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Award Number: DAMD17-98-1-8242

TITLE: Mitosis-specific Negative Regulation of EGF-receptor in Breast Cancer: Molecular Mechanisms, Biological Significance and Therapeutic Application

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REPORT DATE: May 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2001	Final (1 May 98 - 30 Apr 01)

4. TITLE: MITOSIS-SPECIFIC NEGATIVE REGULATION OF EGF-RECEPTOR IN BREAST CANCER: MOLECULAR MECHANISMS, BIOLOGICAL SIGNIFICANCE AND THERAPEUTIC APPLICATION

5. FUNDING NUMBERS
DAMD17-98-1-8242

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color graphics

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The purpose of this project is to study the regulation of EGFR during M-phase. Normally EGFR is negatively regulated during M-phase, however when EGFR is overexpressed it is found to bypass this regulation. We plan to use this negative regulation of EGFR to develop a therapeutic strategy that uses the combination of EGF-PE and taxol as treatment for patients.

In trying to understand the mechanism of action of EGFR we have found specific phosphorylation spots of EGFR in M-phase. We have also shown that using the chimeric toxin EGF-PE and taxol together we can specifically target cells which overexpress EGFR, and protect normal cells, a concept that if proven in vivo can benefit patients in the future.

In addition to the proposed work we have also found that cyclin D1 can be upregulated by β-catenin and that β-catenin can serve as a poor prognostic factor in patients. Furthermore, in trying to further understand β-catenin signaling we have found that β-catenin can interact with and inhibits NF-κB. These studies can provide useful information so we can better understand the signaling involved in breast cancer, and hopefully we can use this to find better treatments for patients.

14. SUBJECT TERMS
Breast Cancer, EGFR, Mitotic Phase, EGF-PE, Taxol

15. NUMBER OF PAGES
50

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

This project is to study the regulation of EGF receptor (EGFR) in breast cancer epithelial cells in M-phase. EGFR is normally negatively regulated during M-phase. Since cells that overexpress EGFR can escape this regulation during M-phase(1), we wanted to develop a strategy to specifically target these cells. Using the combination of EGF-Pseudomonas (EGF-PE)(2), to target EGFR expressing cells, and nocadazole or taxol to synchronize the cells in M-phase, we should be able to target only cells that overexpress EGFR. After testing this concept both *in vitro* and *in vivo* we should be able to move to clinical trial if this strategy proves to work. In the future this should be able to benefit breast cancer patients with EGFR-overexpressing tumors.

Body

A. Statement of Work

Task 1: To identify the specific phosphorylation site(s) and the kinase(s), which mediate the desensitization of EGFR in the mitotic phase of the cell cycle

Task 2: To generate two tetracycline-inducible EGFR stable transfectants. One is transfected with the wild-type human EGFR; the other is transfected with the human EGFR cDNA with the regulatory sites mutated.

Task 3: To determine the possible biological implications for those non-regulated EGFR mutant cells.

Task 4:To compare the cytotoxic effects of the chimeric EGF-toxin between the cell lines with basal EGFR expression and the ones with the EGFR overexpressed after treating cells with the M phase specific agents (nocadazole or taxol).

Task 5: To test the therapeutic effect of EGF-PE *in vivo*.

B. Studies and Results

For the past three years we have been working on understanding the regulation of EGFR during M-phase. We have also been working on a way to specifically target EGFR overexpressing cells, while protecting normal cells. This work may provide a useful way to treat breast cancer patients. The work for each task will be discussed separately below.

Task 1:

We have been working on identifying the phosphorylation sites of EGFR, which mediate the desensitization of EGFR in M-phase. By performing phosphopeptide and phosphoamino acid analysis to identify the M-phase specific phosphorylation sites in both MDA-MB-468 and HER-5 cells lines. We have observed nine different phosphorylation sites specific for M-phase, six of which were found in both cell lines. We have tried to obtain the sequence however we have not been able to do so. We have also shown, in collaboration with Dr. Dihua Yu in our institution, that cdc2 does not bind to EFGR (Fig 1), which is different from neu, previously shown in our lab(3)

Task 2:

We have been working on the tetracycline induced EGFR cell lines, using the cell line NR-6 an EGFR null cell line as the parental cells(4). We have been able to obtain a stable cell line with tet-on expression construct, which can activate the luciferase reporter gene. We have not been able to obtain a stable cell line with the wild type or the mutant EGFR expression vectors.

Task 3:

We have not yet been able to test the biological implications of these cell lines with mutation in the EGFR receptor, since as stated in task 2, we have not been able to obtain the cell lines with wild type and mutant EGFR.

Task 4:

We have compared the cytotoxic effects of the chimeric EGF-toxin, EGF- Psedemonas Exotoxin A (EGF-PE). Using cells with genetically similar background, Her-5, NR-6 and SW3T3, all variants of SW3T3 cells with varying levels of EGFR(4), we found that the cells which overexpress EGFR are more susceptible to killing by the EGF-PE. We found similar results when we used the cell line MDA-MB-468, a breast cancer cell line with a high level of EGFR and compared it to HBL-100, a

mammary breast epithelial cell line with a basal level of EGFR. This manuscript has been sent to *Molecular Carcinogenesis* and is currently under revision. I have provided a copy of this manuscript to provide detail for this work.

Task 5:

The work from task 4 needs to be tested *in vivo,* however that has not been completed yet.

In addition to the work on the proposed experiment we also reported last year that cyclin D1 a downstream target of EGFR can be upregulated by β-catenin. We found that cyclin D1 is transcriptionally upregulated by β-catenin through co-transfection of β-catenin and a cyclin D1 reporter into human embryonic kidney cells. We also found that there is a correlation between cyclin D1 expression and β-catenin activity in 8 different breast cancer cell lines. Also we wanted to see whether this is also the case for breast cancer patients, and found that there is a correlation between β-catenin and cyclin D1 expression and this is associated with poor patient prognosis. This work was published in *Proceeding of the National Academy of Sciences*, a reprint is attached for further description of the work.

This past year we have continued to work on β-catenin since it is important in breast cancer. We found very exciting results and have subsequently focused on this project. Recently it was published in *Nature* that GSK-3β can induce NF-κB activity independently of IκB-α(5). We were interested in the mechanism of how GSK-3β can upregulate NF-κB and wanted to look at it's downstream target β-catenin. Normally GSK-3β along with APC and Axin form a complex, which negatively regulates β-catenin(6). Also it has been found that APC can act as nuclear export protein which shuttles β-catenin out of the nucleus where it is then subsequently degraded(7).

We found that when we co-transfect β-catenin and p65 (a subunit of NF-κB) using a κB-reporter, known to be activated by both NF-κB and TNF, that β-catenin can downregulate NF-κB reporter activity in a dose dependant manner. We also found that 293 cells, which are stably transfected with β-catenin, cannot activate the reporter in the presence of TNF to the same extent as the parental cells. We next wanted to know whether β-catenin and NF-κB can interact with each other, so we performed a co-immunoprecipitation assay to precipitate the subunits of NF-κB (p65 and p50). We

found that β-catenin can bind to both p65 and p50, regardless of TNF treatment in vitro. Also using an immunofluorescence assay we found both β-catenin and NF-κB can co-localize in vivo.

Next we wanted to see what effect APC has on NF-κB activity. We found that in SW480 cells which have a mutated and inactive APC(8) that NF-κB transcriptional activity is low, possibly due to the high level of β-catenin. We transfected in wild type APC and found that there is an increase in NF-κB activity in a dose dependant manner. Therefore we concluded that β-catenin can interact with and repress NF-κB activity and this repression can be de-repressed by it upstream negative regulator APC. This work was submitted to *Nature* as a brief communications paper, however due to the fact that they reject more than 90% without review, it was sent back. We are currently trying to add more data to submit elsewhere. I have attached a copy of this manuscript for further description and for figures of this work.

Key research accomplishments the past three years:

(1) We identified six M-phase specific phosphorylation spots of the EGFR by phosphopeptide mapping
(2) We completed the first step in generating the tetracycline-inducible EGFR cell lines
(3) We found that EGF-PE could specifically target EGFR overexpressed cells when nocadazole or taxol was combined to enrich the M-phase population of the treated cells.
(4) We found that cdc2 does not bind to EGFR.
(5) We have found that an EGFR induced gene, cyclin D1, can also be upregulated by β-catenin.
(6) We have found that β-catenin is a prognostic marker for breast cancer.
(7) We found that β-catenin and NF-κB can interact with each other.
(8) We found that β-catenin can inhibit NF-κB activity.
(9) We found that APC can release NF-κB from the inhibition by β-catenin.

Reportable outcomes:

The original principle investigator, S. -Y. Lin graduated and has received his Ph.D. in December 1999; S. Miller has started to serve as PI since January 2000.

Four manuscripts have been published with acknowledgement of this grant.

(1.) **Lin, S. -Y.**, Xia, W., Wang J.C., Kwong K. Y., Sphon, B., Wen, Y., Pestell, R.G., and Hung, M. -C.β-catenin, a novel prognostic marker for breast cancer: its role in cyclin D1 expression and cancer progression. *Proc. Natl. Acad. Sci. U.S. A.* 97:4262-4266, 2000.

Zhou, B. P., Hu M. C. -T., **Miller, S**. A., Yu, Z., Xia, W., **Lin, S. -Y.**, and Hung M. -C. *Her-2/neu* blocks tumor necrosis factor-induced apoptosis via the AKT/NF-κB Pathway. *J. Biol. Chem.* 275:8027-8031, 2000.

Wen, Y., Yan, D. -H., Spohn, B., Deng, J., **Lin, S. -Y.**, and Hung, M. -C. tumor suppression and sensitization to tumor necrosis factor α-induced apoptosis by an interferon-inducible protein, p202, in breast cancer cells. *Cancer Research.* 60:42-46, 2000.

Shao, R., Hu, M. C. -T., Zhou, B. P., **Lin, S. -Y.**, Chiao, P. J., von Linderen, R., Spohn, B., and Hung, M. -C. *J. Biol. Chem.* 274: 21495-21498, 1999.

Conclusion

The studies can help us understand the mechanism of the regulation of EGFR. EGFR is negatively regulated during M-phase, however this negative regulation can be lost during transformation. We have found a potential new therapeutic approach for the treatment of cancer patients, which can specifically target cells that have been transformed with EGFR overexpression, while protecting normal cells. This treatment should reduce toxicity to the patient and may prove to be beneficial in the future. In addition we have found that β-catenin is a poor prognostic factor for breast cancer. We have further tried to elucidate the mechanism of action of β-catenin in order to help understand the reasons for the poor outcome of the patients. We have found β-catenin can upregulate cyclin D1. Also we have found that β-catenin can interact with and inhibit NF-κB. These findings can lead us to a better understanding the molecules involved in breast cancer and hopefully a better understanding of how to treat breast cancer patients.

References:

1. N. Kiyokawa, E. K. Lee, D. Karunagaran, S. Y. Lin, M. C. Hung, *J Biol Chem* **272**, 18656-65 (1997).

2. C. H. Lee *et al.*, *Protein Eng* **6**, 433-40 (1993); C. W. Liao, T. H. Hseu, J. Hwang, *Appl Microbiol Biotechnol* **43**, 498-507 (1995).

3. N. Kiyokawa *et al.*, *Oncogene* **15**, 2633-41. (1997).

4. M. C. Hung, A. L. Schechter, P. Y. Chevray, D. F. Stern, R. A. Weinberg, *Proc Natl Acad Sci U S A* **83**, 261-4 (1986); C. A. Schneider, R. W. Lim, E. Terwilliger, H. R. Herschman, *Proc Natl Acad Sci U S A* **83**, 333-6 (1986).

5. K. P. Hoeflich *et al.*, *Nature* **406**, 86-90 (2000).

6. B. M. Gumbiner, *Curr Opin Cell Biol* **7**, 634-40 (1995).

7. B. R. Henderson, *Nat Cell Biol* **2**, 653-660 (2000).

8. I. Nishisho *et al.*, *Science* **253**, 665-9 (1991).

Figure 1: Cdc2 does not bind to EGFR

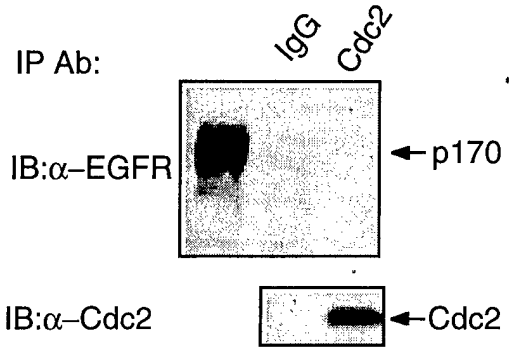

Figure 1. A co-immunoprecipitation assay was preformed by precipitating with the cdc2 antibody. Then a western blot was done to detect for EGFR. The first lane shows cell lysate, without IP, to show EGFR is present in the cell. The second lane shows IgG negative control. The third lane is the IP with cdc2. The bottom panel shows that cdc2 is present, thus distinct from cdc2-neu, EGFR and cdc2 do not seem to bind together under the same conditions.

The specific targeting on epidermal growth factor receptor overexpressed cancer cells by the combination of EGF-Pseudomonas Exotoxin and paclitaxel

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This work was supported by National Institutes of Health Grants RO1-CA58880 and RO1-CA77858 (to M. -C. Hung) and a predoctoral fellowship from the Department of Defense Breast Cancer Research Program DAMD17-98-1-8242 (to S.-Y.L. and S.A.M.).

Abbreviated title: EGF-PE and paclitaxel treatment target EGFR overexpressing cells.

Abbreviations used: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PE, Pseudomonas Exotoxin

Key words: Breast Cancer, Mitotic phase, desensitization

Abstract

Epidermal Growth Factor Receptor (EGFR) overexpression can be found in many different types of cancers, and has been linked to poor prognosis. EGF-Pseudomonas Exotoxin (EGF-PE) is a toxin that has been developed to preferentially target EGFR overexpressing cells, however it can also kill normal cells which still express a basal level of EGFR. Targeting overexpression has been explored in clinical trials. We have previously shown that EGFR does not respond to EGF stimulation during M phase, but this negative regulation can be overcome by EGFR overexpression. This has prompted us to hypothesize that normal cells, which express a basal level of EGFR, at M phase would be resistant to EGF-PE, however the EGFR overexpressing cancer cells should still be sensitive to EGF-PE. We have tested the hypothesis by using either paclitaxel or nocodazole to synchronize cells into M phase, and showed that the survival rate of normal cells indeed increases in response to EGF-PE at M phase. Thus, treatment of either paclitaxel or nocodazole can protect normal cells, EGFR low expressing cells, from EGF-PE while still targeting EGFR overexpressing cells. This strategy maintains the preferential killing of EGF-PE for EGFR overexpressing cancer cells, but protects normal cells from being killed by EGF-PE.

Introduction

The epidermal growth factor receptor (EGFR) plays an important role in cell proliferation, migration and differentiation. The receptor is a 170 kDa glycoprotein with intrinsic kinase activity [1, 2]. Overexpression of EGFR has been shown to be involved in the progression of many types of cancers such as breast [3], ovarian [4], lung [5], colon [6], and more. In breast cancer it is overexpressed in 14-42% of cancers [7]. It is often correlated with poor clinical outcome [8-11]. Therefore EGFR is a very good target for breast cancer therapy and clinical trials targeting EGFR using monoclonal antibodies are currently underway [12].

EGFR can be regulated by phosphorylation. It is known to be differentially regulated throughout the cell cycle [13-15]. Previously, we have shown that EGFR is negatively regulated during the M phase of the cell cycle. In M phase there is a decrease in tyrosine phosphorylation and a reduced affinity for ligand binding which results in the inability of EGFR to respond to EGF induced signals. However, this negative regulation can be overcome by overexpression of the receptor suggesting a mechanism by which overexpression of EGFR may help in leading to transformation of the cell [15].

Since there is a low affinity for the EGF ligand during M phase in normal cells, cells expressing a basal level of EGFR, at M phase normal cells should be resistant to a EGF chimeric toxin. In this study we treated cells with either nocodazole or paclitaxel, both are agents which can synchronize the cells into M phase. We then treated the cells with EGF- Pseudomonas Exotoxin (EGF-PE). EGF-PE is a chimeric toxin consisting of the epidermal growth factor (EGF), a known ligand for EGFR, fused to a Pseudomonas Exotoxin. This toxin will preferentially target EGFR overexpressing cells, however it can also target normal cells which express a basal level of EGFR,

though at a lower extent [16, 17]. Therefore use of this toxin alone could have some toxicity when given to patients. Our approach, however, should protect normal cells from EGF-PE. In this study we show that when using this toxin in combination with either nocodazole or paclitaxel we are able to synchronize cells into M phase and normal cells expressing a low level of EGFR are resistant to the toxin, while EGFR overexpressing cells are still sensitive to be killed. Thus it provides a proof of concept that normal cells at M phase are resistant to EGF-PE, but EGF-PE is still toxic to EGFR overexpressing cells.

Materials and Methods

Materials: EGF-PE was provided by J. Hwang (Institute of Molecular Biology, Academia Sinica, Taiwan). Paclitaxel was purchased from Bristol-Myers Squibb Co., Wallingford, CT. Nocodazole, was purchased from Sigma. MTT was purchased from Sigma.

Cell Culture: Cell lines Swiss3T3, MDA-MB-468, and HBL-100 were obtained from American Type Culture Collection. The Her-5 cell line is a line derived from the cell line NR-6 by stable transfection of EGFR provided to us by Dr. H-J Kung (Case Western Reserve University). NR-6 is a variant of Swiss 3T3, which lacks EGFR [18, 19]. Cells were grown in Dulbecco's Modified Eagle's Medium mixed with F-12 (1:1 life technologies, Inc.) supplemented with 10% Fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere.

Cell synchronization and EGFR-PE treatment: Cells were treated with either 0.6 μg/ml of nocodazole or 0.1 μM of paclitaxel for one day and then treated with 1μg/ml of EGF-PE for HER5 cells, SW3T3 cells, or NR-6 or 3ng/ml of EGF-PE for the MDA-MB-468 cells and HBL-100 cells. Cells were treated with EGF-PE for 1-3 days. G2/M populations were determined by FACS

analysis.

MTT assay: Following EGF-PE treatment cells were incubated with 20µl of MTT solution (5mg/ml in PBS) for 2 hours at 37°C. 100µl of MTT lysis buffer was then added and cells were incubated overnight at 37°C. Absorbance was measured at 570nm.

Results and Discussion

Since EGFR is negatively regulated during M phase of the cell cycle we wanted to test how normal cells when synchronized in M phase would react to EGF-PE as compared to cells which overexpress EGFR. Cells which overexpress EGFR receptor can overcome this negative regulation so we expected that EGFR overexpressing cells would be killed with treatment of this toxin, however normal cells would be less sensitive. We used cells that have genetically similar backgrounds, NR-6, HER-5, and Swiss 3T3 (SW3T3) mouse fibroblasts, all variants of SW3T3 cells. All 3 cell lines have a varying level of EGFR expression. NR-6 cells do not express EGFR, while Her-5 cells are SW3T3 cells that have been stably transfected with EGFR, so that they overexpress the EGFR [15]. SW3T3 cells have a low level of EGFR. Based on our hypothesis, Her-5 cells should be able to take up the EGF-PE toxin during M phase since they overexpress EGFR, while SW3T3 cells should not be able to take up the toxin due to low affinity for the ligand because of negative regulation of the receptor. NR-6 should not take up the toxin because it lacks EGFR.

First, we treated the cells with nocodazole, 0.6 µg/ml, for 1 day, to synchronize the cells into M phase, and then we treated the cells with EGF-PE. To measure viability of the cells we did an MTT assay and compared the results with cells that have not been treated with nocodazole but were

treated with EGF-PE. EGFR overexpressing cells, Her-5 were killed by the toxin regardless of whether or not they were synchronized into M phase. On the other hand SW3T3 cells, which have a low level of EGF-PE showed 70% viability when treated with nocodazole first as opposed to 40% when not treated with nocodazole first (Fig. 1A). The percent of cells which are arrested in G2/M for this cell line is only 37%, therefore we would expect an even greater difference in survival of the cells treated with nocodazole if a greater percentage of cells were synchronized in M phase. As expected there is no difference in the NR-6 cells, which do not have the EGFR receptor, both treated and untreated show high viability, indicating EGF-PE is specific for cells expressing EGFR (Fig. 1A). These results suggest that cells which overexpress EGFR are sensitive to EGF-PE, while cells which express a low level, i.e., normal cells, are resistant to the toxin when synchronized in M phase.

We found similar results when we compared MDA-MB-468 cells, a breast cancer cell line which overexpresses EGFR, and compared it to HBL100 cells, a mammary epithelial cell line expressing a low level of EGFR. Regardless of whether or not MDA-MB-468 cells were first treated with nocodazole we found that they were sensitive to EGF-PE. These results are similar to the high EGFR expressing Her5 cells. However the HBL100 cells were less sensitive to the toxin when first synchronized in M phase, as the low expressing SW3T3 cells were (Fig. 1B).

Since paclitaxel is widely used in the clinic to treat cancer patients and is known to enrich M phase populations in order to kill cancer cells [20]. We wanted to test if we could get similar results using paclitaxel, as we did with nocodazole. We first used the mouse fibroblast cells lines, Her-5, SW3T3, and NR-6, and we treated them with 0.1μM paclitaxel. We then treated the cells with EGF-PE. Similar results were obtained as with nocodazole treatment. With only 32% of the cells in M phase we still see a significant difference in the viability of the SW3T3 cells. However, Her-5

cells are killed regardless of pretreatment with paclitaxel. The toxin does not affect NR-6 cells (Fig. 2A).

When treating the MDA-MB-468 cells and HBL100 cells with paclitaxel prior to treatment with EGF-PE, again, while all the cells die in the MDA-MB 468 cells, the HBL100 cells show resistance to the toxin. These results suggest that treatment with paclitaxel will protect normal cells from EGF-PE while killing the EGFR overexpressing cells (Fig 2 B).

