Sigma (St. Louis, MO). Taxol was purchased from Mead Johnson, Inc. (Princeton, NJ). Recombinant p34^{Cdc2}-cyclin B1 was purchased from New England Biolabs, Inc. (Beverly, MA).

Transmission Electron Microscopy

Cells were washed and fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer overnight at 4°C. Cells were postfixed in cacodylate-buffered 1% osmium tetroxide, dehydrated, and embedded in epon. Thin sections were poststained with uranyl acetate and lead

citrate and viewed on a JOEL JEM 1200 Ex transmission electron microscope.

DNA Fragmentation Assay

Approximately 1×10^7 cells were incubated on ice in 100 μ l of DNA isolation buffer (10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100) for 30 min and then added with 100 μ l of PBS. After centrifugation, the low molecular weight DNA was extracted from the supernatant with phenol/chloroform and precipitated with ethanol. Ten micrograms of DNA from each sample was analyzed by 1.8% agarose gel electrophoresis.

Flow Cytometry Analysis

Cells were harvested by trypsinization and fixed in 1% formaldehyde on ice for 20 min and washed once with PBS. For multiparameter flow cytometry analysis, the cells were incubated in terminal deoxynucleotidyl transferase (TdT) solution (0.1 M sodium cacodylate, 1 mM CoCl₂, 0.1 mM dithiothreitol, 0.05 mg/ml bovine serum albumin, 10 U TdT, and 0.5 mM biotin-16-dUTP) at 37°C for 30 min, transferred to 100 μ l of staining solution (4 $\times$ SSC, 5% dry milk, 0.1% Triton X-100, and 2.5 ng/ μ l Avidin-FITC), and incubated in the dark at room temperature for 30 min. Finally, the cells were stained in 500 μ l of propidium iodide (PI) solution (0.5 μ g/ml of PI and 0.1% RNase A). For single-label flow cytometry analysis, cultured cells were harvested and fixed in a similar manner and then stained in 500 μ l of PI solution. Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cell cycle profile was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA), and FITC signal was analyzed using Epics Elite Software (Coulter Corp., Miami, FL).

Immunocomplex-Kinase Assay

Cells (3×10^6) were lysed with the immunoprecipitation (IP) buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris at pH 7.4, 1 mM ethylene glycol-bis-tetraacetic acid, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40). Forty micrograms of protein from each sample was incubated at 4°C for 3 hr with 1 μ g of antibodies and for another 3 hr after addition of protein A-agarose. The immunoprecipitates were washed twice with IP buffer and once with kinase buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 10 mM MgCl₂, and 0.5 mM DTT) and resuspended in 40 μ l kinase buffer containing 1 μ g of histone H1, 25 μ M of ATP, and 2.5 μ Ci of γ -³²P]ATP. Following 30 min incubation at 30°C, the reaction was terminated by adding 40 μ l of 2 $\times$ SDS sample buffer (125 mM Tris [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue). Samples were resolved on 12% SDS-PAGE and analyzed by autoradiography.

Immunoprecipitation and Immunoblotting

Immunoprecipitation was performed as described in Immunocomplex-Kinase Assay, and immunoblot analyses were performed as described previously (Yu et al., 1990).

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated using RNeasyB reagent (Tel-Test Inc., Friendswood, TX). Reverse transcription was performed using SuperScript Preamplification System (Life Technologies, Inc., Gaithersburg, MD). Ten percent of the RT products (2 μ l) were used for 30 cycles of PCR (94°C 30 s, 56°C 30 s, 72°C 45 s) using GeneAmp System 9600 (Perkin Elmer, Norwalk, CT). The sequence of forward primer for the β -globin gene is GCACGTGGATCCTGAGAACTTCAG (both pCMVcdc2-dn and pCMVcdk2-dn contain this β -globin gene sequence at the 5' of inserts). The sequence of reverse primers are CGAGCTGACCCAGCAATACTTCT for Cdc2 and CAGGAGGATTT CAGGAGCTCGGTA for Cdk2, respectively.

Northern Blot Analysis

Twenty micrograms of RNA was denatured with glyoxal buffer, separated by electrophoresis on a 1% agarose gel in 10 mM NaPO₄ (pH 6.8) buffer, and blotted onto a nylon membrane. The membrane was hybridized at 65°C with the ³²P-labeled p21^{Cip1} cDNA probe for detection of p21^{Cip1} or with a β -actin cDNA probe as an internal loading control.