This approach is a potential new therapeutic strategy to target cancer cells, which overexpress EGFR. Paclitaxel, which is widely used in the clinic in combination with EGF-PE may provide a way to specifically target EGFR overexpressing cancer cell and at the same time minimize normal cell killing by the EGF-PE toxin due to the enrichment of M phase cells by paclitaxel. EGF-PE is selective for EGFR expressing cells, however alone it may be toxic to normal cells. Use of these agents, paclitaxel and EGF-PE together may provide a way to achieve specific targeting while reducing toxicity to the patient.

References

1. Wells A. EGF receptor. Int J Biochem Cell Biol 1999;31:637-43.

2 Rusch V, Mendelsohn J, Dmitrovsky E. The epidermal growth factor receptor and its ligands as therapeutic targets in human tumors. Cytokine Growth Factor Rev 1996;7:133-41.

3. Nicholson S, Wright C, Sainsbury JR, et al. Epidermal growth factor receptor (EGFr) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure. J Steroid Biochem Mol Biol 1990;37:811-4.

4. Gullick WJ, Marsden JJ, Whittle N, Ward B, Bobrow L, Waterfield MD. Expression of epidermal growth factor receptors on human cervical, ovarian, and vulval carcinomas. Cancer Res 1986;46:285-92.

5. Ohsaki Y, Tanno S, Fujita Y, et al. Epidermal growth factor receptor expression correlates with poor prognosis in non-small cell lung cancer patients with p53 overexpression. Oncol Rep 2000;7:603-7.

6. Porebska I, Harlozinska A, Bojarowski T. Expression of the tyrosine kinase activity growth factor receptors (EGFR, ERB B2, ERB B3) in colorectal adenocarcinomas and adenomas. Tumour Biol 2000;21:105-15.

7. Hoskins KWeber BL. Recent advances in breast cancer biology. Curr Opin Oncol 1995;7:495-500.

8. Nicholson S, Sainsbury JR, Halcrow P, Chambers P, Farndon JR, Harris AL. Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. Lancet 1989;1:182-5.

9. Sainsbury JR, Malcolm AJ, Appleton DR, Farndon JR, Harris AL. Presence of epidermal growth factor receptor as an indicator of poor prognosis in patients with breast cancer. J Clin Pathol

1985;38:1225-8.

10. Harris AL, Nicholson S, Sainsbury JR, Farndon J, Wright C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. J Steroid Biochem 1989;34:123-31.

11. Hawkins RA, Killen E, Whittle IR, Jack WJ, Chetty U, Prescott RJ. Epidermal growth factor receptors in intracranial and breast tumours: their clinical significance. Br J Cancer 1991;63:553-60.

12. Baselga J, Pfister D, Cooper MR, et al. Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin [In Process Citation]. J Clin Oncol 2000;18:904.

13. Newberry EP, Pike LJ. Cell-cycle-dependent modulation of EGF-receptor-mediated signaling. Biochem Biophys Res Commun 1995;208:253-9.

14. Pillay I, Sharma SV. In vivo and in vitro serine/threonine phosphorylations of epidermal growth factor receptor upon entry into the cell cycle. Cell Growth Differ 1995;6:39-49.

15. Kiyokawa N, Lee EK, Karunagaran D, Lin SY, Hung MC. Mitosis-specific negative regulation of epidermal growth factor receptor, triggered by a decrease in ligand binding and dimerization, can be overcome by overexpression of receptor. J Biol Chem 1997;272:18656-65.

16. Lee CH, Lee EC, Tsai ST, Kung HJ, Liu YC, Hwang J. An EGF-pseudomonas exotoxin A recombinant protein with a deletion in toxin binding domain specifically kills EGF receptor bearing cells. Protein Eng 1993;6:433-40.

17. Liao CW, Hseu TH, Hwang J. A target-specific chimeric toxin composed of epidermal growth factor and Pseudomonas exotoxin A with a deletion in its toxin-binding domain. Appl Microbiol Biotechnol 1995;43:498-507.

18. Hung MC, Schechter AL, Chevray PY, Stern DF, Weinberg RA. Molecular cloning of the neu

gene: absence of gross structural alteration in oncogenic alleles. Proc Natl Acad Sci U S A 1986;83:261-4.

19. Schneider CA, Lim RW, Terwilliger E, Herschman HR. Epidermal growth factor-nonresponsive 3T3 variants do not contain epidermal growth factor receptor-related antigens or mRNA. Proc Natl Acad Sci U S A 1986;83:333-6.

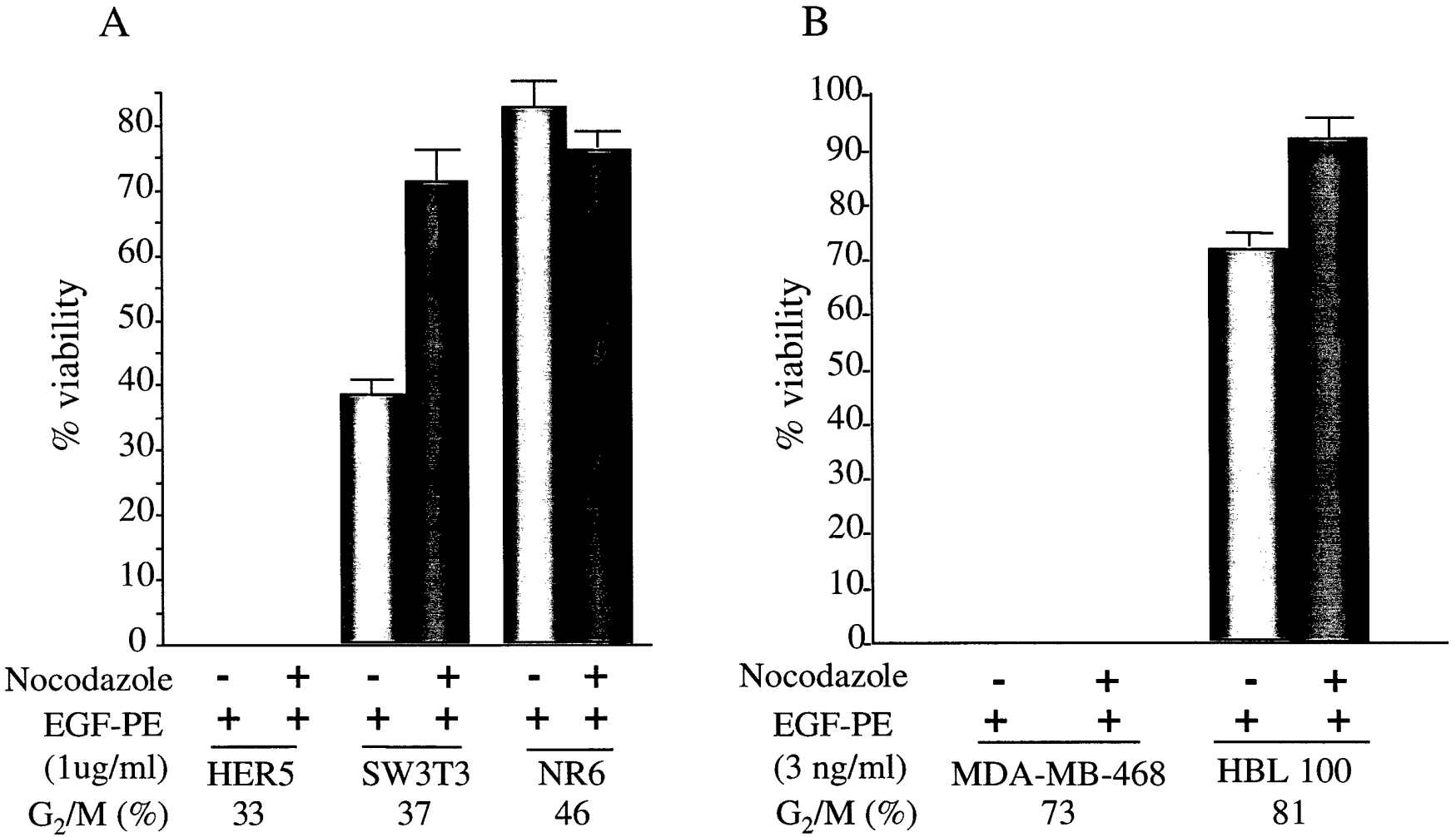
20. Yu D, Jing T, Liu B, et al. Overexpression of ErbB2 blocks Taxol-induced apoptosis by upregulation of p21Cip1, which inhibits p34Cdc2 kinase. Mol Cell 1998;2:581-91.

Figure Legends

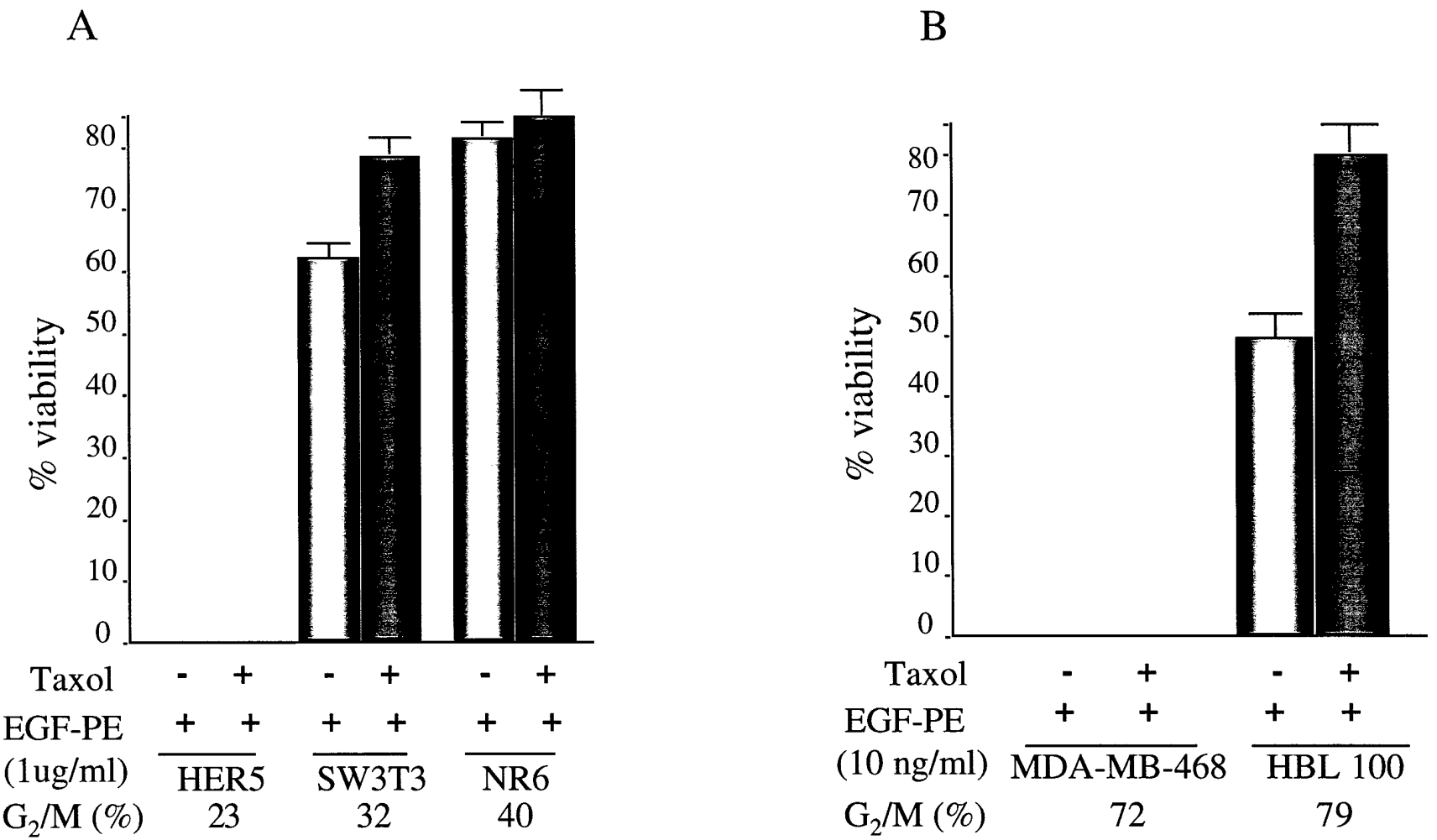
Figure 1. Nocadazole protects EGFR low expressors from killing by EGF-PE. (A). Three mouse fibroblast cell lines with the same genetic background except the EGFR expression levels (HER5: High, SW3T3: Low, NR-6: EGFR null) were treated with (+) or without (-) nocadazole for one day and then treated with 1 μg/ml of EGF-PE. Percent viability is the ratio between the amount of cells in the EGF-PE treated cells compared to cells not treated with EGF-PE. (B) The EGFR overexpressed breast cancer cell line (MDA-MB-468) and the low-expressor (HBL100) were treated as (A) except 3 ng/ml of EGF-PE was used instead.

Figure 2. Paclitaxel protects EGFR low-expressors from killing by EGF-PE. (A) Three mouse fibroblasts cell lines with the same genetic background expect the EGFR expression levels (HER5: High, SW3T3: Low, NR-6: EGFR null) were treated with (+) or without (-) paclitaxel for one day and then treated with 1 μg/ml of EGF-PE. The EGFR overexpressed breast cancer cell line (MDA-MB-468) and the low-expresser (HBL100) were treated as (A) except 10 ng/ml of EGF-PE was used instead.

Nocodazole protects EGFR low-expressors from killing by EGF-PE



Taxol protects EGFR low-expressors from killng by EGF-PE



β-catenin interacts with and inhibits NF-κB

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NF-κB is regulated by IκB-α, which binds to NF-κB and inhibits its nuclear translocation[1]. When IκB-α is phosphorylated by upstream kinases, NF-κB, a heterodimer of p65 and p50, is released [2]. Recently, it was shown that NF-κB is also regulated by an unidentified mechanism involving GSK-3β[3].

To investigate this regulation of NF-κB, we examined the effect of β-catenin, a downstream target of GSK-3β, on NF-κB activity. β-catenin, which is regulated by GSK-3β and APC[4], translocates from the cytoplasm to the nucleus, similar to NF-κB[5,6]. We examined the effects of β-catenin on a κB-driven luciferase reporter that is activated by p65 or TNF. Co-transfection of HEK 293 cells with a fixed dose of p65 and increasing doses of wild type β-catenin or constitutively active mutant S37Aβ-catenin[7] results in a dose dependent suppression of luciferase activity (Fig. 1a, left) suggesting that β-catenin inhibits p65 transcriptional activity. Similarly, this inhibitory effect is also seen with TNF treatment, which activates NF-κB, in 293-β-catenin[8] cells, stably transfected with β-catenin S45Y (Fig. 1a. middle) and in transient transfected 293 cells (data not shown). Also there is a reduction in NF-κB DNA binding in the stable transfectants, which further supports this notion (Fig. 1a right). Thus, expression of β-catenin has an inhibitory effect on NF-κB activity.

To determine whether β-catenin directly interacts with NF-κB, we preformed a co-immunoprecipitation (IP) to precipitate subunits of NF-κB. Both p65 and p50 were able to bind to β-catenin (Fig 1b) regardless of TNF treatment, which reduced the binding of IκB-α to NF-κB due to IκB-α degredation[1] (Fig 1b). To determine whether this interaction occurs *in vivo*, we examined the subcellular location of p65 and β-catenin by immunofluorescence staining. Following TNF treatment, p65 (red) travels from the

cytoplasm to the nucleus. Conversely, β-catenin (green) is found both in the cytoplasm and nucleus regardless of treatment. Importantly, co-localization of these two molecules is observed when the stainings are merged together (yellow) (Fig. 1c) suggesting that interaction between β-catenin and p65 may also occur *in vivo*.

Recently, APC was shown to function as a nuclear export protein shuttling β-catenin from the nucleus to the cytoplasm [5]. To further investigate the role of β-catenin we examined the effect of APC on NF-κB activity. Using SW480 cells, in which APC is mutated [9] and β-catenin expression is high, we found that introduction of wild type APC significantly enhances NF-κB activity in a dose dependant manner, whereas inactive mutant APC does not (Fig. 1d). The immunofluorescence staining indicates that β-catenin disappears in the presence of APC and GFP but not with GFP plus vector (Fig. 1e). Consistent with the luciferase assay, this suggests that APC shuttles β-catenin from the nucleus to the cytoplasm for degradation, releasing the β-catenin-mediated suppression of NF-κB (Fig.1d). Thus, NF-κB activity is regulated by β-catenin upstream regulator APC.

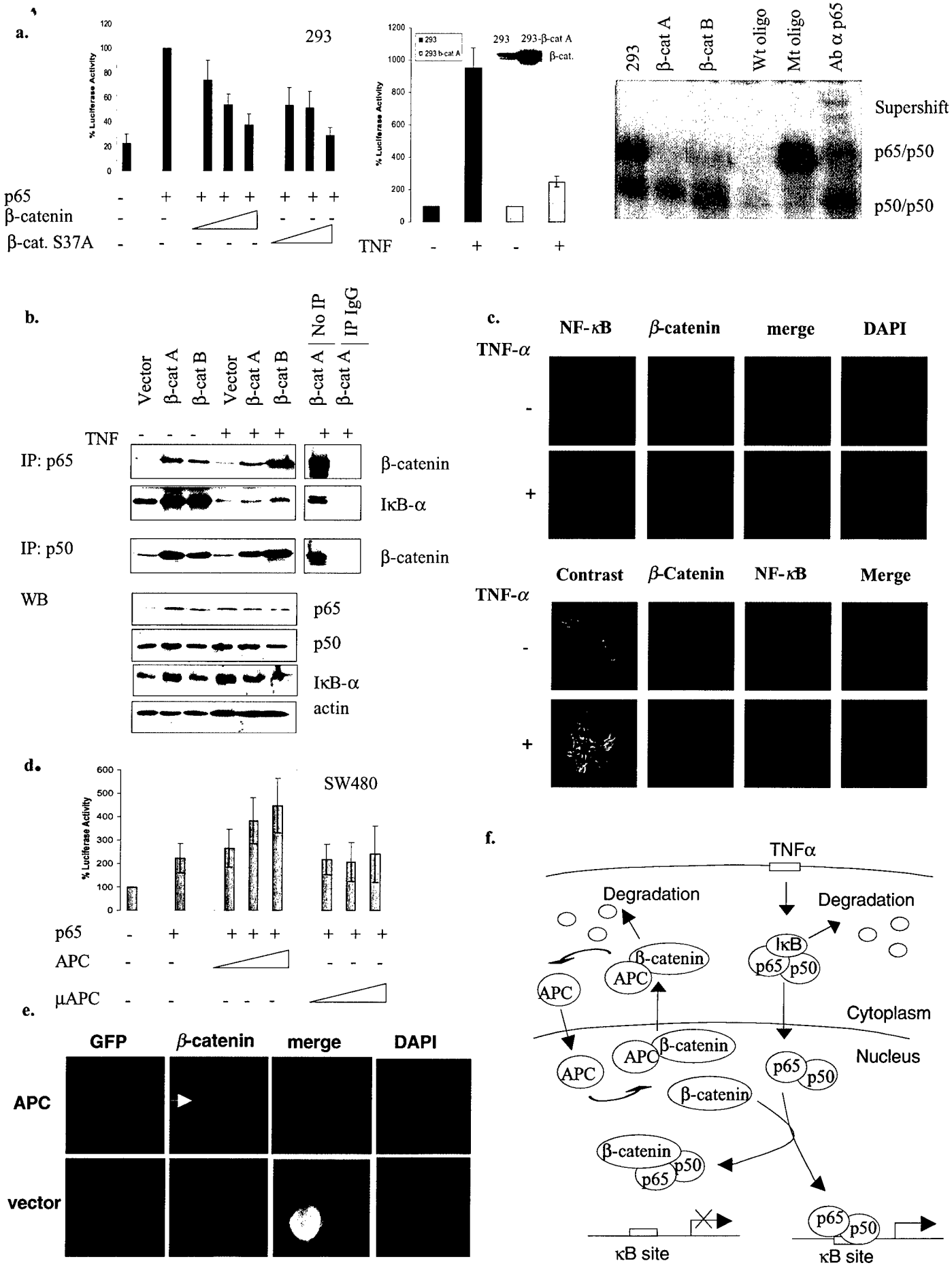
In summary, we show that β-catenin plays a role the regulation of NF-κB. β-catenin interacts with NF-κB components, and inhibits NF-κB activity; this inhibition is further regulated by APC. Therefore, we propose NF-κB can be repressed by β-catenin and derepressed by APC (Fig. 1f).

Fig. 1. β-catenin interacts with and inhibits NF-κB, which is further regulated by APC a. β-catenin inhibits NF-κB-driven-reporter. Left, 293 cells were transfected with κB-luc.(0.1μg), p65 (0.2μg), and β-catenin, or constitutively active mutant β-catenin S37A, resistant to degradation (β-cat S37A) (0.6, 1.2, 1.8 μg). Middle, 293 S45Y β-catenin stable transfectants, also resistant to degradation, (293-β-cat A) were transfected with kB-luc (0.1ug) and treated 48 hours later with 20ng/ml TNF for 8 hours. Insert shows β-catenin level. pRL-TK-luc.(0.1μg) was used as internal control in all transfection assays except when otherwise indicated. Right, Nuclear extracts (5 μg) were used in electrophoretic mobility shift assay (EMSA) with an oligonucleotide probe containing the κB binding site as described [10]. The p65/p50 heteodimer is shown. Eighty-fold of cold wild type or mutant NF-κB oligonucleotides were used for competition in the nuclear extracts of TNF-treated 293 cells. An antibody against p65 was used for supershifting the complex as indicated. **b**. β-catenin interacts with NF-κB (p65 and p50) *in vitro*. Immunoprecipitation (IP) was performed with 2μg/ml antibodies against p65 or p50 (normal IgG as a negative control), then subjected to immunoblot with antibody against β-catenin, or IκB-α as a positive control. β-cat-A lysate was used for immunoblot as positive control (No IP). Immunoblot indicates no significant change in p65, p50, and IκB-α level. **c**. β-catenin and p65 co-localize *in vivo*. The immunofluorescence-staining was performed with primary antibodies against p65 or β-catenin, and secondary antibodies conjugated with TRITC or FITC respectively. The top panel represents an individual cell, while the bottom panel shows a population of cells. **d**. β-catenin-mediated inhibition of NF-κB is released by APC. SW480 cells (APC

mutated and β-catenin active) were transfected with plasmids of κB-luc.(0.1μg), p65 (0.2μg), and wt APC (or μAPC) (0.5, 1.0, 1.5 μg), pRL-TK-luc was not used in this assay due to a regulatory effect of APC on tk promoter. **e**. Introduction of APC into SW480 cells eliminates β-catenin. SW480 cells were transfected with APC and GFP or GFP and vector. The immunofluorescence-staining was performed as above. The arrow indicates negative staining for β-catenin in the GFP-positive in the APC transfected cells. **f**. Model of β-catenin-mediated inhibition of NF-κB. Error bars are the mean +/- standard error.

References:

1. Baeuerle, P. A. & Baltimore, D. *Science* **242**, 540-6 (1988).
2. Ghosh, S., May, M. J. & Kopp, E. B.*Annu Rev Immunol* **16**, 225-60 (1998).
3. Hoeflich, K. P. et al. *Nature* **406**, 86-90 (2000).
4. Gumbiner, B. M. *Curr Opin Cell Biol* **7**, 634-40 (1995).
5. Henderson, B. R. *Nat Cell Biol* **2**, 653-660 (2000).
6. Johnson, C., Van Antwerp, D. & Hope, T. J. *Embo J* **18**, 6682-93 (1999).
7. Orford, K., Crockett, C., Jensen, J. P., Weissman, A. M. & Byers, S. W. *J Biol Chem* **272**, 24735-8. (1997).
8. Lin, S. Y. et al. *Proc Natl Acad Sci U S A* **97**, 4262-6 (2000).
9. Nishisho, I. et al. *Science* **253**, 665-9 (1991).
10. Zhou, B. P. et al. *J Biol Chem* **275**, 8027-31. (2000).


a.
293
% Luciferase Activity
p65
β-catenin
β-cat. S37A
293
293-β-cat A
β-cat.
TNF
293
β-cat A
β-cat B
Wt oligo
Mt oligo
Ab α p65
Supershift
p65/p50
p50/p50
b.
Vector
β-cat A
β-cat B
Vector
β-cat A
β-cat B
No IP
IP IgG
β-cat A
β-cat A
TNF
IP: p65
β-catenin
IκB-α
IP: p50
β-catenin
WB
p65
p50
IκB-α
actin
c.
NF-κB
β-catenin
merge
DAPI
TNF-α
Contrast
β-Catenin
NF-κB
Merge
d.
SW480
% Luciferase Activity
p65
APC
μAPC
e.
GFP
β-catenin
merge
DAPI
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Degradation
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IκB
p65
p50
β-catenin
APC
Cytoplasm
Nucleus
κB site
κB site

[CANCER RESEARCH 60, 42–46, January 1, 2000]

Advances in Brief

Tumor Suppression and Sensitization to Tumor Necrosis Factor α-induced Apoptosis by an Interferon-inducible Protein, p202, in Breast Cancer Cells[1]

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Abstract

p202, an IFN-inducible protein, interacts with several important regulatory proteins, leading to growth arrest or differentiation. In this report, we demonstrate that, in addition to inhibiting *in vitro* cell growth, p202 can also suppress the tumorigenicity of breast cancer cells *in vivo*. Furthermore, we found that p202 expression could sensitize breast cancer cells to apoptosis induced by tumor necrosis factor α treatment. One possible mechanism contributing to this sensitization is the inactivation of nuclear factor-κB by its interaction with p202. These results provide a scientific basis for a novel therapeutic strategy that combines p202 and tumor necrosis factor α treatment against breast cancer.

Introduction

IFNs possess a wide variety of biological properties such as antivirus, antiproliferation, immunoregulation, antiangiogenesis, and antineoplasia and have been used in clinical treatment of certain cancers (1). Here, we examined the possibility of using an IFN-inducible protein, p202 (2), as a potential therapeutic substitute for IFNs. p202 is a M_r 52,000 nuclear phosphoprotein known to be a negative transcription modulator that, in most cases, inhibits transcription of its target genes by physically interacting with certain transcription activators (3–8). Like IFN treatment, constitutive expression of p202 causes G_1-S cell cycle arrest in murine fibroblast cells (9, 10). Consistent to that observation, we demonstrated previously that the enforced expression of p202 could significantly retard the *in vitro* growth of prostate cancer cells in both cell culture and soft agar (10). However, it is not known whether p202 expression could exert an antitumor effect on cancer cells. In this report, we demonstrated for the first time that p202 expression was able to inhibit tumorigenicity of human breast cancer cells *ex vivo*. Furthermore, p202 expression can sensitize breast cancer cells to apoptosis induced by TNF[4]-α and that correlates with inactivation of NF-κB by a NF-κB/p202 interaction. These results suggest a potential combined therapy using p202 and TNF-α against breast cancer.

Received 7/27/99; accepted 11/11/99.

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[1] This work is partially supported by NIH Grants RO-1 CA 58880 and CA 77858, M. D. Anderson Breast Cancer Research Program, and Faculty Achievement Award (to M-C. H). Y. W. is the recipient of a predoctoral fellowship from the United States Army Breast Cancer Research Training Grant Program (Grant DAMD 17-99-1-9264 to M-C. H.). S-Y. L. is a predoctoral fellow supported by the Department of Defense Army Breast Cancer Research Program, Grant DAMD17-98-1-8242.

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[4] The abbreviations used are: TNF, tumor necrosis factor; NF, nuclear factor; CMV, cytomegalovirus; PEI, polyethylenimine; FACS, fluorescence-activated cell sorter.

Materials and Methods

Cell Culture, Transfection, and Colony-forming Assay. MDA-MB-453 and MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection. Cells were maintained in DMEM/F-12 (HyClone Laboratories, Inc.) supplemented with 10% (v/v) fetal bovine serum. Cells were transfected with a p202 expression vector (CMV-p202) or the control vector pcDNA3 (Invitrogen) using lipofectin (Life Technologies, Inc.) and selected in 500 μg/ml G418 (Geneticin; Life Technologies, Inc.). Western blotting using an anti-p202 polyclonal antibody (11) identified p202 stable transfectants.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay and FACS Analysis. These standard assays were done as described previously (12).

[^{3}H]Thymidine Incorporation Assay and Soft-Agar Assay. These standard assays were done as described previously (12).

Tumorigenicity Assay. Female athymic nude mice (*nu/nu*), 4–5 weeks of age, were used in this *ex vivo* experiment. Briefly, MCF-7 cells were transfected with CMV-p202 (10 μg) using PEI. Twenty-four h after transfection, cells (3×10^6) were harvested in 0.2 ml of PBS and injected into the mouse mammary fat pads. 17-β-Estradiol pellets (0.72 mg/pellet, 60-day release; Innovative Research of America, Inc.) were implanted s.c. into the mice 1-day before cell injection. The presence of estrogen is essential for MCF-7 cells to grow in mice. The size of the tumors was measured with a caliper every week, and the tumor volume was calculated using a formula: $V = 1/2 \times S^2 \times L$, where V = volume, S = the short length of the tumor, and L = the long length of the tumor in cm.

Immunoprecipitation and Immunoblotting. MDA-MB-453 (453) and 453-p202 cells were treated with 10 and 20 ng/ml of human TNF-α (R & D Systems, Inc., Minneapolis, MN) for 30 min. Cells with or without TNF-α treatment were extracted in RIPA lysis buffer without SDS on ice. Exacts were sonicated and cleared by centrifugation at 4°C. For immunoprecipitation, equivalent aliquots of cell lysates (1 mg of total protein) were incubated with 1 μg of anti-p65 antibody (Santa Cruz Biotechnology) for 4 h with gentle rotation at 4°C. Protein A-Sepharose beads (50 μl) was added for an additional 1 h. The beads were extensively washed with ice-cold RIPA buffer, and the precipitate was dissolved in a sample buffer for electrophoresis and Western blot.

Results and Discussion

To investigate a potential growth-inhibitory effect of p202 on breast cancer cells, we performed a colony-forming assay by transfecting a p202 expression plasmid driven by CMV promoter (CMV-p202) or a control vector (pcDNA3) containing neomycin-resistance gene into two human breast cancer cell lines, MDA-MB-453 (453) and MCF-7. After 3 weeks of G418 selection, the number of G418-resistant colonies was scored. A dramatic reduction in the number of G418-resistant colonies was seen in cells (MCF-7 and 453) transfected with p202 as compared with that with the control plasmid, pcDNA3 (Fig. 1*a*, *left panel*). There was at least a 75% reduction in colony number in both p202-transfected cell lines (Fig. 1*a*, *right panel*). These data suggest that p202 expression may be associated with antiproliferation

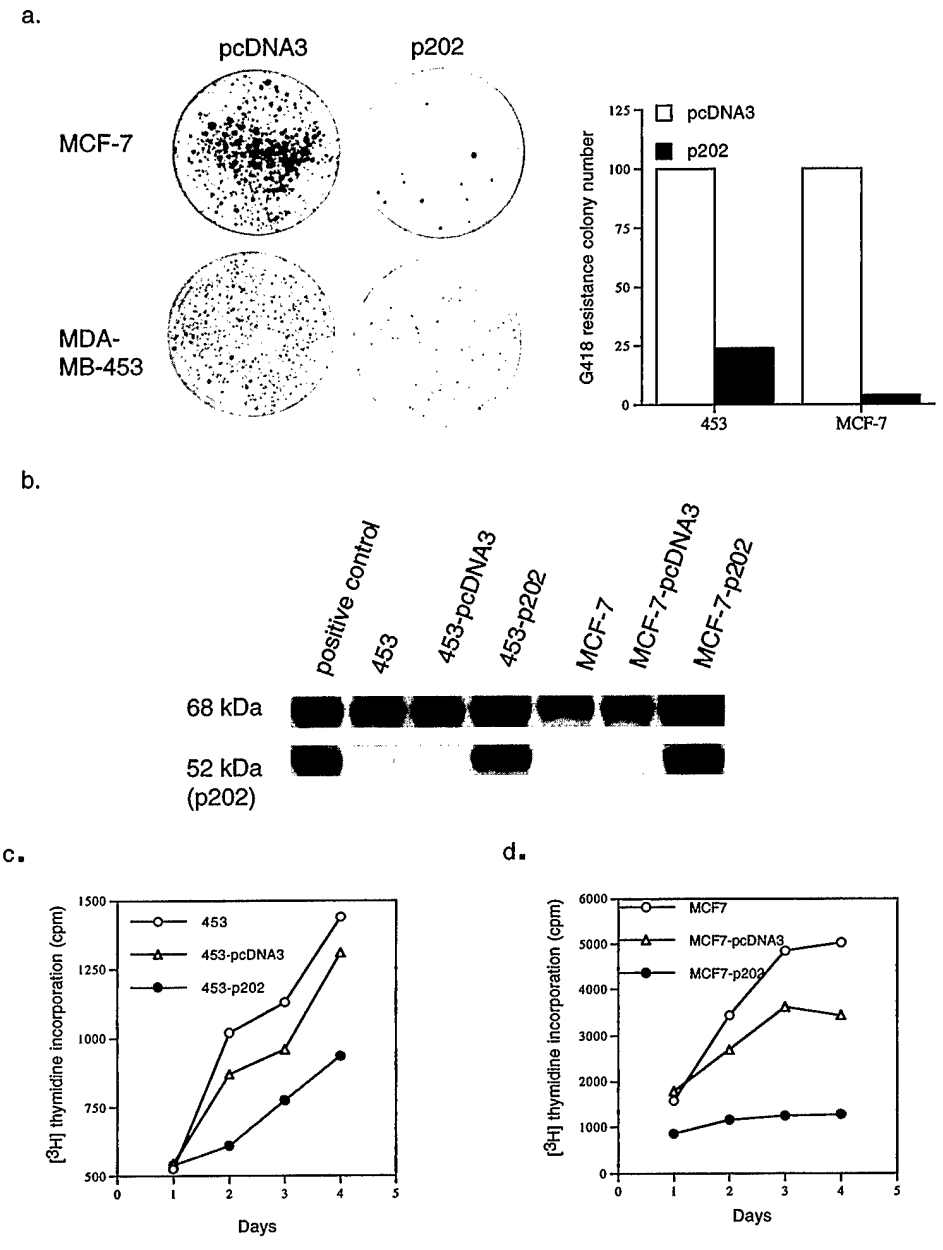

Fig. 1. Expression of p202 inhibits the proliferation of MDA-MB-453 and MCF-7 breast cancer cells. *a*, colony-forming assay. MDA-MB-453 and MCF-7 cells were transfected with either a control vector (pcDNA3) or a p202 expression vector. The colony number obtained from pcDNA3 transfection was set as 100%. *b*, Western blot analysis of the p202 stable transfectants. The M_r 52,000 protein represents p202, and the nonspecific M_r 68,000 protein cross-reacting with the antibody was used as an equal loading control. *c* and *d*, [^{3}H]thymidine incorporation assays. DNA synthesis rate was measured by the amount of [^{3}H]thymidine incorporated into the cells at each time point. The measurement was conducted in quadruplicates, and the variations within each quadruplicate are too small to be of any significance.

and/or proapoptotic activity in these breast cancer cells. To further characterize the biological effects of p202 expression on these cells, we attempted to isolate several lines of p202-expressing stable clones. Using Western blot with a p202-specific antibody (11), we were able to identify one p202-expressing stable clone (of 20) from each cell line, *i.e.*, MDA-MB-453-p202 (453-p202) and MCF-7-p202 (Fig. 1*b*). The low frequency of p202-expressing clones obtained from the G418-resistant colony supports the idea that p202 expression may cause an antiproliferation and/or proapoptotic effect on these cells. To assess these two p202-mediated biological effects, we first measured and compared the mitogenic activity between the p202 stable lines and the control cell lines using [^{3}H]thymidine incorporation assay. The p202-expressing cells (453-p202 and MCF-7-p202) exhibited a reduced DNA synthesis rate as compared with their respective control cell lines, *i.e.*, 453 and 453-pcDNA3; MCF-7 and MCF-7-pcDNA3 (Fig. 1, *c* and *d*). Similarly, the p202-expressing cells also showed a slower growth rate than the control cells as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Thus, our data strongly suggest that p202 functions as a growth inhibitor in breast cancer cells.

To test whether p202 expression in breast cancer cells may also suppress their *in vitro* transformation phenotype defined by the ability of these cells to grow in soft agar, we then measured the number of colonies formed in soft agar by the p202-expressing cells and the control cells. As shown in Fig. 2, *a* and *b*, both 453-p202 and MCF-7-p202 exhibited >60% reduction (after 3 weeks of incubation) in colony number than those of the parental and pcDNA3 transfectant. The difference in number was not attributable to the slower growth rate of the p202-expressing cells than that of the control cells (Fig. 1, *c* and *d*), because a prolonged (6 weeks) incubation of the same plates did not yield more colonies. Rather, it represents a real loss of anchorage-independent growth, *i.e.*, an *in vitro* transformation phenotype, of these p202-expressing cells.

One of the most critical biological properties determining the potential application of a tumor suppressor gene in cancer therapy is its ability to reduce tumorigenicity *in vivo*. To test a possible antitu-

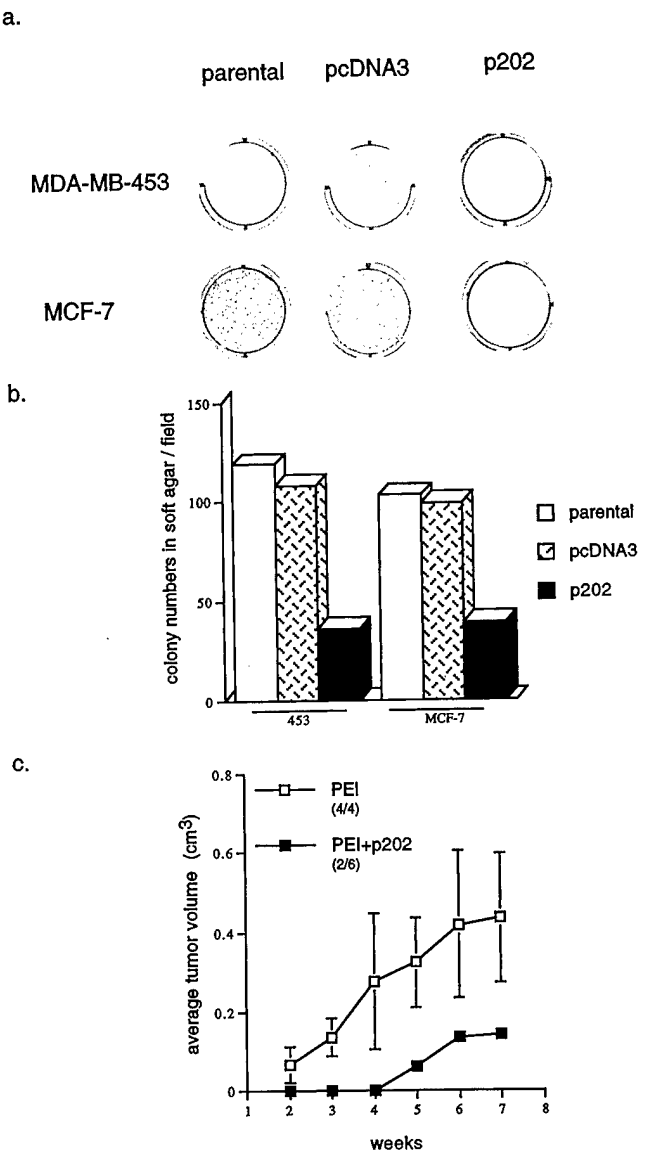

Fig. 2. p202 inhibits the transformation phenotype of breast cancer cells. *a*, colony formation in soft agar. MDA-MB-453, 453-pcDNA3, 453-p202, MCF-7, MCF-7-pcDNA3, and MCF-7-p202 cell lines were subjected to anchorage-independent growth in soft agar. *b*, number of colonies formed in soft agar as shown in *a*. The number represents the average of five random microscopic fields from each cell line. *c*, p202 *ex vivo* experiment. MCF-7 cells were transfected with p202 expression vector using PEI. After 24 h, cells were harvested, and the p202 (*PEI+p202*) or mock (*PEI*) transfected cells (3×10^6 cells/injection) were injected into the mammary fat pad of female nude mice. 17-β-Estradiol pellets were implanted s.c. into the mice 1-day before inoculation. Tumor formation was monitored every week. *Bars*, SE.

mor activity of p202, we performed an *ex vivo* tumorigenicity assay in an orthotopic breast cancer model. Briefly, CMV-p202/PEI or PEI alone (PEI is a polymer vector used for transfection) was transfected into MCF-7 cells before injection into the mammary fat pads of the estrogen-supplemented nude mice. The p202 transfection caused a drastic reduction of tumorigenesis of MCF-7 cells as compared with that of the mock transfection (PEI alone; Fig. 2*c*). Our data demonstrated, for the first time, that the p202 expression is associated with an antitumor activity in animals.

In an attempt to identify therapeutic agents that may cooperate with p202 to synergize the antitumor effect on breast cancer cells, we used FACS analysis (Fig. 3) to determine a potential synergism in inducing apoptosis. We found that the p202-expressing cells were more susceptible to TNF-α-induced apoptosis than the control cells, *i.e.*, after treatment with TNF-α (0, 10, 20 ng/ml) for 48 h, more 453-p202 cells were undergoing apoptosis (sub-G_1 population) than the parental 453 cells and 453-pcDNA3 control cells in a dose-dependent manner (Fig. 3*a*). Likewise, MCF-7-p202 cells were also found to be more sensitive to TNF-α-induced apoptosis than the parental MCF-7 cells in a dose-dependent manner (Fig. 3*b*). These results suggested that p202 expression could sensitize cells to TNF-α-induced apoptosis.

One possible mechanism of the p202-mediated sensitization to TNF-α-induced apoptosis is that p202 could antagonize the antiapoptotic function of NF-κB (13–15). To test that hypothesis, we tested whether p202 expression could affect the NF-κB-mediated transcription activation in response to TNF-α treatment. We cotransfected CMV-p202 and a NF-κB-activatable promoter-reporter construct (κB-luc), *i.e.*, an IκB promoter-driven luciferase gene, into 453 cells in the presence of TNF-α (Fig. 4*a*). As expected, κB-luc was readily activated in the presence of TNF-α. However, this TNF-α-induced transcription activation was repressed by p202 in a dose-dependent manner. To test whether p202 acted on the NF-κB molecule to elicit such transcription repression, we cotransfected CMV-p202 with a Rel-A (a p65 subunit of NF-κB) cDNA expression vector and κB-luc. As shown in Fig. 4*b*, whereas p202 expression alone has no effect on κB-luc, it could greatly repress NF-κB (Rel-A)-activated IκB pro-

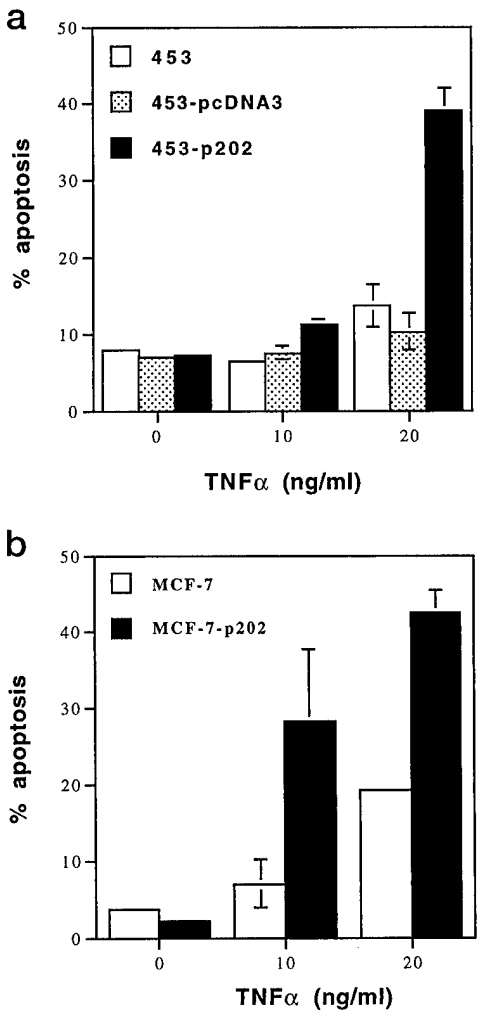

Fig. 3. p202 sensitizes breast cancer cells to apoptosis induced by TNF-α in a dose-dependent manner. *a*, 453, 453-pcDNA3, and 453-p202 cell lines were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. *Bars*, SE. *b*, MCF-7 and MCF-7-p202 were treated with TNF-α (0, 10, and 20 ng/ml) for 48 h. Cells were fixed and stained with propidium iodide. Apoptosis was quantitated by FACScan cytometer. *Bars*, SE.