Luciferase Assays

MDA-MB-435 cells were cotransfected with pCMV-lacZ and the pWVP-Luc reporter gene with either the pCMVneo control vector or with the pCMVVerbB2 expression vector using cationic liposome. Forty hours later, cells were lysed with 500 μ l of 1 $\times$ lysis buffer (Promega, Madison, WI) for 10 min at room temperature. After centrifugation, 100 μ l of supernatant was used for a β -gal assay to determine transfection efficiency, and 15–30 μ l of supernatant was added to 100 μ l of reconstituted luciferase assay reagent in the luciferase assay kit (Promega). Light emission was detected by a luminometer.

Use of GST-p21 for Inhibition of Cdc2-Cyclin B1 Kinase Activity

The GST-p21 fusion protein or GST protein was isolated from BL21 (DE3) bacteria transformed with either pGEX-2T-WAF1-S or pGEX-2T as previously described (El-Deiry et al., 1993). Immunoprecipitates of anti-cyclin B1 or anti-Cdk2 from MDA-MB-435 total cell lysates or recombinant Cdc2-cyclin B1 kinases were incubated at 30°C for 30 min in 20 μ l of kinase buffer with 10 μ g of GST-p21 or GST protein. Samples were then processed for kinase assay.

Antisense Oligonucleotides

Phosphorothioate oligodeoxynucleotides (5 μ g) and cationic liposome, DC-Chol:DOPE (75 nmol) were incubated at 37°C for 15 min. The oligonucleotide-liposome mixture was diluted with serum-free medium and added to the cells for 36 hr. Cells were then treated with or without 0.02 μ M Taxol for various times. We used antisense oligonucleotides based on the p21^{Cip1} coding sequence complementary to the region of the initiation codon (p21-AS, 5'-CCCAGCCGGTCTGACATGGCGCC-3') and the scrambled p21^{Cip1} antisense oligonucleotides as control (p21-S, 5'-CCGCACGGAGCGCTGCGTTCTACC-3'). These oligonucleotides were purchased from Genosys Inc. (Woodlands, TX).