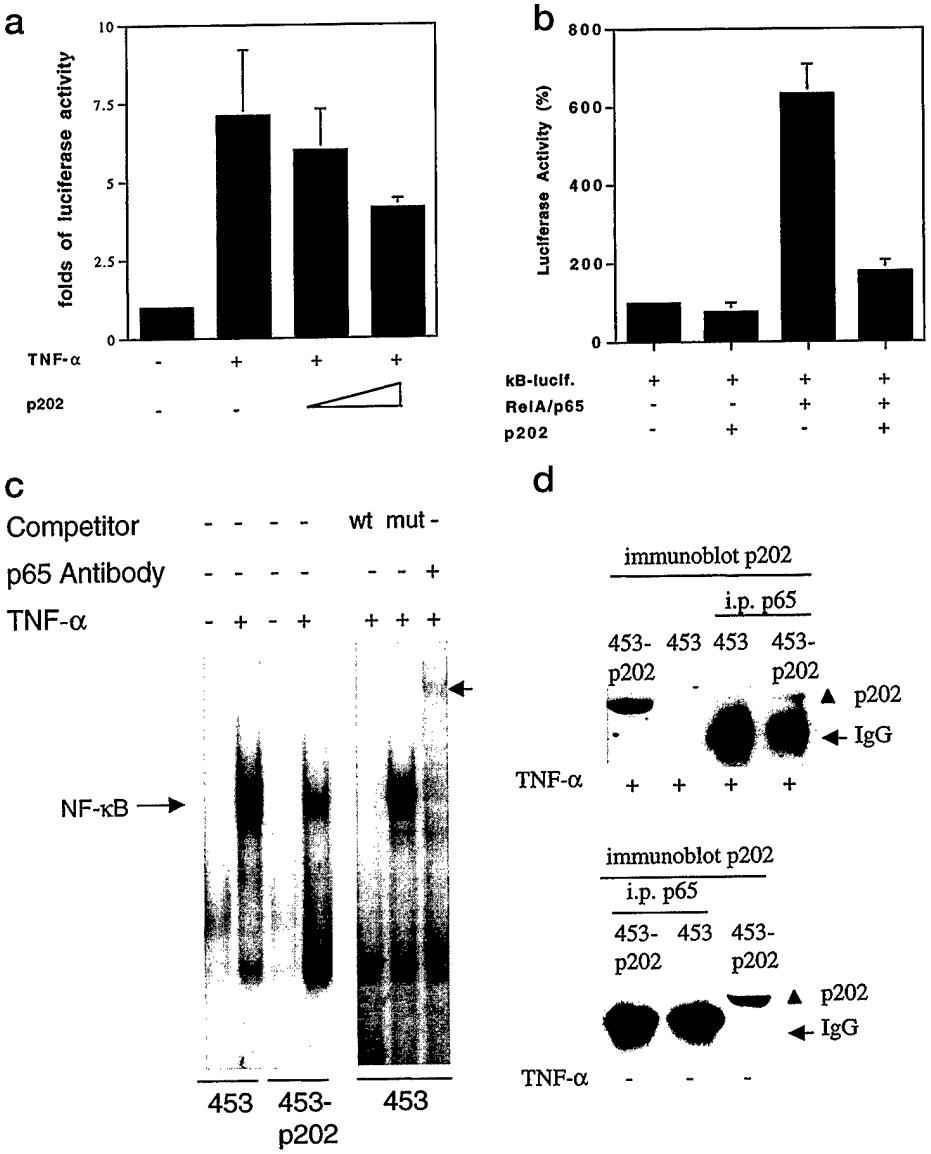

Fig. 4. The interaction and inactivation of NF-κB by p202 is responsible for the p202-mediated sensitization to TNF-α-induced apoptosis. *a*, p202 expression represses NF-κB-mediated transcription activation in response to TNF-α. IκB-Luciferase reporter gene (0.2 μg) and CMV-p202 (0, 0.8, or 2 μg) were cotransfected into MDA-MB-453 cells. Thirty-six h after transfection, cells were either left untreated or stimulated with TNF-α (20 ng/ml) for 6 h. The fold difference in IκB-Luciferase expression was calculated with respect to IκB-Luciferase expression in the absence of TNF-α and p202. *b*, p202 expression represses Rel-A (p65)-activated transcription. MDA-MB-453 cells were cotransfected with κB-luc and ± NF-κB (p65) expression vector. The inhibitory activity of p202 on the induction of IκB promoter activity by p65 was assessed by cotransfection with p202 expression vector. Luciferase activity was measured 48 h after transfection. The data represent an average of two independent experiments after normalization; *bars*, SE. *c*, gel-shift assay. 453 and 453-p202 nuclear extracts, used in this assay, were isolated from TNF-α-treated cells (20 ng/ml of for 30 min). *Left panel*, the activated NF-κB (p65/p50) induced by TNF-α is indicated by an *arrow*. *Right panel*, competition assay was performed in the presence of a 70-fold excess of wild-type or mutant oligonucleotides containing NF-κB binding site. A polyclonal Rel-A antibody supershifted the NF-κB complex to a slower-migrating position, as indicated by an *arrow*. *d, top panel*, p202 is physically associated with p65. 453 and 453-p202 cells were treated with or without TNF-α (20 ng/ml for 30 min). Cell lysates (1 mg) were used in the subsequent immunoprecipitation with anti-p65 antibody. Immunoprecipitated complexes were analyzed by SDS-PAGE, followed by immunoblotting with p202 antibody. *Bottom panel*, immunoblots of p202 protein using untreated 453 and 453-p202 cells serve as negative and positive controls, respectively. ▲, p202 band. IgG band is also indicated.

moter activity. These results suggest that the transcriptional repression of TNF-α-mediated gene expression by p202 may be attributable to the inactivation of NF-κB by p202.

This hypothesis was further supported by a subsequent observation that p202 expression was associated with a reduced level of the active NF-κB (p65/p50) molecule as measured by a gel-shift assay (Fig. 4*c*, *left panel*). As expected, the level of active NF-κB was found to be significantly increased in both the p202-expressing (435-p202) and the parental (453) cells treated with TNF-α (20 ng/ml). However, the level of activated NF-κB was greatly reduced in 453-p202. Using either a wild-type or mutant NF-κB DNA binding sequence as a competitor, we showed that the DNA/protein complex was indeed NF-κB specific in that only wild-type, but not mutant, sequence could compete with the NF-κB/DNA complex. Moreover, the fact that this complex could be supershifted in the presence of an anti-p65 antibody (Fig. 4*c*, *right panel*) further confirms the identity of this DNA/protein complex being NF-κB-specific. Thus, these data support the idea that p202 expression may impede the formation of active p65/p50 heterodimer. That, in turn, represses transcriptional activation induced by NF-κB.

It is possible that p202 may interact with p65, forming a p202/p65 complex, which may significantly reduce the concentration of free p65 in p202-expressing cells. To test that possibility, we performed a coimmunoprecipitation assay. As shown in Fig. 4*d, upper panel,* with TNF-α treatment, p202 could be coimmunoprecipitated with p65 by an anti-p65 antibody in 453-p202 nuclear extract but not 453 extract. As a control, no detectable p202 was observed in either cell line without TNF-α treatment (Fig. 4*d, lower panel*). These data strongly indicate that p202 and p65 are physically associated in the same complex upon TNF-α stimulation. The p65 protein level is comparable between 453 and 453-p202 cells with TNF-α treatment (data not shown), indicating that p202 may not regulate p65 expression.

The above observation presents a possible scenario that TNF-α-induced NF-κB activation could be antagonized by p202 via a p202/p65 interaction. That, in turn, causes subsequent transcriptional repression of genes, the activation of which requires active NF-κB. Although it has been reported previously that p202 could bind both p50 and p65 *in vitro* and p50 *in vivo* (6), our data are the first demonstration of an *in vivo* association between p202 and p65 upon TNF-α stimulation. Taken together, our results provide a possible mechanism that accounts for the p202-mediated sensitization to TNF-α-induced apoptosis in breast cancer cells.

Inflammatory cytokines, *e.g.,* TNF family members, can transduce apoptotic signals in certain tumor cells and have been tested in a number of clinical trials (16). Despite the promising data in animal models, unsatisfactory results have been observed in many clinical trials (17). It might be attributable to the resistance of many cancer cells to TNF-α-induced apoptosis, presumably, by the activation of NF-κB and the subsequent induction of survival factors that counteract apoptosis. In this report, we demonstrated that p202 expression not only exerted strong growth retardation and tumor suppression activities in breast cancer cells but also is able to sensitize these cells to TNF-α-induced apoptosis, and that sensitization is associated with inactivation of NF-κB via a p202/p65 interaction. Thus, our data implicate a potential therapeutic application of a combined treatment of TNF-α and p202 gene therapy for cancer patients.

References

1. Gutterman, J. U. Cytokine therapeutics: lessons from interferon α. Proc. Natl. Acad. Sci. USA, *91:* 1198–1205, 1994.
2. Choubey, D., Snoddy, J., Chaturvedi, V., Toniato, E., Opdenakker, G., Thakur, A., Samanta, H., Engel, D. A., and Lengyel, P. Interferons as gene activators–indications for repeated gene duplication during the evolution of a cluster of interferon-activatable genes on murine chromosome 1. J. Biol. Chem., *264:* 17182–17189, 1989.
3. Choubey, D., Li, S-J., Datta, B., Gutterman, J. U., and Lengyel, P. Inhibition of E2F-mediated transcription by p202. EMBO J., *15:* 5668–5678, 1996.
4. Choubey, D., and Gutterman, J. U. Inhibition of E2F-4/DP-1-stimulated transcription by p202. Oncogene, *15:* 291–301, 1997.
5. Datta, B., Li, B., Choubey, D., Nallur, G., and Lengyel, P. p202, an interferon-inducible modulator of transcription, inhibits transcriptional activation by the p53 tumor suppressor protein, and a segment from the p53-binding protein 1 that binds to p202 overcomes this inhibition. J. Biol. Chem., *271:* 27544–27555, 1996.
6. Min, W., Ghosh, S., and Lengyel, P. The interferon-inducible p202 protein as a modulator of transcription: inhibition of NFκB, c-Fos, and c-Jun activities. Mol. Cell. Biol., *16:* 359–368, 1996.
7. Datta, B., Min, W., Burma, S., and Lengyel, P. Increase in p202 expression during skeletal muscle differentiation: inhibition of MyoD protein expression and activity by p202. Mol. Cell. Biol., *18:* 1074–1083, 1998.
8. Choubey, D., and Lengyel, P. Binding of an interferon-inducible protein (p202) to the retinoblastoma protein. J. Biol. Chem., *270:* 6134–6140, 1995.
9. Lembo, D., Angeretti, A., Benefazio, S., Hertel, L., Gariglio, M., Novelli, F., and Landolfo, S. Constitutive expression of the interferon-inducible protein p202 in NIH3T3 cells affects cell cycle progression. J. Biol. Regul. Homeostatic Agents, *9:* 42–46, 1995.
10. Yan, D-H., Wen, Y., Spohn, B., Choubey, D., Gutterman, J. U., and Hung, M-C. Reduced growth rate and transformation phenotype of the prostate cancer cells by an interferon-inducible protein, p202. Oncogene, *18:* 807–811, 1999.
11. Choubey, D., and Lengyel, P. Interferon action: cytoplasmic and nuclear localization of the interferon-inducible 52-kD protein that is encoded by the *Ifi202* gene from the gene 200 cluster. J. Interferon Res., *13:* 43–52, 1993.
12. Shao, R., Karunagaran, D., Zhou, B. P., Li, K., Lo, S-S., Deng, J., Chiao, P., and Hung, M-C. Inhibition of nuclear factor-κB activity is involved in E1A-mediated sensitization of radiation-induced apoptosis. J. Biol. Chem., *272:* 32739–32742, 1997.
13. Wang. C. Y., Mayo, M. W., and Baldwin, A. S., Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB [see comments]. Science (Washington DC), *274:* 784–787, 1996.
14. Beg, A. A., and Baltimore, D. An essential role for NF-κB in preventing TNF-α-induced cell death [see comments]. Science (Washington DC), *274:* 782–784, 1996.
15. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB [see comments]. Science (Washington DC), *274:* 787–789, 1996.
16. Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A., Jr., and Shepard, H. M. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. Science (Washington DC), *230:* 943–945, 1985.
17. Frei, E. D., and Spriggs, D. Tumor necrosis factor: still a promising agent. J. Clin. Oncol., *7:* 291–294, 1989.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 11, Issue of March 17, pp. 8027–8031, 2000
Printed in U.S.A.

HER-2/neu Blocks Tumor Necrosis Factor-induced Apoptosis via the Akt/NF-κB Pathway*

(Received for publication, October 26, 1999, and in revised form, January 10, 2000)

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Overexpression of *HER-2/neu* correlates with poor survival of breast and ovarian cancer patients and induces resistance to tumor necrosis factor (TNF), which causes cancer cells to escape from host immune defenses. The mechanism of *HER-2/neu*-induced TNF resistance is unknown. Here we report that *HER-2/neu* activates Akt and NF-κB without extracellular stimulation. Blocking of the Akt pathway by a dominant-negative Akt sensitizes the *HER-2/neu*-overexpressing cells to TNF-induced apoptosis and inhibites IκB kinases, IκB phosphorylation, and NF-κB activation. Our results suggested that *HER-2/neu* constitutively activates the Akt/NF-κB anti-apoptotic cascade to confer resistance to TNF on cancer cells and reduce host defenses against neoplasia.

Overexpression of the *HER-2/neu* (*ErbB2*) oncogene correlates with poor prognosis in breast and ovarian cancer patients because it enhances the metastatic potential of cancer cells and induces resistance to Taxol and TNF[1] (1–5). Cancer cells that overexpress *HER-2/neu* are therefore an excellent target for the development of anticancer therapies. For instance, an anti-*HER-2/neu* antibody (Herceptin™) has been used clinically as a potent growth inhibitor of such breast cancer cells (6), and previous research has shown that overexpression of *HER-2/neu* up-regulates $p21^{Waf1}$ and leads to resistance by these cancer cells to Taxol (3). Still, the mechanism of *HER-2/neu*-mediated TNF resistance in cancer cells remains unclear. The *HER-2/neu* gene encodes a 185-kDa transmembrane receptor tyrosine kinase with homology to members of the EGF receptor family. Unlike the other EGF receptors, *HER-2/neu* has an intrinsic tyrosine kinase activity that activates receptor-mediated signal transduction in the absence of ligand. Although EGF can bind to EGF receptor to induce receptor dimerization and activate phosphatidylinositol 3-kinase (PI3K) (7), it is not known whether *HER-2/neu* homodimer can activate the PI3K pathway without extracellular stimulation. Activation of PI3K generates PtdIns-3,4-P2, which in turn recruits and activates a downstream serine/threonine kinase, Akt. Activated Akt phosphorylates specific targets such as Bad (8), pro-caspase-9 (9), and transcription factor FKHRL1 (10, 11), with the result of promoting cell survival. Thus, the Akt signaling pathway has a critical role in anti-apoptosis that may contribute to the pathogenesis of cancer (12, 13).

In this study, we examined the activation of Akt in breast tumor specimens and breast cancer cell lines for its anti-apoptotic roles in *HER-2/neu*-overexpressing breast cancer cells. We found that Akt was constitutively activated in *HER-2/neu*-overexpressing breast cancer cells and that Akt activity was required for these cells resistance to TNF-induced apoptosis. We showed that *HER-2/neu*-overexpressing cancer cells became sensitive to apoptosis when the Akt pathway was blocked by the dominant-negative Akt. Furthermore, we found that Akt activity was required for the activation of both IKK-α and -β, for IκB phosphorylation, and for NF-κB activation. Our results provide a molecular explanation for the finding that *HER-2/neu*-overexpressing breast cancer cells are more resistant to TNF-induced apoptosis, leading to poor prognosis and shortened survival of patients.

EXPERIMENTAL PROCEDURES

Cell Lines and Cultures—All breast cancer cell lines and NIH3T3 cells were grown in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. *HER-2/neu*-transformed NIH3T3 cells were generated by transfecting the cells with membrane point-mutated human *HER-2/neu* cDNA. Transformed cells were cloned from the transformed foci in three rounds of selection. The DN-Akt transfectants in MDA-MB453 and *HER-2/neu*-transformed 3T3 cells were established by transfecting these cells with HA-tagged Akt (K179M) cDNA. The transfectants were grown under the same conditions, except that 600 μg/ml of G418 was added to the culture medium.

Apoptosis Assay—Cells treated with or without TNF were collected at the time interval as indicated and washed once with ice-cold PBS, and apoptosis was analyzed by either a flow cytometry assay or DNA fragmentation, as described previously (14, 15).

Electrophoretic Mobility Shift Assay—Cell nuclear extracts from samples treated with or without TNF for 30 min were prepared as described previously (14, 15). The nuclear extract (5 μg) was incubated with 1 μg of poly(dI-dC) (Amersham Pharmacia Biotech) on ice for 20 min, and a ^{32}P-labeled double-stranded oligonucleotide containing the κB site of the human immunodeficiency virus was added. Binding of the probe was carried out at room temperature for 20 min. The resulting complexes were resolved in 4% nondenaturing polyacrylamide gel.

Immunoprecipitation—Cells were washed twice with PBS, scraped into 500 μl of lysis buffer, and incubated on ice for 20 min. After centrifugation at 14,000 × *g* for 10 min, 500 μg of each supernatant was preincubated with 2 μg of rabbit immunoglobulin G and 50 μl of protein

* This work was supported by Grants R01-CA58880 and R01-CA77858 (to M.-C. H.) and Cancer Core Grant 16672 from the NCI, National Institutes of Health, by the Nellie Connally Breast Cancer Research Fund, and by a Faculty Achievement Award at M. D. Anderson Cancer Center (to M.-C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These two authors are recipients, respectively, of postdoctoral and predoctoral fellowships from the United States Department of Defense (DOD) Breast Cancer Research Training Grant (DAMD17-99-1-9264).

§ Predoctoral fellow of the DOD Breast Cancer Research Program (DAMD17-98-1-8242).

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[1] The abbreviations used are: TNF, tumor necrosis factor; EGF, epidermal growth factor; IKK, IκB kinase; HA, hemagglutinin; luc, luciferase; PBS, phosphate-buffered saline; DN-Akt, dominant-negative Akt; p-Akt, phosphorylated Akt; ER, estrogen receptor; NF-κB, nuclear factor-κB; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; 3T3 cells, NIH3T3 cells.

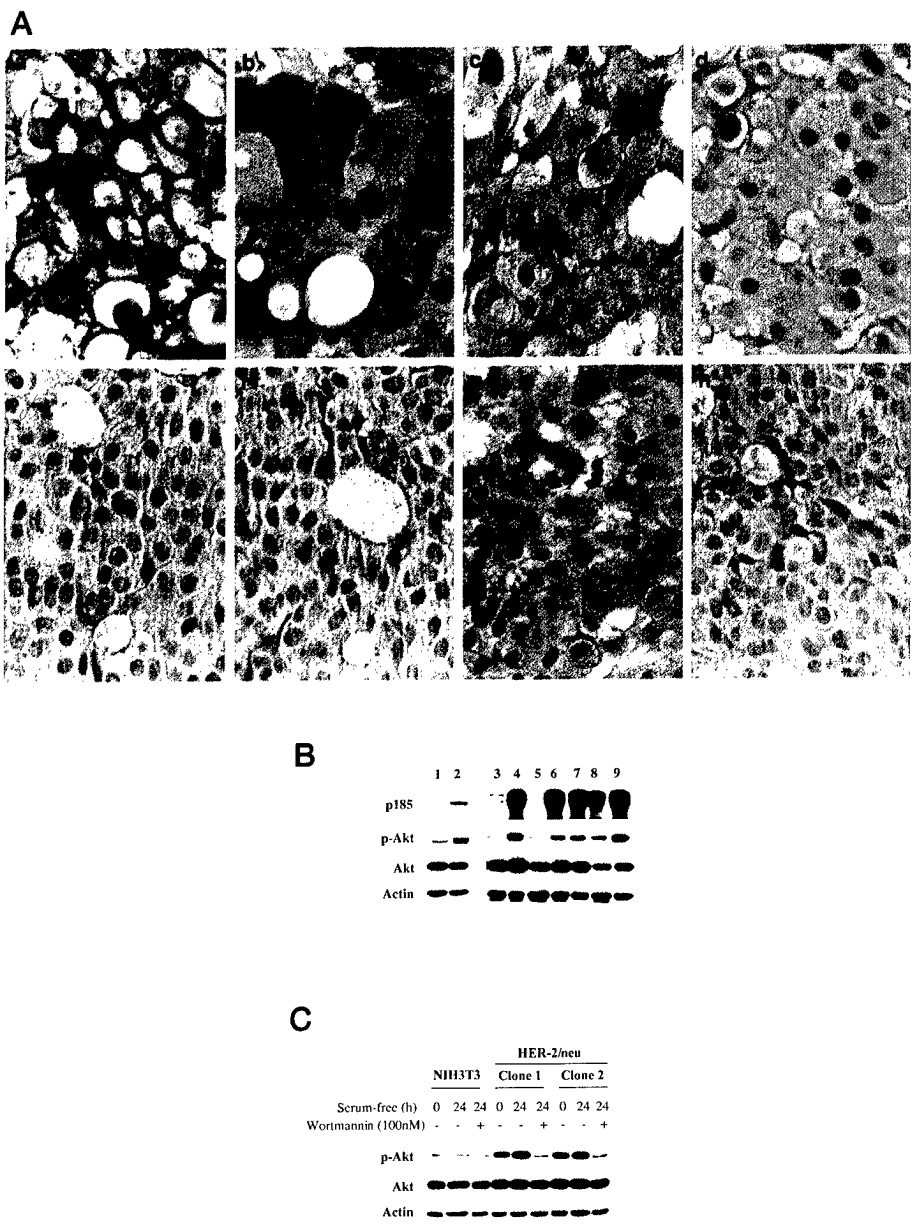

FIG. 1. ***HER-2/neu* activates Akt.** *A*, ten tissue sections from the *HER-2/neu*$^+$ adenocarcinoma (*a–d*) and 10 sections from the *HER-2/neu*$^-$ adenocarcinoma (*e–h*) were stained with antibodies specific to *HER-2/neu* (*a, e*), p-Akt (*b, f*), Akt (*c, g*), or normal rabbit serum (*d, h*) followed by immunostaining with an anti-rabbit IgG antibody conjugated with peroxidase. Antibodies were obtained from DAKO and New England Biolabs. *B*, nine human breast cancer cell lines were starved for 24 h without serum. Whole-cell lysates (50 μg each) were subjected to Western blot analyses using antibodies specific to *HER-2/neu*, Akt, p-Akt, and actin (Roche Molecular Biochemicals). *Lanes 1–9*, respectively: MCF-7, MCF-7/*HER-2*, MDA-MB435, BT483, MDA-MB231, MDA-MB453, MDA-MB361, SKBR3, and BT474. *C*, *HER-2/neu*-transformed NIH3T3 cells were established by transfecting human *HER-2/neu* cDNA into NIH3T3 cells. After being cultured for 3 weeks, the *HER-2/neu*-transformed clones (foci) were isolated and characterized by the transformed phenotypes and overexpression of *HER-2/neu*. Two *HER-2/neu*-transformed clones and parental cells were cultured in medium containing 10% fetal bovine serum or serum-free medium for 24 h, with or without wortmannin (100 nM), a PI3K inhibitor, before harvest. Whole-cell lysates were analyzed by Western blots using antibodies against Akt, p-Akt, and actin.

G for 1 h at 4 °C. Endogenous IKK-α was immunoprecipitated overnight with 2 μg of anti-IKK-α antibody (Santa Cruz) and 50 μl of protein G. The immunocomplex was washed five times with lysis buffer, dissolved in loading buffer, and subjected to SDS-PAGE.

Western Blot—The protein samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. The immunoblots were visualized by an enhanced chemiluminescence (ECL) kit obtained from Amersham Pharmacia Biotech.

Immunocomplex Kinase Assay—Cell extracts were prepared from samples treated with or without TNF, and immunocomplex kinase assays were performed as described previously (15).

Transient Transfections—Approximately 0.2 × 10^6 cells of either MDA-MB453 or its DN-Akt transfectants were cotransfected in 6-well plates with pcDNA3-*lacZ* and either wild-type NF-κB luciferase (κB-luc) or mutant NF-κB luciferase (mut/κB-luc). After 40 h of transfection, TNF was added to the culture medium as indicated, and both TNF-treated and untreated cultures were continued to incubate for another 8 h. The luciferase activity of each sample was measured with the luciferase assay kit (Promega) and normalized with a β-galactosidase assay.

RESULTS AND DISCUSSION

Because overexpression of *HER-2/neu* induced resistance to TNF (4, 5), and the Akt pathway is known to enhance cell survival, we examined whether expression of *HER-2/neu* correlated with activation of Akt in breast cancers. We compared the levels of activated Akt (phosphorylated Akt) (p-Akt) of 10 *HER-2/neu*-positive and 10 *HER-2/neu*-negative human breast tumors by immunostaining them with an antibody specific to p-Akt. Although no p-Akt signal was detected in the 10 *HER-2/neu*-negative tumors, 7 of 10 *HER-2/neu*-positive tumors showed strong p-Akt staining, suggesting that expression of *HER-2/neu* correlates significantly with Akt activation ($p < 0.01$). As control, all samples were Akt-positive when they were stained with an anti-Akt antibody. Representative stainings of p-Akt are shown in Fig. 1*A*. To confirm our observation of a correlation between *HER-2/neu* expression and Akt activation in the clinical samples, we used Western blotting with an anti-p-Akt antibody to analyze p-Akt in nine breast cancer cell lines that showed various expression levels of *HER-2/neu*. The level of p-Akt paralleled the cell's *HER-2/neu* expression (Fig.

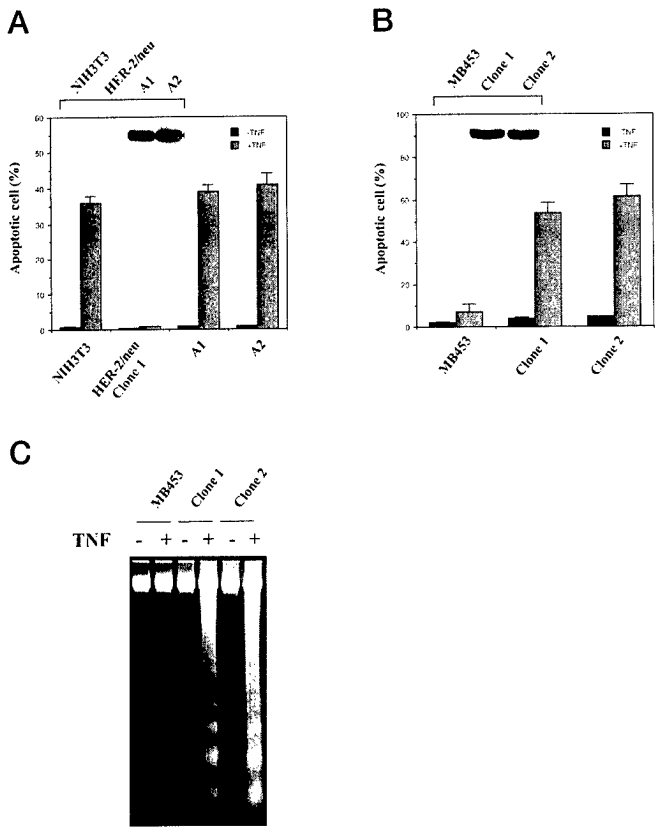

FIG. 2. ***HER-2/neu* inhibits TNF-induced apoptosis through Akt.** DN-Akt stable transfectants were generated by transfecting *HER-2/neu*-transformed NIH3T3 and the MDA-MB453 cells with DN-Akt cDNA (HA-tagged Akt, K179M). Several stable cell clones were isolated after G418 selection (600 μg/ml). *A*, NIH3T3, *HER-2/neu*-transformed 3T3 and its DN-Akt-transfected cell lines (clones *A1* and *A2*) were cultured in low-serum (1% serum) medium with TNF (40 ng/ml) or without it for 48 h. Apoptotic cells were measured by flow cytometry using a fluorescence-activated cell sorter (FACS) with propidium iodide staining (mean ± S.E. in three separate experiments). Equal amounts of cell lysates were analyzed by Western blot for expression of DN-Akt in these cell clones (*insert*). *B*, MDA-MB453 cells and their DN-Akt transfectants (*Clone 1* and *Clone 2*) were cultured in low-serum medium with (20 ng/ml) or without TNF for 48 h. Expression of DN-Akt in these clones was analyzed as described for *A* (*insert*). The apoptotic cells were quantitated by FACS (mean ± S.E. in three separate experiments) or determined by DNA fragmentation (*C*).

1*B*), indicating that activation of Akt correlates well with expression of *HER-2/neu* in breast cancer cells. Moreover, this correlation remained the same in the absence of serum, suggesting that activation of Akt corresponds to the level of *HER-2/neu*, independent of stimulation of growth factors or cytokines in the serum (*i.e.* constitutive activation). To create a model system, and to rule out the possibility that some other mechanisms might contribute to concurrent activation of Akt and overexpression of *HER-2/neu*, we compared Akt activation between the *HER-2/neu*-transformed NIH3T3 cell clones with that of their parental cells. In the absence of serum, Akt was activated constitutively in the *HER-2/neu*-transformed cells but not in the parental cells (Fig. 1*C*), which confirmed that Akt was activated by the intrinsic tyrosine kinase activity of *HER-2/neu* in the absence of extracellular stimulation. Furthermore, activation of Akt was blocked by wortmannin, an inhibitor of PI3K, suggesting that this *HER-2/neu*-mediated Akt activation occurs through PI3K.

If the *HER-2/neu*–induced resistance to TNF is caused primarily by activation of Akt and not by other mechanisms, blocking this Akt pathway should render the cells sensitive to TNF-induced apoptosis. Therefore, to inhibit the Akt pathway, we transfected a DN-Akt (kinase-dead) DNA into the *HER-2/neu*-transformed NIH3T3 (*HER-2/neu*-3T3) cells. Upon TNF treatment, the DN-Akt transfectants of *HER-2/neu*-3T3 and NIH3T3 cells were about 20-fold more sensitive to apoptosis than the *HER-2/neu*-3T3 cells (Fig. 2*A*). Expression levels of DN-Akt in these cell clones are indicated in the *insert* to Fig. 2*A*. To confirm the Akt anti-apoptotic effect in the *HER-2/neu*-overexpressed human breast cancer cells, we transfected DN-Akt DNA into *HER-2/neu*-overexpressing MDA-MB453 cells and obtained several independent DN-Akt-overexpressing cell clones (Fig. 2*B*, *insert*). Similarly, the DN-Akt transfectants (clones 1 and 2) of MDA-MB453 cells became about 10-fold more sensitive to TNF-induced apoptosis than the parental cells (Fig. 2*B*). Apoptosis induced by TNF was further verified by DNA fragmentation assay (Fig. 2*C*). Thus, *HER-2/neu* was found to block TNF-induced apoptosis via the PI3K/Akt pathway.

PI3K has recently been shown to be involved in the activation of transcription factor NF-κB (16, 17), which is a p50/p65 (RelA) heterodimer regulated by its inhibitory protein, IκB (18, 19). Clinical evidence indicates that loss of estrogen receptor (ER) correlates strongly with overexpression of *HER-2/neu* (20), which is consistent with our previous finding that ER down-regulates *HER-2/neu* expression (21). Analogously, NF-κB is often activated constitutively in ER-negative breast cancer cells (22). Thus, we hypothesized that activation of Akt by *HER-2/neu* may turn on NF-κB, which inhibits TNF-induced apoptosis (23–25). To test whether overexpression of *HER-2/neu* can activate NF-κB, we assayed the NF-κB DNA binding and transcriptional activation activities in *HER-2/neu*-3T3 and NIH3T3 cells and found NF-κB DNA binding activity higher in the *HER-2/neu*-3T3 cells than in the NIH3T3 cells, in a serum-independent manner (Fig. 3*A*, *lanes 4* and *5*). As controls, NF-κB DNA binding activities were strongly activated by TNF treatment (Fig. 3*A*); these activities were abrogated by the competing wild-type κB oligonucleotides (data not shown; see below). Furthermore, activation of the transcriptional activity of NF-κB in the *HER-2/neu*-3T3 cells without serum was confirmed by luciferase assay (Fig. 3*B*). Similar results were obtained in rat *HER-2/neu*-transformed NIH3T3 and SW3T3 cells (data not shown). These data strongly suggested that overexpression of *HER-2/neu* activates NF-κB constitutively. To determine whether sensitization of TNF-induced apoptosis in the DN-Akt transfectants occurs through inhibition of NF-κB, we measured NF-κB activities in the transfectants and MDA-MB453 cells. As shown in Fig. 3, *C* and *D*, TNF-induced NF-κB DNA binding and transcription activities in the DN-Akt transfectants were significantly inhibited (3–5-fold). That these inhibitions were not caused by down-regulation of p65 or p50 by TNF is demonstrated by the finding of no change in the p65 and p50 levels of these cells in the absence or presence of TNF (Fig. 3*C*, *bottom panel*).

To investigate whether DN-Akt inhibits IκB phosphorylation and degradation, we analyzed the expression and phosphorylation patterns of IκB-α in the DN-Akt transfectants and MDA-MB453 cells before and after TNF treatment. As shown in Fig. 4*A*, only one IκB-α band was observed in the DN-Akt transfectants before or after the TNF treatment, whereas two bands were detected in the TNF-treated parental cells. The upper band may be the phosphorylated form of IκB-α (p-IκB-α), because it disappeared after treatment with calf intestine phosphatase (*CIP*, Fig. 4*B*). TNF has been demonstrated to activate IκB kinases (IKKs), which in turn phosphorylate IκB, which is then degraded and activates NF-κB (26, 27). To examine whether DN-Akt blocks activation of IKKs, we compared the kinase activities of IKK-α and -β in the DN-Akt transfectants

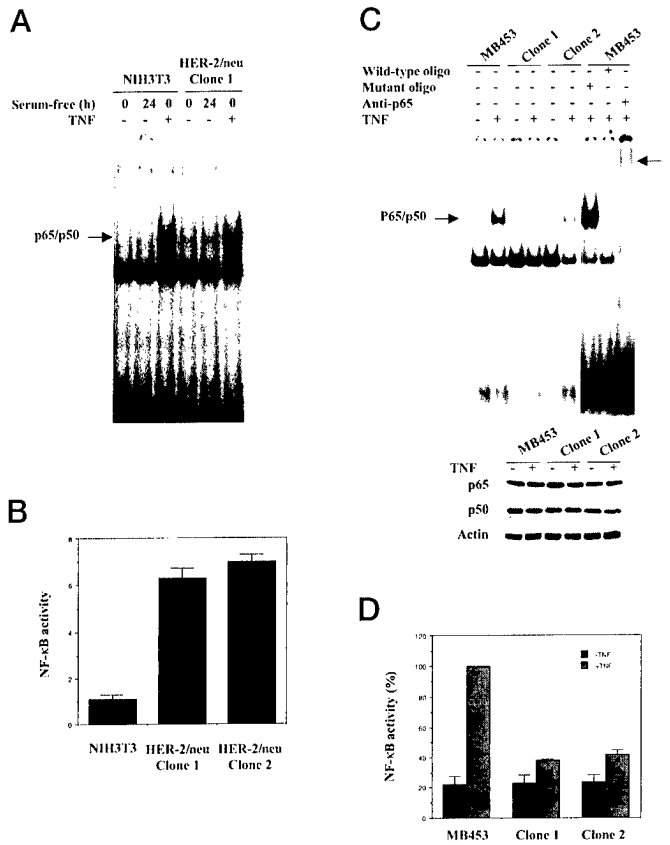

FIG. 3. ***HER-2/neu* activates NF-κB.** *A*, NIH3T3 cells and *HER-2/neu*-transformed 3T3 cells were cultured in serum-containing or serum-free medium for 24 h. Nuclear extracts (5 μg each) were used to determine NF-κB DNA binding activities by an electrophoretic mobility shift assay using an oligonucleotide probe containing the κB binding site. Nuclear extracts from the TNF-stimulated cells were included as positive controls. *B*, NIH3T3 and *HER-2/neu*-transformed 3T3 cells were cotransfected with 0.2 μg of pcDNA3-*lacZ* plus 1.8 μg of either wild-type or mutant NF-κB luciferase (κB-luc or mut/κB-luc) plasmids. Forty-eight h post-transfection, luciferase activities were determined and normalized by β-galactosidase activities. NF-κB activities were calculated by the luciferase activities of κB-luc *versus* mut/κB-luc (mean ± S.E. in three separate experiments). *C*, DN-Akt blocks activation of NF-κB induced by TNF. The MDA-MB453 cells and DN-Akt transfectants (*Clone 1* and *Clone 2*) were treated with (20 ng/ml) or without TNF for 5 min, and NF-κB DNA binding activities were determined as described above for *panel A*. Cold wild-type or mutant NF-κB oligonucleotides were included as controls in the TNF-induced MDA-MB453 cells. An anti-p65 antibody (Santa Cruz) was also included in the assay; the supershifted complex is indicated by an *arrow*. As control, 50 μg of each cell lysate was assayed for the expression of p65 or p50 by Western blots using anti-p65 or anti-p50 antibody (*bottom panel*). *D*, MDA-MB453 cells and DN-Akt transfectants were cotransfected with pcDNA3-*lacZ* plus κB-luc or mut/κB-luc plasmids as described above. After 40 h of transfection, the cells were treated with or without TNF (20 ng/ml) for 8 h and then harvested. NF-κB activities were determined as above.

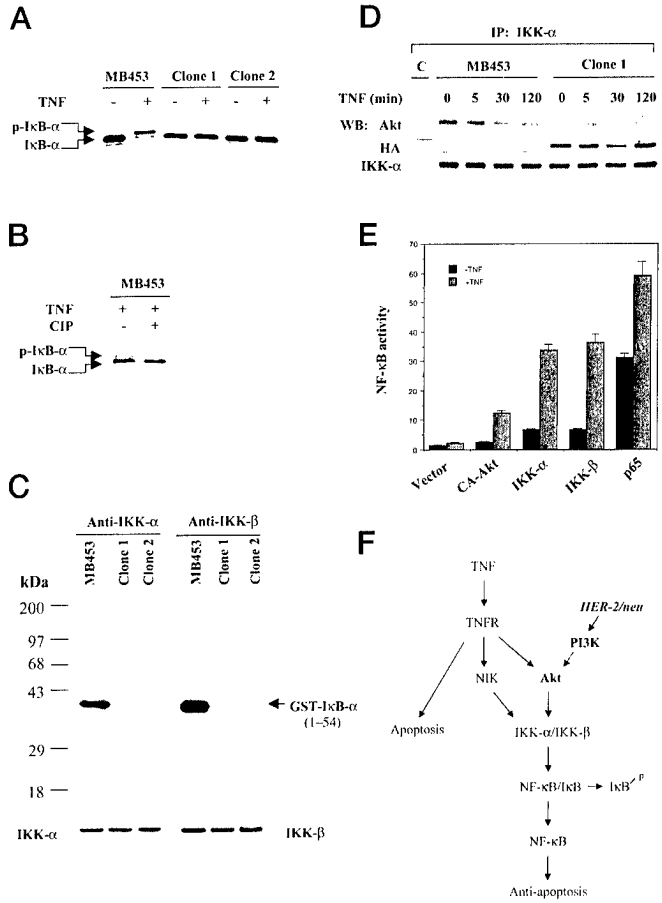

FIG. 4. ***HER-2/neu* activates the Akt/NF-κB signaling cascade.** *A*, MDA-MB453 and DN-Akt transfectants were starved for 12 h without serum and treated with TNF (20 ng/ml) or without it for 5 min before harvesting. Equal amounts of whole-cell lysates were subjected to SDS-PAGE (12%) and transferred to nitrocellulose membranes. IκB-α was detected by Western blot using an anti-IκB-α antibody (Santa Cruz). *B*, phosphorylation of IκB-α induced by TNF could be dephosphorylated by a phosphatase. Cell lysates (500 μg each) from the TNF-treated MDA-MB453 cells were immunoprecipitated with anti-IκB-α, and the precipitates were then incubated with or without 20 units of calf intestine phosphatase (*CIP*) at 37 °C for 30 min before Western blot analysis with anti-IκB-α. *C*, MDA-MB453 and DN-Akt transfectants were starved and treated with TNF (10 min). Endogenous IKK-α and -β were immunoprecipitated with anti-IKK-α and anti-IKK-β antibodies, respectively, and their kinase activities were determined by immunocomplex kinase assays using GST-IκB-α (amino acid 1–54) as a substrate as described previously (15). *D*, MDA-MB453 and DN-Akt transfectants (*Clone 1*) were treated with TNF (20 ng/ml) for different time periods. The endogenous IKK-α was immunoprecipitated with an anti-IKK-α antibody, then analyzed by Western blot (*WB*) using an anti-Akt, anti-HA, or anti-IKK-α antibody. *E*, NF-κB activity in the DN-Akt transfectants could be partially restored by overexpression of p65, IKK-β, IKK-α, or a constitutively active Akt (*CA-Akt*). The DN-Akt transfectants (clone 1) were cotransfected with pcDNA3-*lacZ* (0.2 μg) and either κB-luc or mut/κB-luc (0.6 μg each) plus p65, IKK-β, IKK-α, or CA-Akt plasmid (1.2 μg each). Forty h post-transfection, the cells were treated with or without TNF, and the NF-κB activities were determined as described above for *panel B* in Fig. 3 (mean ± S.E. in four separate experiments). *F*, model of *HER-2/neu* activation of the Akt/NF-κB pathway that blocks TNF-induced apoptosis. *TNFR*, TNF receptor; *NIK*, NF-κB-inducing kinase.

with those in the parental cells after TNF treatment, using immunocomplex kinase assays. The endogenous IKK-α and -β kinase activities were readily detected in the MDA-MB453 cells, whereas their activities were inhibited in the DN-Akt transfectants (Fig. 4*C*), suggesting that Akt activity is required for activation of IKKs by TNF. Furthermore, we showed that in the DN-Akt transfectants, DN-Akt and the endogenous Akt associate specifically with IKK-α *in vivo* regardless of TNF treatment (Fig. 4*D*). To further confirm that Akt is an activator upstream of IKKs, we transfected the DNA of p65 (RelA), IKK-α or -β, or a constitutively active Akt into the DN-Akt transfectants to restore TNF-induced NF-κB activities in these cells. Overexpression of each of these proteins significantly overrode the inhibitory effect of DN-Akt and restored activation of NF-κB by TNF (Fig. 4*E*), indicating that Akt is indeed upstream of both IKKs. Taken together, our results suggested that Akt activity is essential for NF-κB activation by *HER-2/neu* and TNF. A model we propose to illustrate the parallel *HER-2/neu*- and TNF-induced anti-apoptotic pathways is shown in Fig. 4*F*. While we were preparing this manuscript, NF-κB was reported to be a target of Akt (28, 29), confirming

our finding that *HER-2/neu* activates the NF-κB anti-apoptotic pathway through Akt.