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Appendices

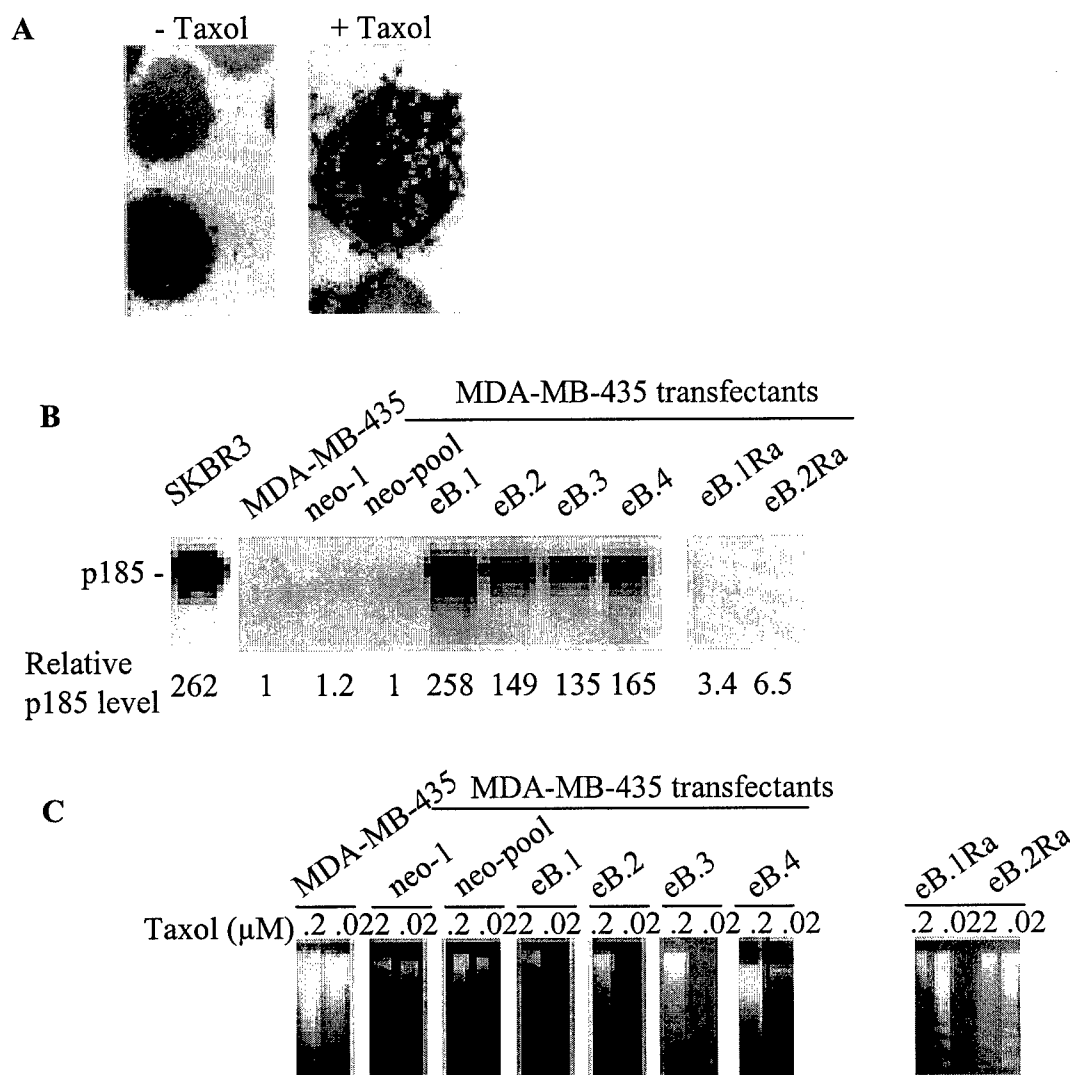


Figure 1. Overexpression of p185^{ErbB2} inhibited Taxol-Mediated Apoptosis.

(A) Morphology of MDA-MB-435 cells cultured in media without or with 0.02 μM Taxol for 24hrs under transmission electronmicroscopy (TEM). Magnification of the originals was 2000x.

(B) Western blot analysis of p185^{ErbB2} in the indicated cell lines. The relative p185^{ErbB2} level represents the amount of p185 in each cell line as fold of that of the MDA-MB-435 cells and determined by quantitation with the Personal Densitometer 50371 (Molecular Dynamics, Sunnyvale, CA).

(C) DNA fragmentation assays showing that overexpression of p185^{ErbB2} in MDA-MB-435 transfectants inhibited Taxol-induced apoptosis. The low molecular weight DNAs were isolated from the indicated cell lines cultured in media containing indicated concentrations of Taxol (0.02 or 0.2μM) and processed for DNA fragmentation assays.

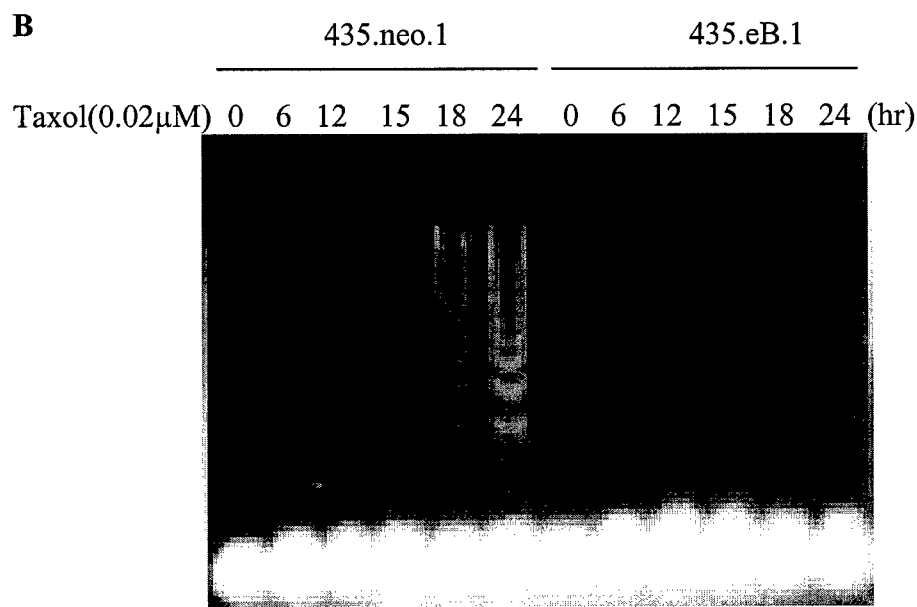
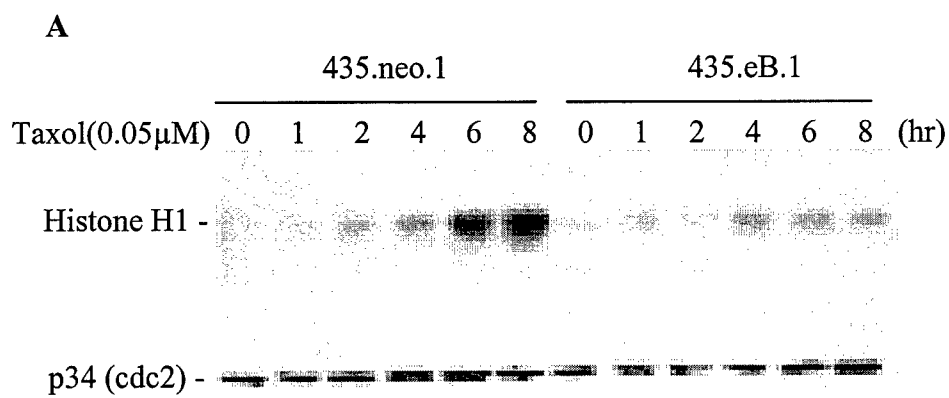