In general, activation of the Akt signaling pathway requires extracellular survival factors (mitogenic stimuli) such as EGF, insulin, platelet-derived growth factor, thrombin, heregulin, and nerve growth factor. To our knowledge, this is the first evidence that *HER-2/neu* activates the Akt/NF-κB pathway without extracellular stimulation. Our study also details a molecular mechanism of TNF resistance that may provide an interpretation for the *HER-2/neu*-overexpressing cancer cells, escape from host immune defenses, and the contribution of this mechanism to the poor survival of cancer patients with *HER-2/neu* overexpression. Understanding the *HER-2/neu*-mediated anti-apoptotic pathway may open an avenue for developing novel anticancer therapies for *HER-2/neu*-overexpressing breast and ovarian cancers.

REFERENCES

1. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987) *Science* **235,** 177–182
2. Hung, M.-C., Zhang, X., Yan, D. H., Zhang, H. Z., He, G. P., Zhang, T. Q., and Shi, D. R. (1992) *Cancer Lett.* **61,** 95–103
3. Yu, D., Jing, T., Liu, B., Yao, J., Tan, M., McDonnell, T. J., and Hung, M.-C. (1998) *Mol. Cell* **2,** 581–591
4. Hudziak, R. M., Lewis, G. D., Shalaby, M. R., Eessalu, T. E., Aggarwal, B. B., Ullrich, A., and Shepard, H. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5102–5106
5. Lichtenstein, A., Berenson, J., Gera, J. F., Waldburger, K., Martinez-Maza, O., and Berek, J. (1990) *Cancer Res.* **50,** 7364–7370
6. Pegram, M. D., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Tripathy, D., Baly, D., Baughman, S. A., Twaddell, T., Glaspy, J. A., and Slamon, D. J. (1998) *J. Clin. Oncol.* **16,** 2659–2671
7. Hu, P., Margolis, B., Skolnik, E. Y., Lammers, R., Ullrich, A., and Schlessinger, J. (1992) *Mol. Cell. Biol.* **12,** 981–990
8. Peso, L. D., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) *Science* **278,** 687–689
9. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282,** 1318–1321
10. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96,** 857–868
11. Kops., G. J. P. L., de Reiter, N. D., De Vries-smits, A. M. M., Powell, D. R., Bos, J. L., and Burgering, B. M. Th.B. M. (1999) *Nature* **398,** 630–634
12. Frank, T. F., Kaplan, D. R., and Cantley, L. C. (1997) *Cell* **88,** 435–437
13. Downward, J. (1998) *Curr. Opin. Cell Biol.* **10,** 262–267
14. Shao, R., Karunagaran, D., Zhou, B. P., Li, K., Lo, S.-S., Deng, J., Chiao, P. J., and Hung, M.-C. (1997) *J. Biol. Chem.* **272,** 32739–32742
15. Shao, R., Hu, M. C.-T., Zhou, B. P., Lin, S.-Y., Chaio, P. J., von Lindern, R. H., Spohn, B., and Hung, M.-C. (1999) *J. Biol. Chem.* **274,** 21495–21498
16. Beuaud, C., Henzel, W. J., and Baeuerle, P. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 429–434
17. Kane, L. P., Shapiro, V. S., Stokoe, D., and Weiss, A. (1999) *Curr. Biol.* **9,** 601–604
18. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20
19. Karin, M., and Delhase, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9067–9069
20. Tagliabue, E., Menard, S., Robertson, J. F., and Harris, L. (1999) *Int. J. Biol. Markers* **14,** 16–26
21. Russell, K. S., and Hung, M.-C. (1992) *Cancer Res.* **52,** 6624–6629
22. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. (1997) *Mol. Cell. Biol.* **17,** 3629–3639
23. Baltimore, D., and Beg, A. A. (1996) *Science* **274,** 782–784
24. Wanxg, C.-Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* **274,** 784–787
25. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 787–789
26. Maniatis, T. (1997) *Science* **278,** 818–819
27. Verma, I. M., and Stevenson, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11758–11760
28. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* **401,** 82–85
29. Romashkova, J. A., and Makarov, S. S. (1999) *Nature* **401,** 86–90

β-Catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression

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Communicated by Robert A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA, January 19, 2000 (received for review September 16, 1999)

β-Catenin can function as an oncogene when it is translocated to the nucleus, binds to T cell factor or lymphoid enhancer factor family members, and transactivates its target genes. In this study, we demonstrate that cyclin D1 is one of the targets of β-catenin in breast cancer cells. Transactivation of β-catenin correlated significantly with cyclin D1 expression both in eight breast cell lines *in vitro* and in 123 patient samples. More importantly, we found that high β-catenin activity significantly correlated with poor prognosis of the patients and was a strong and independent prognostic factor in breast cancer. Our studies, therefore, indicated that β-catenin can be involved in breast cancer formation and/or progression and may serve as a target for breast cancer therapy.

Cyclin D1 overexpression has been found in ≈50% of patients with breast cancer (1, 2), whereas gene amplification accounted for only 15–20% of these cases (3). Therefore, other mechanisms such as up-regulation of gene transcription must have played a substantial role in the overexpression of cyclin D1. By analyzing the promoter region of cyclin D1, we identified a perfect T cell factor 4 (Tcf4)-binding site (CTTTGATC) located between nucleotides −80 and −73, suggesting the potential involvement of the β-catenin/Tcf4 pathway in the regulation of cyclin D1 expression. β-Catenin was first found to be a cell–cell adhesion molecule. However, recent studies have indicated that β-catenin also could be translocated to the nucleus, where it binds to Tcf/lymphoid enhancer factor (Lef) architecture factor family members and activates genes whose promoters contain the binding sites for Tcf/Lef (4–6).

Several mechanisms have been reported to cause this deregulation, including deletion of the adenomatous polyposis coli (*APC*) gene, mutation of β-catenin, and activation of the Wnt pathway (7). Although deletion of *APC* and mutation of β-catenin have been found in many types of cancers (7), so far no such defects have been reported in breast cancer. However, many studies have indicated a possible role for the Wnt pathway in breast cancer. For example, mouse Wnt1, Wnt3, and Wnt10b have been found to be among the oncogenes activated by the insertion of mouse mammary tumor virus (MMTV) (8, 9). Mammary hyperplasias also have occurred in Wnt1 transgenic mice (10). In addition, several members of the Wnt family have been shown to induce cell proliferation (11, 12). Moreover, the expression of different Wnt members has been reported to correlate with abnormal cell proliferation in human breast tissue, suggesting the possible involvement of Wnt and the β-catenin pathway in breast cancer (13–15).

Materials and Methods

Cell Lines and Transfections. All cell lines were obtained from the American Type Culture Collection and maintained in DMEM/F-12 (HyClone) with 10% (vol/vol) fetal bovine serum. Transient transfections were performed by using DC-Chol liposome provided by Leaf Huang, University of Pittsburgh. In brief, exponentially growing 293 cells and MCF7 cells were cultured in six-well plates and transfected with 0.4 μg of reporter, 0.2 μg of pCMVβGal control, and 1 μg of effector constructs or different amounts of β-catenin expression vectors in the dose-dependent experiment or transfected with the control vector pcDNA3 (Invitrogen). The β-catenin, GSK-3β (16), and dnTcf4 effector plasmids have been described (4). Luciferase assays were performed 40 h after transfection and normalized through β-galactosidase activity. Each assay was performed triplicate. The β-catenin stable cell lines were generated by transfecting the 293 cells with the β-catenin phosphorylation mutant (S45Yβ-catenin). Individual clones were selected for resistance to 500 μg/ml G418 (Geneticin, GIBCO/BRL).

Western Blot Analysis. Cell lysates were separated by SDS/PAGE and transferred onto the nitrocellulose membrane. Protein levels were determined by using antibodies that recognized myc-tagged β-catenin, cyclin D1 (purchased from NeoMarkers, Union City, CA), and α-actin (purchased from Oncogene Science).

Gel Mobility Shift Assays. The gel-shift assays for β-catenin/Tcf4 were performed as described (4). Extracts were prepared from intact nuclei of different breast cancer cell lines. The probe was a double-stranded 15-nt oligomer, CCCTTTGATCTTACC; the control oligomer was CCCTTTGGCCTTACC. The binding reaction contained 5 μg of nuclear protein, 10 ng of radiolabeled probe, and 1 μg of poly(dIdC) in 25 μl of binding buffer (60 mM KCl/1 mM EDTA/1 mM DTT/10% glycerol). Samples were incubated on ice for 30 min, and the probes were added and incubated further at room temperature for 30 min. The β-catenin/Tcf4 bands were confirmed by the competition assays with the excess of cold wild-type or control oligomers and by comparing the complexes derived from the nuclear extract of 293 cells and its β-catenin transfectants.

Immunohistochemical Staining. Immunohistochemical staining was done by using a modification of the avidin–biotin complex technique described previously (17). The results were analyzed and confirmed by two individuals.

Results

Up-Regulation of Cyclin D1-Promoter Activity and the Protein Expression by β-Catenin. We first sought to determine whether cyclin D1 could be transcriptionally regulated by β-catenin. We found that

Abbreviations: Tcf, T cell factor; Lef, lymphoid enhancer factor; APC, adenomatous polyposis coli.

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.060025397. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.060025397

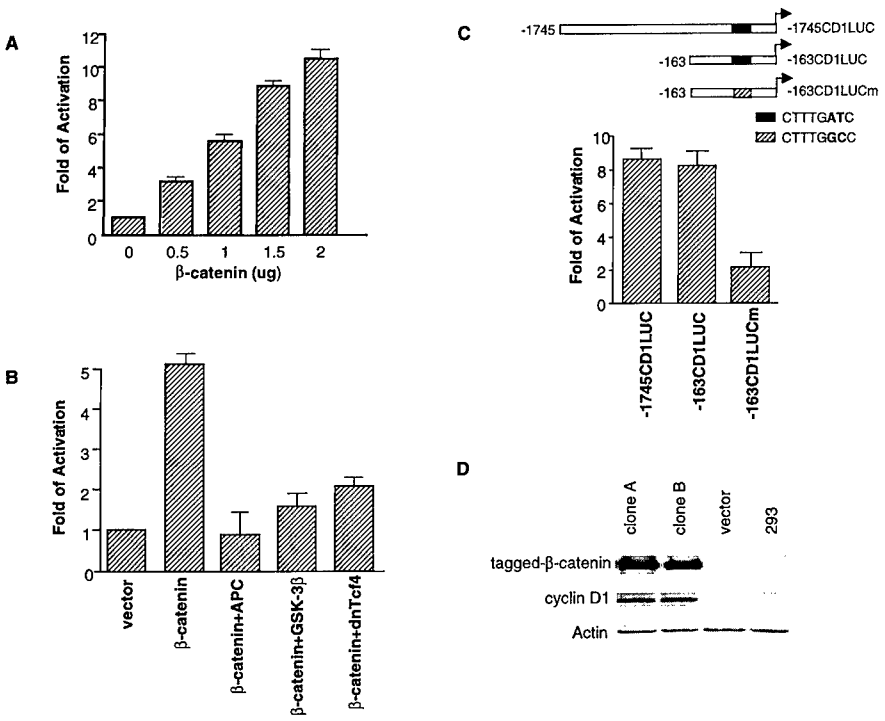

Fig. 1. Up-regulation of cyclin D1 promoter activity and the protein expression by β-catenin. (*A*) The 293 cells were transfected with 0.4 μg of cyclin D1 reporter (−1745CD1LUC) along with increasing amounts of the wild-type human β-catenin expression plasmid. (*B*) Cells were transfected with 0.4 μg of cyclin D1 reporter (−1745CD1LUC) with 1.0 μg of β-catenin expression plasmid and 1.0 μg of different negative β-catenin/Tcf regulators or with control pcDNA3 plasmid. (*C*) Cyclin D1 reporters (0.4 μg) (−1745CD1LUC, −163CD1LUC, and its mutant, 163CD1LUCm, which had an AT to GC change at nucleotides −75 and −74) and 1.5 μg of β-catenin expression plasmid were transfected into 293 cells. (*D*) The whole cell lysate of 293 cells, 293 cells transfected with empty vector, and two constitutively-activated β-catenin stable transfectants were separated by SDS/PAGE and analyzed by Western blotting with antibodies that recognized myc-tagged β-catenin, cyclin D1 (purchased from NeoMarkers), and α-actin (purchased from Oncogene).

transient transfection of exogenous human β-catenin in human embryonic kidney 293 cells could activate a cyclin D1 reporter, containing 1,745 bp of the cyclin D1 promoter, up to 11-fold in a dose-dependent manner (Fig. 1*A*). This activation was blocked when various inhibitors of the β-catenin/Tcf4 pathway were coexpressed such as APC, GSK-3β, and a dominant negative mutant of human Tcf4 (dnTcf4) (Fig. 1*B*) (18). We chose the 293 cell line to perform our studies because of its low background of β-catenin activity and its previous use for studying the response to β-catenin/Tcf4-mediated transcription (19).

To confirm that the Tcf4 site on cyclin D1 promoter was responsible for the activation by β-catenin, we used a deletion construct containing 163 bp of the cyclin D1 promoter as the reporter. Many known transcription factor binding sites had been eliminated from this construct, but it still contained the putative Tcf4 site (−163CD1LUC). As shown in Fig. 1*C*, expression of β-catenin activated this reporter to a similar extent, suggesting that the responsive element remained within this deletion construct. When the Tcf4 site was mutated so that the AT was changed to GC at nucleotides −75 and −73 (163CD1LUCm), β-catenin no longer sufficiently activated the cyclin D1 gene promoter. These data indicated that the putative Tcf4 site located at −80 to −73 was responsible for the β-catenin-mediated transactivation of the cyclin D1 promoter. In addition to transient transfection, we also generated a stable cell line by transfecting the 293 cells with the β-catenin phosphorylation mutant (S45Yβ-catenin). This mutant has been shown to resist degradation and to increase its activity to transactivate β-catenin/Tcf4-dependent transcription (19). As shown in Fig. 1*D*, cyclin D1 protein expression in both individual stable transfectants was substantially increased (lanes 1 and 2) compared with the vector control cells (lane 3) and the parental cells (lane 4).

Correlation Between Cyclin D1 Expression and β-Catenin/Tcf4 Activity in Breast Cancer Cell Lines. After identifying cyclin D1 as the target gene for β-catenin, we next asked whether β-catenin played an important role in up-regulating the expression of cyclin D1 in breast cancer. We first tested this possibility in breast cancer cell lines *in vitro*. Eight breast cancer cell lines were chosen to compare their cyclin D1 expression level and their β-catenin/Tcf4 activity. We used reporter constructs that contained three repeats of wild-type (TOP) or mutant (FOP) Tcf4-binding sites (4) to determine the transactivational activity of endogenous β-catenin/Tcf4. Higher ratios of these two reporter activities (TOP/FOP) indicated a higher β-catenin/Tcf4 activity. As shown in Fig. 2*A* (*Top* and *Middle*), cyclin D1 expression in breast cancer cells highly correlated with the β-catenin/Tcf4 activity. The eight cell lines tested could be roughly divided into three groups. BT549 and HBL100 cell lines, which expressed almost no detectable cyclin D1, had the background transactivating activity of β-catenin/Tcf4 (TOP/FOP = 1). In contrast, MCF-7, which expressed the highest level of cyclin D1 protein, had the most significant β-catenin/Tcf4 activity (TOP/FOP = 10). In the other five cell lines, cyclin D1 expression was consistently moderate, as were β-catenin/Tcf4 activities. By linear regression, we demonstrated that cyclin D1 expression indeed was proportionally correlated with β-catenin/Tcf4 activity (r = 0.97). In addition to the reporter assay, we also confirmed the β-catenin/Tcf4 activity by gel-shift assay. Consistent with reporter activity and cyclin D1 expression levels, β-catenin/Tcf4-binding activity was not detectable for either BT549 or HBL100

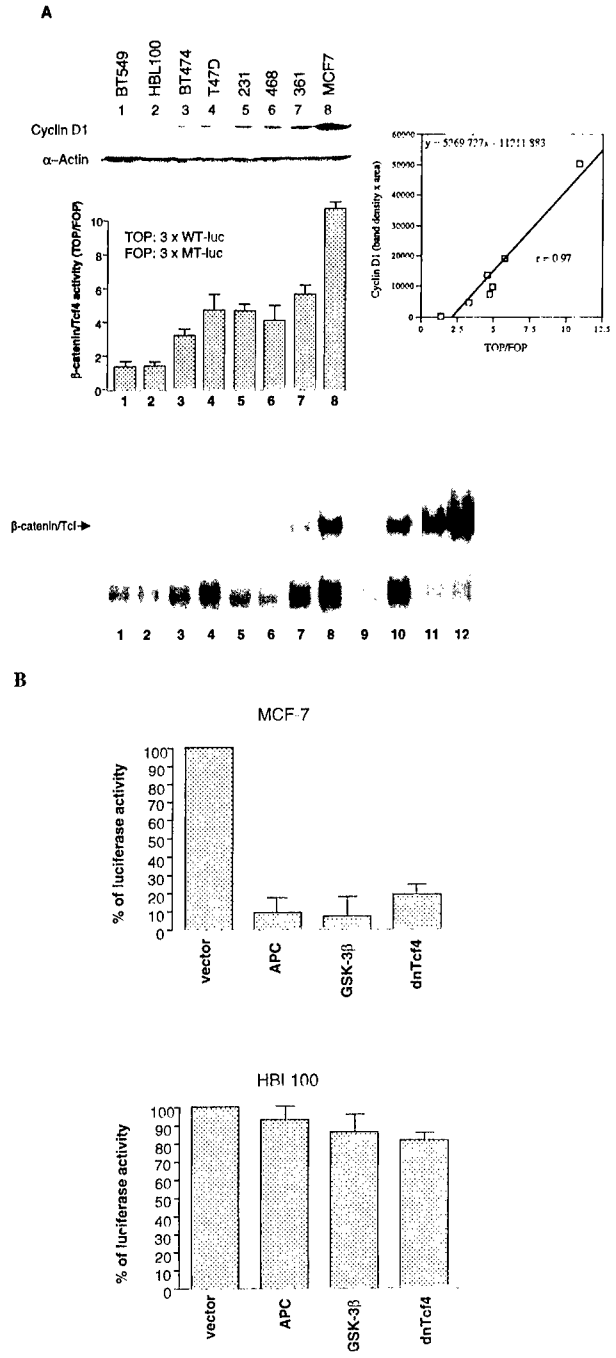

Fig. 2. Correlation between cyclin D1 expression and β-catenin/Tcf4 activity in breast cancer cell lines. (*A*) Cell lysates from different breast cell lines were separated by SDS/PAGE and analyzed by Western blotting with antibodies, which recognized cyclin D1 and α-actin (*Top*). The relative β-catenin/Tcf4 activity in different cell lines were determined by the TOP/FOP luciferase activities in each cells (*Middle*). The density of cyclin D1 bands were quantitated (by NIH IMAGE, an analyzing software) and plotted with β-catenin/Tcf4 activity (TOP/FOP) with the $r = 0.967$ by linear regression. Also, the DNA-binding activities of β-catenin/Tcf4 were determined by gel-shift assay as described previously (*Bottom*) (4). Lanes 1–8 show the β-catenin/Tcf4-binding activity in indicated cell lines. Lanes 9–12 are the controls to demonstrate the specific binding. Lane 9 and 10, the same as lane 8 except 60-fold excess of wild type (lane 9) or the mutant (lane 10) cold oligonucleotide was added; lane 11, nuclear extract was from the 293 vector control line; lane 12, nuclear extract was from 293 β-catenin stable line. (*B*) MCF-7 cells (*Top*) or HBL100 cells (*Bottom*) were cotransfected with cyclin D1 reporter (−1745CD1LUC) with different negative β-catenin/Tcf regulators or with the control pcDNA3 plasmid. The absolute luciferase activity of cyclin D1 reporter alone in MCF-7 cells was ≈7-fold higher than that in HBL100 cells.

cells and was detected most strongly in MCF-7 cells as shown in Fig. 2*A Bottom*.

To further address whether cyclin D1 promoter activity is indeed regulated by β-catenin in these breast cancer cell lines, we cotransfected the cyclin D1 reporter with different negative regulators of the β-catenin/Tcf4 pathway in MCF-7 cells. As shown in Fig. 2*B Top*, the reporter activity of cyclin D1 promoter was significantly reduced. This reduction of activity could be reversed when β catenin was coexpressed (data not shown). In contrast, cyclin D1 reporter activity was not affected by the expression of APC, GSK-3β, or dnTcf4 in HBL100 cells in which both β catenin activity and cyclin D1 expression were low (Fig. 2*B Bottom*). Our data, therefore, support a substantial role for β-catenin in activating cyclin D1 expression in breast cancer cells.

Correlation Between Activated β-Catenin and Cyclin D1 Overexpression and Their Association with Poor Patient Survival Rate. Because cyclin D1 overexpression has been well-documented in patients with breast cancer, we next sought to clinically verify whether β catenin activity truly contributed to the cyclin D1 overexpression in breast cancer tissues. We determined both cyclin D1 expression and β-catenin activity in 123 primary human breast cancer tissues (age: 26–87 years old; medium: 48 years old) by immunohistochemical staining (Fig. 3*A*). We determined β-catenin activity by its subcellular localization (20, 21). It has been well documented that accumulated β-catenin in cytoplasm and/or the nucleus increased when cells had stabilized β-catenin and, consequently, the activated β-catenin/Tcf4 activity. In contrast, β-catenin was localized solely at the plasma membrane of cells when its transactivation activity was low. We also have confirmed the correlations between the β-catenin localization and its transactivation activity in various breast cell lines listed in Fig. 2 (data not shown).

As shown in Table 1, the subcellular localization of β-catenin and cyclin D1 was significantly correlated based on the analysis by Spearman rank correlation ($r = 0.6$, $P < 0.001$). The samples stained as either high β-catenin activity with high cyclin D1 expression (40%) or low β-catenin activity with negative cyclin D1 staining (37%). It is worthwhile to mention that, among the 53 cases staining positive for cyclin D1, 49 cases (92%) were positive for β-catenin activity (stained in cytoplasm/nucleus). Thus, the correlation between these two molecules in the primary tumor samples was consistent with our *in vitro* data from the breast cancer cell lines (Fig. 2*A*). Therefore, we believe that high β-catenin activity may significantly contribute to cyclin D1 overexpression in breast cancer. These results not only supported our molecular data described above but also further strengthened their clinical biological significance.

More importantly, when the prognostic significance was assessed by Kaplan-Meier analysis and log-rank test, we found that both cyclin D1 overexpression and activated β-catenin were associated with a poorer prognosis and were negatively correlated with patient survival rates ($P = 0.033$ and $P < 0.001$, respectively) (Fig. 3*B*).

To determine whether activated β-catenin was independent of other known prognostic factors in prognosis, multivariate analyses for survival rate were also performed. We found that activated β-catenin was a strong prognostic factor that provided additional and independent predictive information on the patient's survival rate even when other prognostic factors (lymph node metastasis, estrogen receptor and progesterone receptor status, and tumor size) were taken into account ($P = 0.001$). Cyclin D1 overexpression was also an independent prognostic factor. However, when multivariate analysis was performed including only cyclin D1 expression and β-catenin activity, cyclin D1 was no longer an independent prognostic factor (the P values for cyclin D1 and β-catenin activity were $P = 0.457$ and $P <$

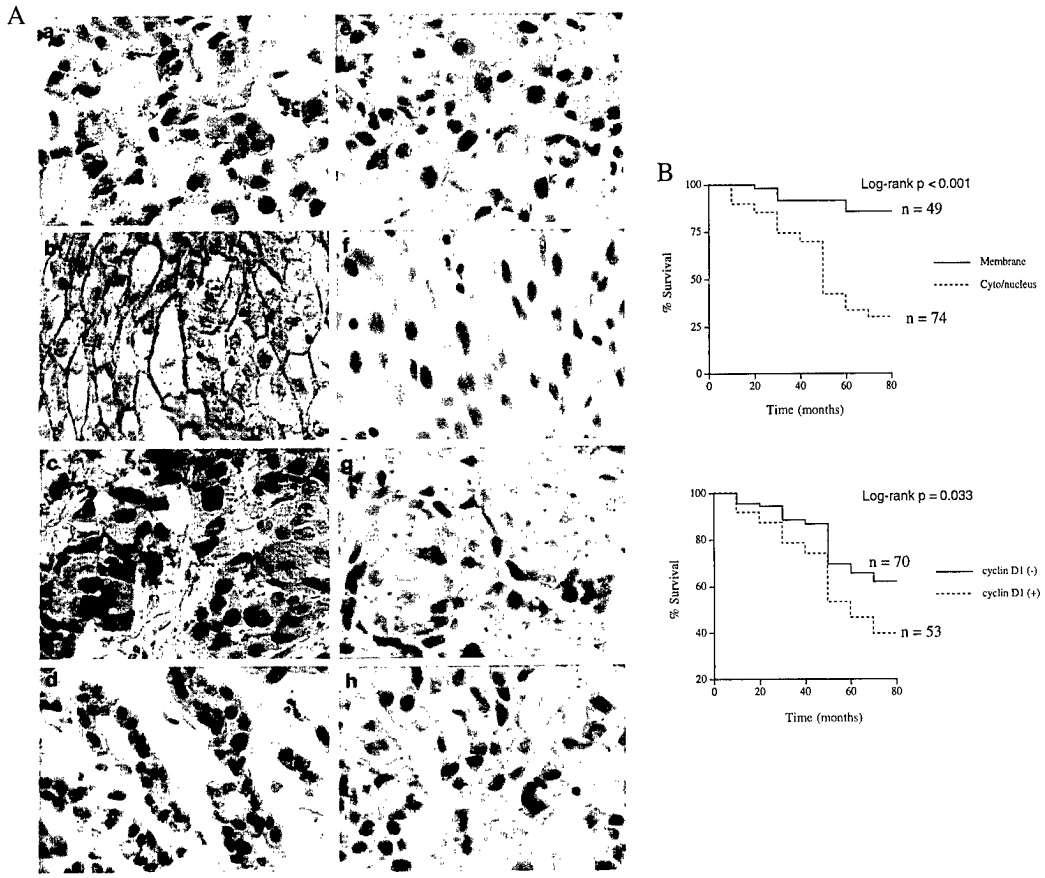

Fig. 3. Correlation between activated β-catenin and cyclin D1 overexpression and their association with poor patient survival rate. (*A*) Breast cancer tissue stained with β-catenin antibody (*a*, cytoplasm/nucleus; *b*, membrane) and cyclin D1 antibody (*c*, overexpression; *d*, negative). The right panels (*e–h*) showed the respective negative controls for a-d using PBS instead of primary antibodies. (*B*) Kaplan-Meier analysis for survival correlated with the subcellular localization of β-catenin (*Top*) and cyclin D1 expression (*Bottom*). The medium of follow-up of patients was 48 months.

0.001, respectively). These results were consistent with the model that cyclin D1 overexpression could be caused by activated β-catenin in breast cancer and consequently correlated to the prognosis.

Discussion

Our studies demonstrated that β-catenin was a poor prognostic marker in human cancer and was implicated in human breast cancer. How β-catenin activity is up-regulated in breast cancer is not clear at this moment. It is possible that activated Wnt pathway may contribute to this up-regulation (13–15). It requires further studies to elucidate the detail mechanisms.

In the past, β-catenin pathway has been studied mainly in colon carcinoma. Almost 100% of colon cancers have either mutated β-catenin or deleted APC, which is expectedly to activate the β-catenin pathway. In fact, during the time period of our studies, two groups identified cyclin D1 as the β-catenin target in colon carcinoma (22, 23). However, it is worthwhile to mention that cyclin D1 overexpression has been found in only ≈30% of colon cancer (24, 25), which might not be consistent with almost 100% deregulation of the β-catenin pathway, suggesting that the overexpression of cyclin D1 in colon cancer may be more complicated than purely up-regulation by β-catenin. Here, we showed that cyclin D1 was one of the targets for β-catenin in breast cancer. More importantly, we demonstrated the significant role of activated β-catenin in breast cancer both by molecular studies in cell culture and by clinical studies on breast tumor samples. Consistent with these findings, our studies provide strong evidence supporting the biological significance and clinical relevance of this pathway in human breast cancer. In contrast to colon carcinoma, the strong correlation between β-catenin activity and cyclin D1 expression was found in both breast cancer cell lines and breast patient tissue samples. Thus, the data presented in this study may open a new direction in the research of breast cancer involving both cancer formation and progression and provide an opportunity for development of potential therapy by blocking the β-catenin/Tcf4 pathway in breast cancer cells.

Table 1. Immunoreactivity of β-catenin and cyclin D1 in surgical specimens of breast cancer

	Cyclin D1 staining		
β-Catenin staining	Negative (n = 70)	Positive (n = 53)	Total
Cytoplasm/nucleus	25 (20%)	49 (40%)	74 (60%)
Membrane only	45 (37%)	4 (3%)	49 (40%)
Total	70 (57%)	53 (43%)	123 (100%)

The 123 surgical specimens of breast cancer were stained with either β-catenin antibodies (purchased from Transduction or Santa Cruz Biotechnology) or cyclin D1 antibody (NeoMarkers) as shown in Fig. 3*A*. The expression patterns of these two molecules in the samples from each patient were determined and summarized. Correlation of subcellular localization of β-catenin and cyclin D1 expression was analyzed by Spearman rank correlation (r = 0.6, $P < 0.001$).

We thank Dr. Paul Polakis for β-catenin expression constructs; Dr. Hans Clevers for dnTcf4, TOP-, and FOP-luciferase constructs; Dr. Li-Kuo Su for APC expression vector; and Dr. Geoffrey M. Cooper for GSK-3β constructs. We also thank Dr. Pierre McCrea and Dr. Li-Kuo Su of our institution for their critical comments on the manuscript. The work is partially supported by National Cancer Institute Grants R01 CA58880 and R01 CA77858, the M.D. Anderson Faculty Achievement Award (to M.-C.H.), and a predoctoral fellowship from Department of Defense Breast Cancer Research Program DAMD17-98-1-8242 (to S.-Y. L). This work was also supported in part by R29CA70897 and R01CA75503 (to R.G.P.). R.G.P. is a recipient of the Irma T. Hirschl award and an award from the Susan G. Komen Breast Cancer Foundation.

1. Gillet, C., Fantl, V., Fisher, C., Bartek, J., Dickson, C., Barnes, D. & Peters, G. (1994) *Cancer Res.* **54,** 1812–1817.
2. Bartkova, J., Lukas, J., Muller, H., Lutzhoft, D., Strauss, M. & Bartek, J. (1994) *Int. J. Cancer* **11,** 353–361.
3. Fantl, V., Smith, R. M., Brookes, S., Dickson, C. & Peters, G. (1993) *Cancer Surv.* **18,** 77–94.
4. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. & Clevers, H. (1997) *Science* **275,** 1784–1787.
5. Morin, P., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. & Kinzler, K. W. (1997) *Science* **275,** 1787–1790.
6. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E. & Polakis, P. (1997) *Science* **275,** 1790–1792.
7. Polakis, P. (1999) *Curr. Opin. Gene Dev.* **9,** 15–21.
8. Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K. & Varmus, H. (1984) *Nature (London)* **307,** 131–136.
9. Roelink, H., Wagenaar, E., Lopes da Silva, S. & Nusse, R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 4519–4523.
10. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T. & Varmus, H. E (1988) *Cell* **55,** 619–625.
11. Blasband, A., Schryver, B. & Papkoff, J. (1992) *Oncogene* **7,** 153–161.
12. Wong, G. T., Gavin, B. J. & McMahon, A. P. (1994) *Mol. Cell. Biol.* **14,** 6278–6286.
13. Dale, T. C., Weber-Hall, S. J., Smith, K., Huguet, E. L., Jayatilake, H., Gusterson, B. A., Shuttleworth, G. O'Hare, M. & Harris, A. L. (1996) *Cancer Res.* **56,** 4320–4323.
14. Lejeune, S., Huguet, E. L., Hamby, A., Poulsom, R. & Harris, A. L. (1995) *Clin. Cancer Res.* **1,** 215–222.
15. Bui, T. D., Rankin, J., Smith, K., Huguet, E. L., Ruben, S., Strachan, T., Harris, A. L. & Lindsay, S. (1997) *Oncogene* **14,** 1249–1253.
16. Pap, M. & Cooper, G. M. (1998) *J. Biol. Chem.* **273,** 19929–19932.
17. Hsu, S., Raine, L. & Fanger, H. (1981) *J. Histochem. Cytochem.* **29,** 577–580.
18. He, T., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L, da Costa, L. T., Morin, P. J., Vogelstein, B. & Kinzler, K. W. (1998) *Science* **281,** 1509–1512.
19. Porfiri, E., Rubinfeld, B., Albert, I., Hovanes, K., Waterman, M. & Polakis, P. (1997) *Oncogene* **15,** 2833–2839.
20. Rimm, D. L., Caca, K., Hu, G., Harrison, F. B. & Fearon, E. R. (1999) *Am. J. Pathol.* **154,** 325–329.
21. Fukuchi, T., Sakamoto, M., Tsuda, H., Maruyama, K., Nozawa, S. & Hirohashi, S. (1998) *Cancer Res.* **58,** 3526–3528.
22. Tetsu, O. & McCormick, F. (1999) *Nature (London)* **398,** 422–426.
23. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. & Ben-Ze'ev, A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 5522–5527.
24. Bartkova, J., Lukas, J., Strauss, M. & Bartek, J. (1994) *Int. J. Cancer* **58,** 568–573.
25. Arber, N., Hibshoosh, H., Moss, S. F., Sutter, T., Zhang, Y., Begg, M., Wang, S., Weinstein, I. B. & Holt, P. R. (1996) *Gastroenterology* **110,** 669–674.

Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 31, Issue of July 30, pp. 21495–21498, 1999

E1A Sensitizes Cells to Tumor Necrosis Factor-induced Apoptosis through Inhibition of IκB Kinases and Nuclear Factor κB Activities*

(Received for publication, April 13, 1999, and in revised form, May 13, 1999)

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The adenovirus E1A protein has been implicated in increasing cellular susceptibility to apoptosis induced by tumor necrosis factor (TNF); however, its mechanism of action is still unknown. Since activation of nuclear factor κB (NF-κB) has been shown to play an anti-apoptotic role in TNF-induced apoptosis, we examined apoptotic susceptibility and NF-κB activation induced by TNF in the E1A transfectants and their parental cells. Here, we reported that E1A inhibited activation of NF-κB and rendered cells more sensitive to TNF-induced apoptosis. We further showed that this inhibition was through suppression of IκB kinase (IKK) activity and IκB phosphorylation. Moreover, deletion of the p300 and Rb binding domains of E1A abolished its function in blocking IKK activity and IκB phosphorylation, suggesting that these domains are essential for the E1A function in down-regulating IKK activity and NF-κB signaling. However, the role of E1A in inhibiting IKK activity might be indirect. Nevertheless, our results suggest that inhibition of IKK activity by E1A is an important mechanism for the E1A-mediated sensitization of TNF-induced apoptosis.

E1A was originally thought to be an oncoprotein that could immortalize primary rodent cells in cooperation with other oncogenes such as *ras* or *E1B* (1). However, recent studies indicate that E1A has strong tumor suppression activities, including suppression of transformation, tumorigenicity, and metastasis (2–5). E1A has also been shown to interact with the tumor suppressor Rb and the transcriptional coactivator p300 in regulating cell differentiation, proliferation, and apoptosis (6, 7). When E1A associates with Rb, it leads to the release of the transcription factor E2F, which promotes cells to enter S phase of the cell cycle. However, when E1A associates with p300 and the tumor suppressor $p19^{ARF}$, it results in the accumulation and stabilization of p53, which induces both p53-dependent and p53-independent apoptosis (7, 8). Moreover, E1A has been shown to sensitize cells to various stimuli causing them to undergo apoptosis. Such stimuli include ionizing irradiation, DNA-damaging agents, serum starvation, and tumor necrosis factor (TNF)[1] (9, 10). Although induction of cellular susceptibility to TNF has been reported to depend on the p300 and Rb binding domains of E1A (10), the mechanism by which E1A sensitizes cells to TNF-induced apoptosis is unknown.

We have shown previously that E1A mediates sensitization of radiation-induced apoptosis through inhibition of NF-κB activity (9). The NF-κB/Rel family of transcription factors plays a crucial role in regulating genes that function in immunologic and inflammatory responses, cell proliferation, and apoptosis (11, 12). This family consists of p65 (RelA), p50 (NF-κB1), c-Rel, RelB, and p52 (NF-κB2) subunits, which can dimerize in various combinations. The primary form of NF-κB is a heterodimer of p50 and RelA subunits and is retained as a latent form in the cytoplasm of resting cells by IκB, an inhibitor of NF-κB (13). NF-κB is activated by stimulation of the IκB kinase (IKK) complex, which phosphorylates IκB and triggers its ubiquitination-dependent degradation (11, 14). This results in nuclear translocation of the activated NF-κB and activation of the target genes. A cytokine-responsive IKK complex containing IKK-α, IKK-β, and IKK-γ subunits has been identified, and the genes encoding these subunits have been cloned (15).

It is intriguing that TNF on the one hand induces cellular apoptosis and on the other hand activates NF-κB, which prevents TNF-induced apoptosis (16, 17). The link between these contradictions is unknown. Mice lacking the RelA gene died embryonically from extensive apoptosis within the liver (18). The TNF-treated RelA−/− mouse embryonic fibroblasts, macrophages, and 3T3 cell lines showed a dramatic decrease in viability when compared with the TNF-treated RelA+/+ cells (19), suggesting that RelA plays an essential role in protecting cells from TNF-induced apoptosis. Moreover, overexpression of a superrepressor IκB mutant in the TNF-resistant cell lines results in the blockage of NF-κB activity and enhancement of TNF-induced apoptosis (20–22), implying that inhibition of NF-κB activation plays a role in TNF-induced apoptosis.

In light of these findings, we hypothesized that E1A may mediate sensitization of cells to TNF through regulation of the NF-κB signaling pathway. Thus, we examined the E1A transfectants of human cancer cell lines for their sensitivity to TNF-induced apoptosis and the role of E1A in regulation of NF-κB

* This work was supported by Grants R01-CA58880 and R01-CA77858 (to M.-C. H.) and Cancer Center Core Grant 16672 from the National Cancer Institute, by the Nellie Connally Breast Cancer Research Fund, and by the Faculty Achievement Award at M. D. Anderson Cancer Center (to M.-C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Recipient of National Institutes of Health Training Grant Predoctoral Fellowship T32CA67759-01.

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[1] The abbreviations used are: TNF, turmor necrosis factor; NF-κB, nuclear factor κB; IKK, IκB kinase; RelA, a p65 subunit of NF-κB; HA, hemagglutinin; CMV, cytomegalovirus; mAb, monoclonal antibody; EMSA, electrophoresis mobility shift assay; PAGE, polyacrylamide gel electrophoresis.

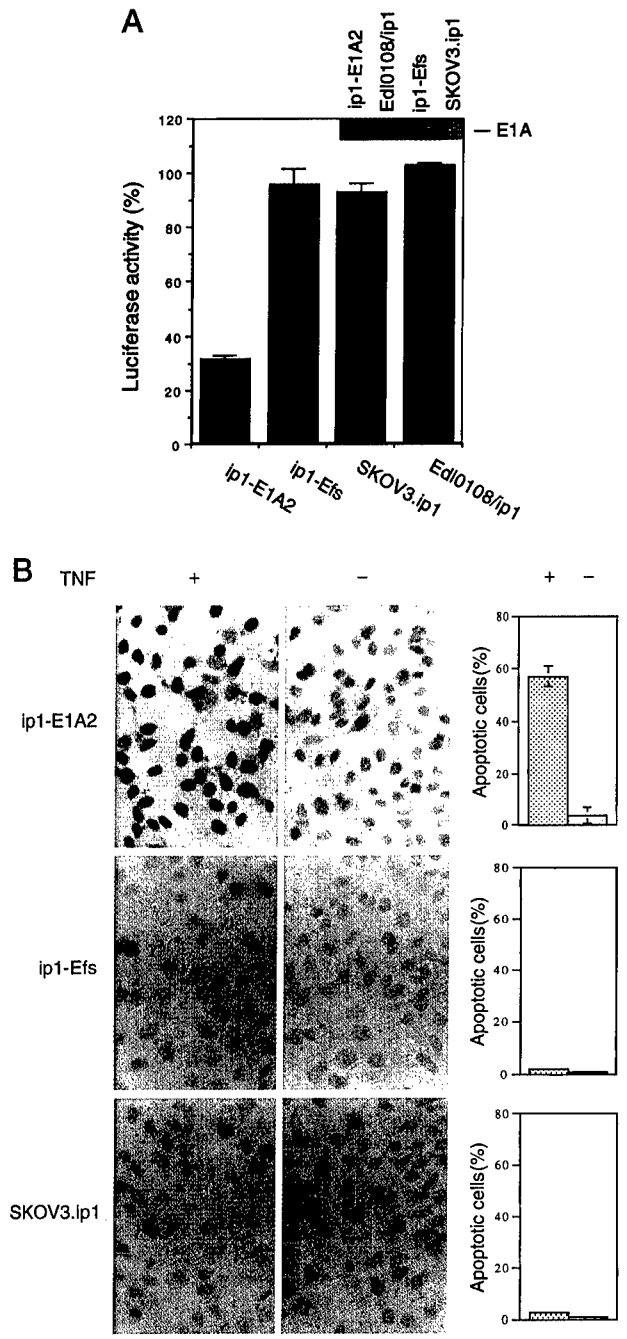

FIG. 1. **ip1-E1A2 cells are susceptible to TNF-induced apoptosis.** *A*, cytotoxicity assays of the various ip1 cell lines were performed. The ip1-Efs, Edl.0108/ip1, and SKOV3.ip1 cells were transfected with a luciferase expression vector (pCMV-luc) for 36 h, treated with human TNF (20 ng/ml) for 12 h, and then harvested. Control cells were harvested 48 h after transfection without TNF treatment. Equal amounts of cell lysates were used for luciferase assays and analyzed by Western blot for the presence of E1A (*inset*) as described under "Experimental Procedures." The percentage of luciferase in the TNF-treated cells represents cell viability and has been normalized against that of the corresponding untreated control cells (100%). The data presented were the mean of three independent experiments, and S.D. was indicated. *B*, apoptotic assays of the ip1-E1A2, ip1-Efs, and SKOV3.ip1 cells were performed. The cells were seeded in an eight-chamber slide (1.6×10^4 cells/chamber) for 4 h before they were treated with or without TNF. The cells were cultured for 3 days followed by a TUNEL assay. Quantitation of apoptotic cells was indicated as a percentage over the total cell number per field (100%), and *error bars* depict S.D. of triplicate samples.