Figure 2. Taxol-induced Activation of p34^{Cdc2} and Apoptosis are inhibited in p185-overexpressing 435.eB1 Cells.

(A) Taxol-mediated p34^{Cdc2} activation is inhibited in 435.eB1 cells. Protein lysates from either 435.neo1 or 435.eB1 cells were collected at the indicated time points after addition of 0.02 μ M Taxol. Equal amounts of protein lysates were immunoprecipitated using the anti-Cdc2 antibodies, and immunocomplexes were subjected to kinase assays. Cdc2 protein levels were determined by Western blotting using anti-Cdc2 antibodies.

(B) Taxol-induced apoptosis is delayed and reduced in 435.eB1 cells compared with that in 435.neo1 cells. The low molecular weight DNA was isolated from either 435.neo1 or 435.eB1 cells at the indicated time points after addition of 0.02 μ M Taxol and processed for DNA fragmentation assays.

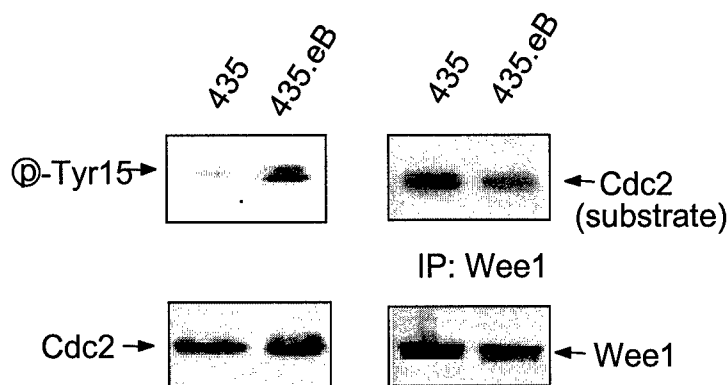


Figure 3. Increased phosphorylation on Cdc2-Tyr15 in p185^{ErbB2}-overexpressing 435.eB transfectants.

Protein lysates were collected from indicated cell lines. Immunoblot analyses of phosphorylation on Cdc2-Tyr15 (left top), p34^{Cdc2} (left bottom), and Wee1 (right bottom) proteins were performed using antibodies recognizing the phosphorylated Tyr15 on p34^{Cdc2}, human p34^{Cdc2}, and human Wee1. Wee1 kinase activity was assayed by immunoprecipitation of Wee1 that then were used for phosphorylation of Cdc2-Cyclin B1 complexes substrate (right top).

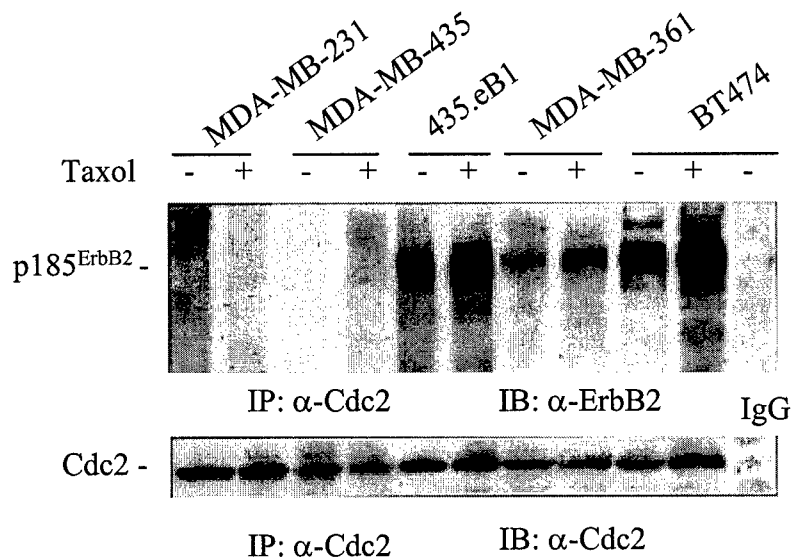


Figure 4. Association of p185^{ErbB2} with p34^{Cdc2} in vivo. The indicated cell lines were cultured in the absence (-) or presence (+) of Taxol (0.02 μ M) for 24 hr. Total protein (500 μ g) was immunoprecipitated for p34^{Cdc2}. Immunocomplexes were resolved on 6-12% gradient SDS-PAGE, Cdc2-associated p185^{ErbB2} and immunoprecipitated p34^{Cdc2} were analyzed by immunoblotting for p185^{ErbB2} or p34^{Cdc2}.

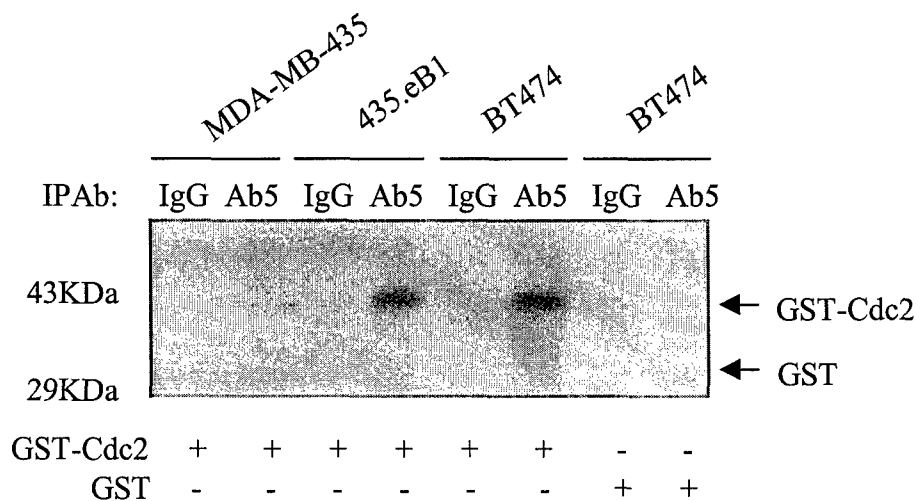


Figure 5. Phosphorylation of GST-Cdc2N50 by p185^{ErbB2} complex. Protein lysates (1mg) from the indicated cell lines were immunoprecipitated using wither antibodies against the extracellular domain p185^{ErbB2} (Ab5) or mouse IgG (control); immunocomplexes were subjected to kinase assays with [³²P]γ-ATP using either GST-Cdc2N50 or GST as substrates.