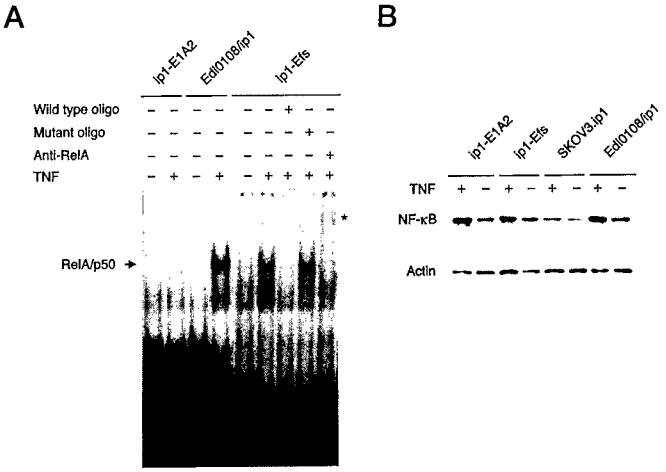

FIG. 2. **E1A suppresses the activation of NF-κB induced by TNF.** *A*, equal amounts of nuclear extracts isolated from the TNF-induced or untreated ip1-E1A2, ip1-Efs, and Edl.0108/ip1 cells were subjected to EMSA with a NF-κB oligonucleotide probe. The cold wild-type and mutant NF-κB oligonucleotides were included as controls in the TNF-induced ip1-Efs EMSA. As a RelA control, an anti-RelA antibody (SC-109, Santa Cruz) was also included in the assay. The RelA-specific supershifted complex was highlighted (*). *B*, NF-κB (RelA) proteins in the TNF-induced or control ip1-E1A2, ip1-Efs, SKOV3.ip1, and Edl.0108/ip1 cells were analyzed by Western blot analysis. Equal amounts of total cell proteins were subjected to SDS-PAGE (10% gel) and detected by Western blot with an anti-RelA antibody (*top panel*). As a control, the same blot was probed with an anti-actin antibody (*bottom panel*).

activation. We found that the cells transfected with E1A, but not E1A mutants, became very sensitive to TNF-induced apoptosis. Furthermore, we found that this E1A-mediated sensitization of TNF-induced apoptosis was due to inhibition of IKK activity, IκB phosphorylation, and NF-κB activation.

EXPERIMENTAL PROCEDURES

Cell Lines and Cultures—The establishment and culture conditions of ip1-E1A2, ip1-Efs, and SKOV3.ip1 cell lines have been described previously (4). To establish the Edl.0108/ip1 cell line, the SKOV3.ip1 cells were transfected with the pE1A mutant DNA whose p300 and Rb binding domains were deleted. All of these stable cell lines were cell clones which were isolated by G418 selection. The culture conditions for the Edl.0108/ip1 cells, a human prostate cancer cell line (PC3), and its transfectants (PC3-E1A1 and PC3-neo) were the same as those for ip1-E1A2, ip1-Efs, and SKOV3.ip1 cells.

Apoptosis Assays—The luciferase-based *in vitro* cell viability assay was performed as described previously (23). Specifically, ip1-E1A2, ip1-Efs, and Edl.0108/ip1 cells were transfected with the cytomegalovirus (CMV) promoter-luciferase expressing vector (pCMV-luc), using liposome as a gene delivery vehicle. About 36 h after transfection, the cells were treated with or without TNF (20 ng/ml). After incubation for an additional 12 h, the cells were lysed, and the luciferase activity was determined. The percentage of luciferase activity of the TNF-treated cells was normalized by using the percentage of luciferase activity of the untreated cells (100%) as base line. Standard deviations were calculated from three independent experiments. The apoptotic cells were also analyzed by the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay as described previously (24). Briefly, the cells were seeded in an eight-chamber slide (1.6×10^4 cells/chamber) for 4 h, and TNF was added to the culture. The cells were then cultured for 3 days, and the TUNEL assay was performed. The percentage of apoptotic cells was quantitated and S.D. were calculated from three independent experiments.

Electrophoretic Mobility Shift Assay (EMSA) and Western Blot Analysis—The cells were treated with TNF (20 ng/ml) for 30 min or untreated (as controls). Cell extracts were prepared, and EMSA for NF-κB was performed as described previously (9). For Western blot analysis, the cells were treated with TNF (20 ng/ml) for 4 min or left untreated (as controls), cell extracts were prepared, and Western blot analysis was performed as described previously (4). Antibodies against RelA (SC-109, Santa Cruz) and IκB-α (SC-371, Santa Cruz) were used.

Dephosphorylation Assay—IκB-α was immunoprecipitated from the cell lysates using an anti-IκB-α antibody (SC-371, Santa Cruz). The precipitates were then incubated with calf intestinal phosphatase (Promega) at 37 °C for 30 min. Subsequently, the samples were dissolved in the loading buffer and subjected to 12% SDS-polyacrylamide gel electrophore-

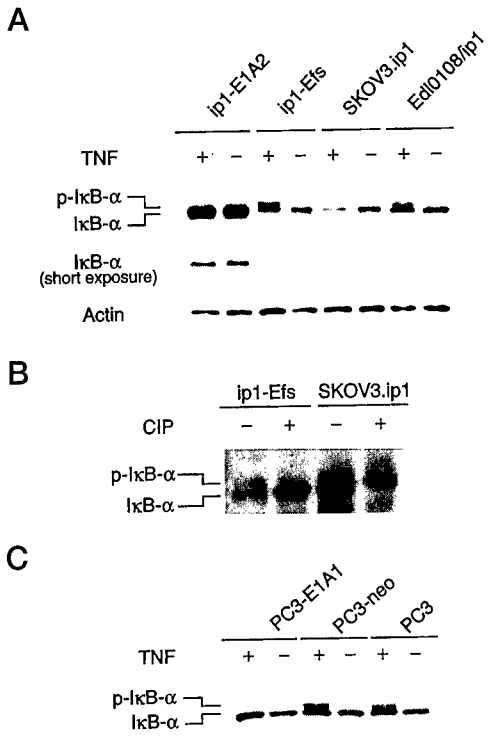

FIG. 3. **E1A inhibits IκB phosphorylation induced by TNF.** *A*, phosphorylation of IκB-α induced by TNF was analyzed by Western blot analysis. The ip1-E1A2, ip1-Efs, SKOV3.ip1, and Edl.0108/ip1 cells were treated with or without TNF (20 ng/ml) for 4 min before harvest. Equal amounts of total cell lysates isolated from these cells were subjected to SDS-PAGE (12% gel), and the phosphorylated IκB-α (*p-IκB-α*) and IκB-α were detected by Western blot with an anti-IκB-α polyclonal antibody (*top two panels*). As a control, the same blot was probed with an anti-actin antibody (*bottom panel*). *B*, phosphorylation of IκB-α induced by TNF could be dephosphorylated by a phosphatase. Equal amount of total cell lysates from the TNF-induced ip1-Efs and SKOV3.ip1 cells were immunoprecipitated with an anti-IκB-α antibody. The precipitates were incubated with or without calf intestine phosphatase (*CIP*) for 30 min at 37 °C, before they were subjected to SDS-PAGE (12% gel). IκB-α was detected by Western blot with an anti-IκB-α antibody. *C*, phosphorylation of IκB-α induced by TNF in PC3 cells was detected by Western blot analysis. The PC3-E1A1, PC3-neo, and PC3 cells were treated with or without TNF, and p-IκB-α and IκB-α were detected by Western blot analysis as described above.

sis (PAGE) and immunoblot analysis using the same anti-IκB-α antibody.

Transient Transfections and Immunocomplex Kinase Assays—The various ip1 cell lines were plated the day before transfection at a density of 2×10^6 cells per 100-mm dish. Cells were transfected with either an IKK-α-FLAG or a HA-IKK-β expression vector or an empty vector (control), using liposome as described above. Cell extracts were prepared 48 h after transfection, and immunocomplex kinase assays were performed as described previously (25).

RESULTS AND DISCUSSION

To test whether E1A could affect cellular susceptibility to cell death induced by TNF, an *in vitro* cell viability assay was performed using a luciferase assay (23). We used a human ovarian cancer cell line derivative, SKOV3.ip1, which was stably transfected with the wild-type Ad5 E1A (ip1-E1A2) or an E1A frameshift Efs mutant (ip1-Efs) that has lost the E1A function, or an E1A deletion mutant (Edl.0108/ip1) whose p300 and Rb binding domains were deleted (10). When the cells were transiently transfected with the luciferase reporter gene (pCMV-luc) and treated with TNF, the luciferase activity was strongly reduced in the ip1-E1A2 cells compared with the parental SKOV3.ip1 and the mutant ip1-Efs cells (Fig. 1*A*). This suggests that E1A sensitizes cells to TNF-induced cell death. However, this E1A-mediated sensitization of TNF-induced cell death was abolished in the Edl.0108/ip1 cells, suggesting that

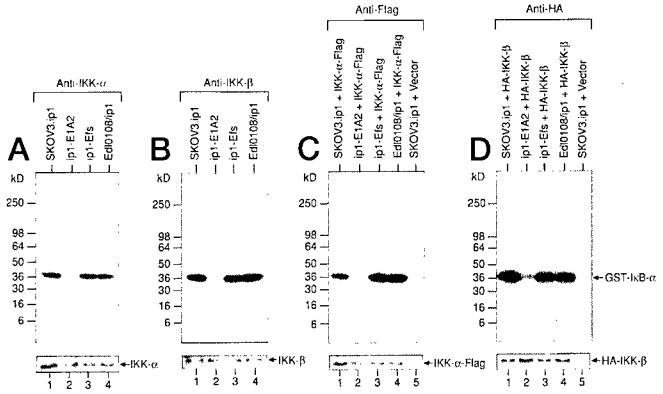

FIG. 4. **E1A down-regulates the activities of IKK-α and -β induced by TNF.** The endogenous IKK-α (*A*) and IKK-β (*B*) activities were examined in the various ip1 cell lines. The SKOV3.ip1, ip1-E1A2, ip1-Efs, and Edl.0108/ip1 cells were treated with human TNF (20 ng/ml) for 10 min before harvest, and equal amounts of cell lysates were used for kinase assays. After immunoprecipitation with either anti-IKK-α or anti-IKK-β antibody, IKK-α (*A*) or IKK-β (*B*) kinase activity was measured by an immunocomplex kinase assay, using GST-IκB-α (1–54) as a substrate. The ectopically expressed IKK-α-FLAG (*C*) and HA-IKK-β (*D*) activities were examined in the various ip1 cell lines. The SKOV3.ip1, ip1-E1A2, ip1-Efs, and Edl.0108/ip1 cells were transfected with either IKK-α-FLAG (*C*) or HA-IKK-β (*D*), and the cells were harvested 48 h after transfection with the stimulation of TNF as described above. As a control, the SKOV3.ip1 cells were transfected with an empty vector (*lane 5* in *C* and *D*) and treated with TNF. After immunoprecipitation with either an anti-FLAG or anti-HA mAb, IKK-α (*A*) or IKK-β (*B*) kinase activity was measured as described above. As controls, the *bottom panels* show Western blots indicating the expression levels of both endogenous IKK-α or IKK-β and the transfected IKK-α-FLAG or HA-IKK-β in the various ip1 cell lines.

the p300 and Rb binding domains of E1A are required for this sensitization. This is consistent with the previous finding that the E1A function in increasing cellular susceptibility to cell death induced by TNF depends on its binding to either p300 or p105Rb (10). As a control, we examined the expression of E1A in these cell lines (ip1-E1A2, ip1-Efs, SKOV3.ip1, and Edl.0108/ip1) by Western blot analysis using an anti-E1A monoclonal antibody (mAb) (M58). These results indicated that the ip1-E1A2 and Edl.0108/ip1 cells expressed a significant amount of E1A, but the parental (SKOV3.ip1) and the mutant (ip1-Efs) cells did not (*inset*, Fig. 1*A*). As another control, we also examined the expression of TNF receptor (TNFR) in these cell lines (ip1-E1A2, ip1-Efs, and SKOV3.ip1) by Western blot analysis using an anti-TNFR1 antibody (R&D System), and showed that the levels of TNFR were similar in these cell lines (data not shown). To confirm the E1A-mediated sensitization of TNF-induced apoptosis, we examined these TNF-treated ip1-E1A2 cells by a TUNEL assay. As shown in Fig. 1*B*, TNF indeed induced apoptotic DNA breakage in the ip1-E1A2 cells. As negative controls, the ip1-Efs and SKOV3.ip1 cells did not show apoptotic phenotypes with the TNF treatment. Moreover, many of the ip1-E1A2 cells treated with TNF showed the morphologic changes associated with apoptosis, including cell shrinking and apoptotic body formation (data not shown). Taken together, these results suggest that E1A sensitizes cells to TNF-induced apoptosis.

Recently, NF-κB has been shown to have an important role in the antiapoptotic pathway (9, 18–22). Although NF-κB plays a role in blocking apoptosis induced by TNF (19–22), the involvement of NF-κB in the E1A-mediated cellular susceptibility to TNF has not been examined. Therefore, the ip1-E1A2, ip1-Efs, and Edl.0108/ip1 cells for their NF-κB DNA binding activities before and after TNF treatment were analyzed by EMSA. Our results showed that TNF induced NF-κB DNA binding activity in the ip1-Efs and Edl.0108/ip1 cells, but not in the ip1-E1A2 cells (Fig. 2*A*). The activated NF-κB complex

induced by TNF in the ip1-Efs cells was eliminated in the presence of excess cold wild-type, but not mutant, NF-κB oligonucleotides, suggesting that the activated NF-κB DNA binding activity is NF-κB DNA specific (Fig. 2*A*). To confirm the presence of the RelA subunit of NF-κB, we performed the same EMSA in the presence of an anti-RelA polyclonal antibody. A RelA-specific super-shifted complex was detected, indicating that the binding complex is indeed an activated NF-κB (Fig. 2*A*), presumably the RelA/p50 heterodimer as reported previously (9). Thus, these results indicate that E1A is capable of inhibiting NF-κB activation induced by TNF. To test whether inhibition of NF-κB activity correlates with inhibition of NF-κB protein expression, whole-cell extracts from the ip1-E1A2, SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells with or without TNF treatment were examined by Western blot analysis using an anti-RelA antibody. While NF-κB (RelA) protein expression was not inhibited by E1A, its expression was slightly enhanced with TNF stimulation (Fig. 2*B*). Although this increase of NF-κB protein by TNF may partially contribute to the TNF-induced NF-κB activity, it does not account for the inhibition of TNF-induced NF-κB activity by E1A. Our data suggest that E1A inhibits NF-κB activation but not its protein expression.

To further study how E1A might inhibit the TNF-induced NF-κB activity, we investigated whether E1A could down-regulate NF-κB activity through inhibition of IκB phosphorylation. The changes of phosphorylation and expression of IκB-α in the ip1-E1A2, SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells treated with TNF for 4 min were examined by Western blot analysis. As shown in Fig. 3*A*, there was only one IκB-α band observed in the ip1-E1A2 cells with or without treatment with TNF. The same band was also detected in the non-TNF-treated SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells. However, two IκB-α bands were detected in the TNF-treated SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 control cells (Fig. 3*A*); the upper band should be the phosphorylated form of IκB-α. To confirm this, we immunoprecipitated IkB-α from the cell extracts with an anti-IκB-α antibody. Then the precipitated pellets were treated with or without calf intestinal phosphatase and subjected to immunoblotting with the same anti-IκB-α antibody. The results showed that the upper band disappeared after the calf intestinal phosphatase treatment (Fig. 3*B*), indicating that the upper band is indeed the phosphorylated form of IκB-α, and E1A inhibits TNF-induced IκB-α phosphorylation. The finding that the level of IκB-α protein was elevated in the ip1-E1A2 cells might be due to slower degradation of the nonphosphorylated IκB-α protein (Fig. 3*A*). Furthermore, the E1A-mediated suppression of IκB phosphorylation induced by TNF was confirmed by using a human prostate cancer cell line PC3 and its E1A transfectants (Fig. 3*C*) and a human ovarian cancer cell line 2774 (data not shown).

It has been well documented that TNF induces the activation of IKK, which in turn phosphorylate IκB-α with the subsequent activation of NF-κB (11, 14). To examine whether E1A could regulate IKK activity, the ip1-E1A2, SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells were treated with TNF, and the endogenous IKK-α and IKK-β activities were determined by immunocomplex kinase assays. The endogenous IKK-α (Fig. 4*A*) and IKK-β (Fig. 4*B*) activities were readily detected in the SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells, whereas the activities of both IKKs in the ip1-E1A2 cells were inhibited. To confirm that E1A could inhibit the activities of IKK-α and IKK-β expressed ectopically, we transiently transfected an IKK-α-FLAG or a HA-IKK-β expression vector or an empty vector (control) into the ip1-E1A2, SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells. The activities of IKK-α and IKK-β in these transfected cells were measured after treatment with TNF. Our results showed that the activities of IKK-α (Fig. 4*C*) and IKK-β (Fig. 4*D*) were significantly suppressed in the ip1-E1A2 cells compared with the same vector transfected in the SKOV3.ip1, ip1-Efs, and Edl.0108/ip1 cells, suggesting that E1A inhibits the TNF-induced IKK-α and IKK-β activities. Moreover, this inhibition was abrogated in the Edl.0108/ip1 cells, implying that the p300 and Rb binding domains are essential for the E1A function in blocking IKK activity in the presence of TNF. Thus, taken together, our results strongly suggest that E1A down-regulates the TNF-induced NF-κB signaling pathway through inhibition of IKK activity, and this mechanism contributes significantly to the E1A-mediated sensitization of cells to TNF-induced apoptosis.

In order to discern the role of E1A in inhibition of IKK activity, direct binding between E1A and IKK was examined by immunoprecipitation and Western blot analysis. An E1A expression vector (pE1A) was cotransfected with either IKK-α-FLAG or a HA-IKK-β into 293T cells. Whole cell lysates were immunoprecipitated with an anti-E1A mAb (M58), and the immunoprecipitates were subjected to Western blot analysis with either an anti-FLAG or anti-HA mAb. While M58 mAb immunoprecipitated E1A protein (~43 kDa) from the cell lysates, neither anti-FLAG nor anti-HA mAb detected IKK-α-FLAG or HA-IKK-β coimmunoprecipitated with E1A in Western blot analysis (data not shown). Similarly, cell lysates were immunoprecipitated with either an anti-FLAG or anti-HA mAb, and the immunoprecipates were then subjected to Western blot analysis with M58 mAb. Again, no detectable E1A protein was coimmunoprecipitated with either IKK-α-FLAG or HA-IKK-β (data not shown). These results indicated no direct binding between E1A and IKK, suggesting that E1A may act on IKK indirectly. Further investigation of the direct cellular target(s) of E1A in this signaling pathway is necessary to elucidate the mechanism underlying the E1A-regulated IKK activity and apoptosis.

Acknowledgment—We are grateful to Dr. Wei-Ya Xia for technical assistance.

REFERENCES

1. Ruley, H. E. (1990) *Cancer Cells* **2,** 258–268
2. Yu, D., and Hung, M. C. (1998) *Cancer Metastasis Rev.* **17,** 195–202
3. Chang, J. Y., Xia, W., Shao, R., Sorgi, F., Hortobagyi, G., Huang, L., and Hung, M. C. (1997) *Oncogene* **14,** 561–568
4. Yu, D., Wolf, J. K., Scanlon, M., Price, J. E., and Hung, M. C. (1993) *Cancer Res.* **53,** 891–898
5. Chang, J. Y., Xia, W., Shao, R., and Hung, M. C. (1996) *Oncogene* **13,** 1405–1412
6. Arroyo, M., and Raychaudhuri, P. (1992) *Nucleic Acids Res.* **20,** 5947–5959
7. Stanchina, E. D., McCurrach, M. E., Zindy, F., Shieh, S. Y., Ferbeyre, G., Samuelson, A. V., Prive, C., Roussel, M. F., Sherr, C. J., and Lowe, S. (1998) *Genes Dev.* **12,** 2434–2442
8. Chiou, S. K., and White, E. (1997) *J. Virol.* **71,** 3518–3525
9. Shao, R., Karunagaran, D., Zhou, B. P., Li, K., Lo, S. S., Deng, J., Chiao, P., and Hung, M. C. (1997) *J. Biol. Chem.* **272,** 32739–32742
10. Shisler, J., Duerksen-Hughes, P., Hemiston, T. M., Wold, W. S. M., and Gooding, L. R. (1996) *J. Virol.* **70,** 68–77
11. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J. W., Young, D. B., Barbosa, M., and Mann, M. (1997) *Science* **278,** 860–866
12. Wang, C. Y., Mayo, M. W., Komeluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. (1998) *Science* **281,** 1680–1683
13. Simeonidis, S., Stauber, D., Chen, G., Hendrickson, W. A., and Thanos, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 49–54
14. Traenchner, E. B. M., Pahl, H. L., Henkel, T., Schmidt, K. N., Wilk, S., and Baeuerle, P. A. (1995) *EMBO J.* **14,** 2876–2883
15. Scheidereit, C. (1998) *Nature* **395,** 225–226
16. Jiang, Y., Woronicz, J. D., Liu, W., and Goeddel, D. V. (1999) *Science* **283,** 543–546
17. Van Antwerp, D. J., Martin, S. J., Verma, I. M., and Green, D. R. (1998) *Trends Cell Biol.* **8,** 107–111
18. Beg, A. A., Sha, W., Wronson, R., Ghosh, S., and Baltimore, D. (1995) *Nature* **376,** 167–170
19. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784
20. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) *Science* **274,** 784–788
21. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 787–789
22. Bakker, T. R., Reed, D., Renno, T., and Jongeneel, C. V. (1999) *Int. J. Cancer* **80,** 320–323
23. Boeckman, F. A., and Aizenman, E. (1996) *J. Pharmacol. Exp. Ther.* **279,** 515–523
24. Deng, J., Xia, W., and Hung, M. C. (1998) *Oncogene* **17,** 2167–2175
25. Hu, M. C.-T., Qiu, W. R., Wang, X., Meyer, C. F., and Tan, T.-H. (1996) *Genes Dev.* **10,** 2251–2264