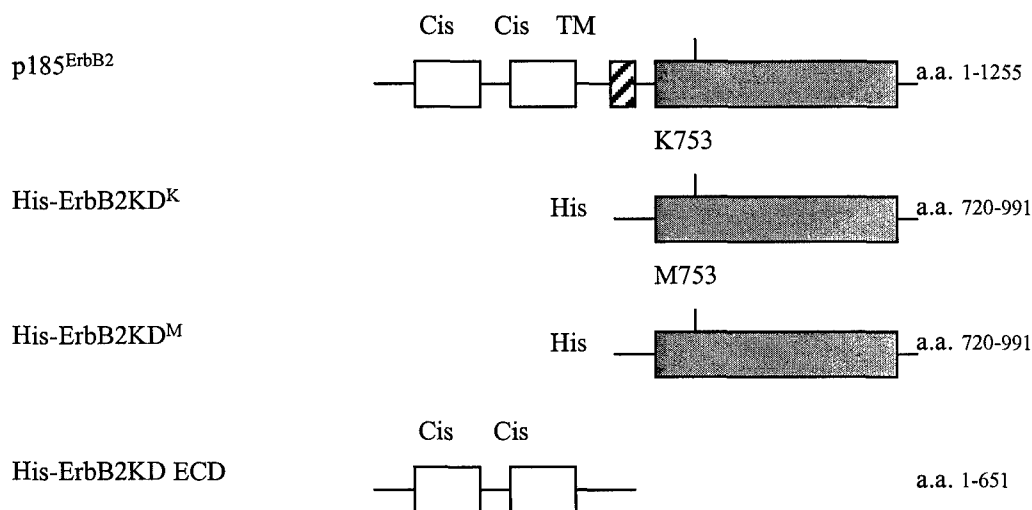
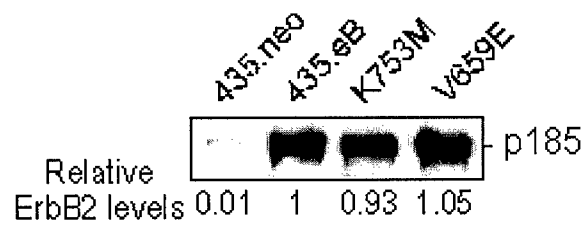


Figure 6. Diagram of the wild-type full-length p185^{ErbB2}, His-tagged wild-type kinase domain of ErbB2 (His-ErbB2KDK^K), mutant kinase domain of ErbB2 (His-ErbB2KDM^M), and wild-type extracellular domain of ErbB2 (His-ErbB2KDECD). The amino acids (a. a.) relative to the wild-type full-length p185^{ErbB2} included in each recombinant protein are indicated at right.

Figure 7

A



B

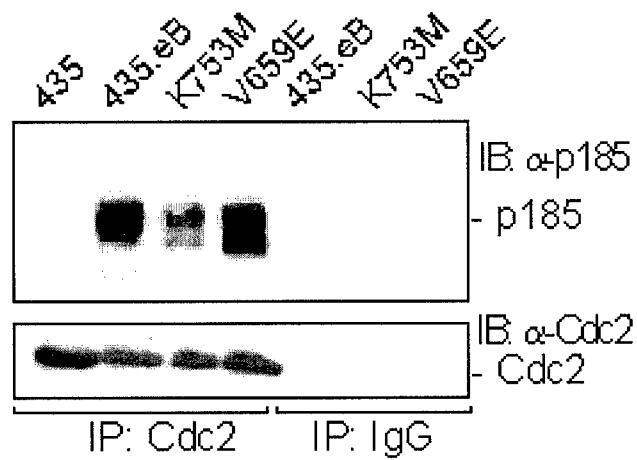
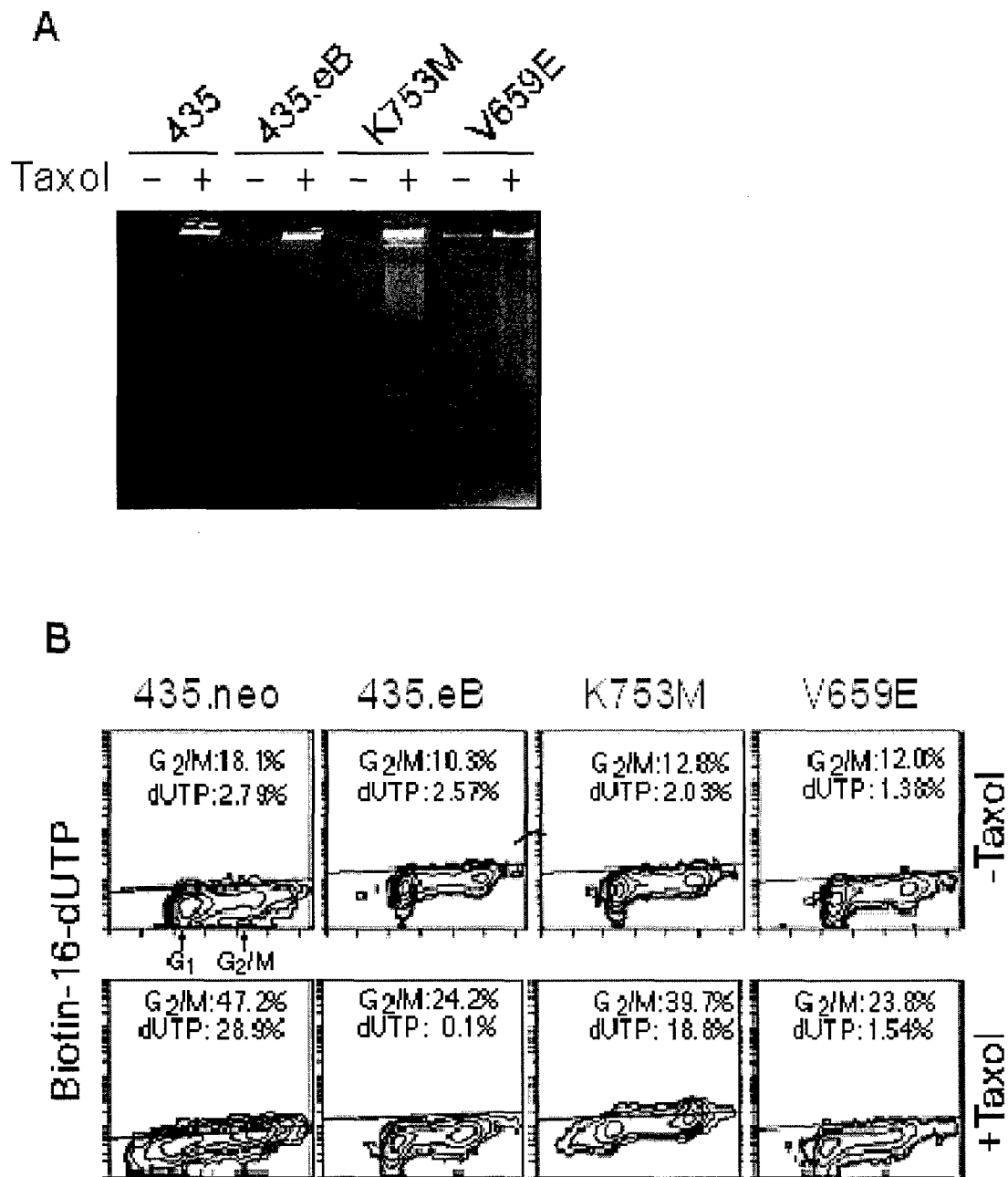


Figure 8



CURRICULUM VITAE

Name: Keng-hsueh Lan

Title/ Affiliation: Graduate Research Assistant
The University of Texas MD Anderson Cancer Center
Dept. of Surgical Oncology
Houston, Texas 77030

Birth Date/ Place: 03/25/71 Taipei, Taiwan

Citizenship: Taiwan

EDUCATION:

1997 National Taiwan University, College of Medicine, Taipei,
Taiwan, Doctor of Medicine
1999-present Graduate School of Biomedical Sciences, University of
Texas-Houston

Academic and Professional Appointments

1997-98 Medical officer, Air Force, Republic of China.
1996-97 Intern doctor, National Taiwan University Hospital,
Taipei, Taiwan

RESEARCH EXPERIENCE

1995 Summer student in Dr. Chia-Li Yu's laboratory at
National Yang-Ming University, Taipei, Taiwan
1998 Research assistant in Prof. Omata's laboratory at the
University of Tokyo, Tokyo, Japan
1999 Summer student in Prof. Pei-Jer Chen's laboratory at
Taiwan University, Taipei, Taiwan

Award

US Army Breast Cancer Research Program Training Grant -- predoctoral



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

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21 Feb 03

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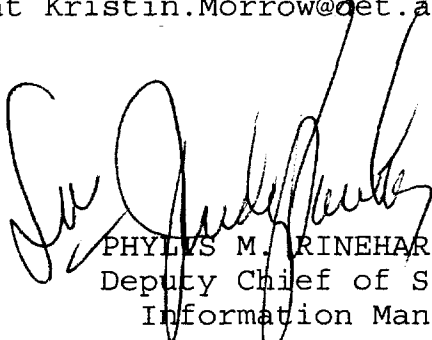
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