

Tumor Suppression and Sensitization to Tumor Necrosis Factor α -induced Apoptosis by an Interferon-inducible Protein, p202, in Breast Cancer Cells¹

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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- κ B 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 anti-virus, 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 G₁-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 TNF⁴- α and that correlates with inactivation of NF- κ B by a NF- κ B/p202 interaction. These results suggest a potential combined therapy using p202 and TNF- α against breast cancer.

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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 μ g/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).

[³H]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 μ g) 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. Extracts 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 μ g of anti-p65 antibody (Santa Cruz Biotechnology) for 4 h with gentle rotation at 4°C. Protein A-Sepharose beads (50 μ l) 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. 1a, left panel). There was at least a 75% reduction in colony number in both p202-transfected cell lines (Fig. 1a, right panel). These data suggest that p202 expression may be associated with antiproliferation

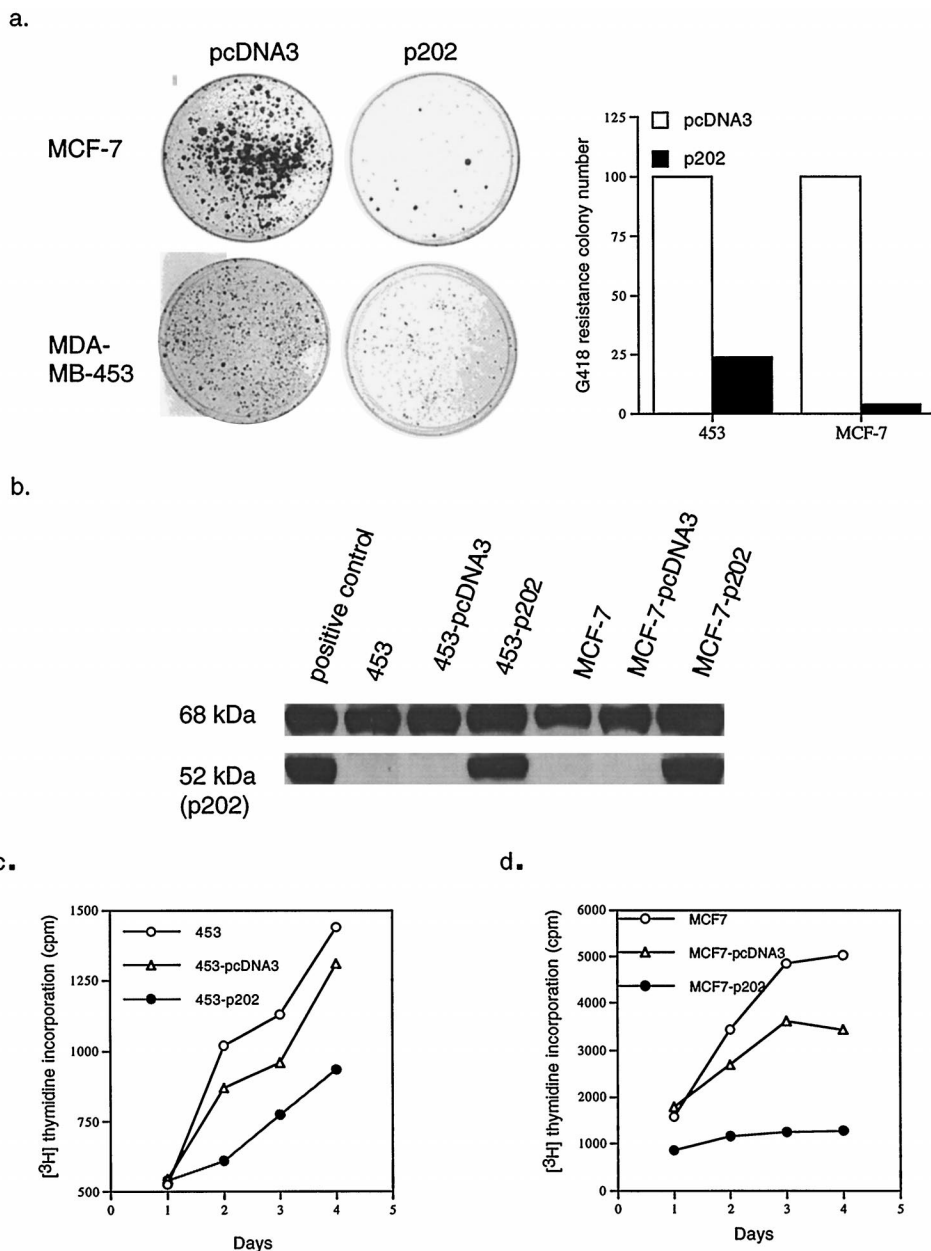


Fig. 1. Expression of p202 inhibits the proliferation of MDA-MB-453 and MCF-7 breast cancer cells. *a*, colony-forming assay. MDA-MB-453 and MCF-7 cells were transfected with either a control vector (pcDNA3) or a p202 expression vector. The colony number obtained from pcDNA3 transfection was set as 100%. *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*, [³H]thymidine incorporation assays. DNA synthesis rate was measured by the amount of [³H]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 [³H]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-

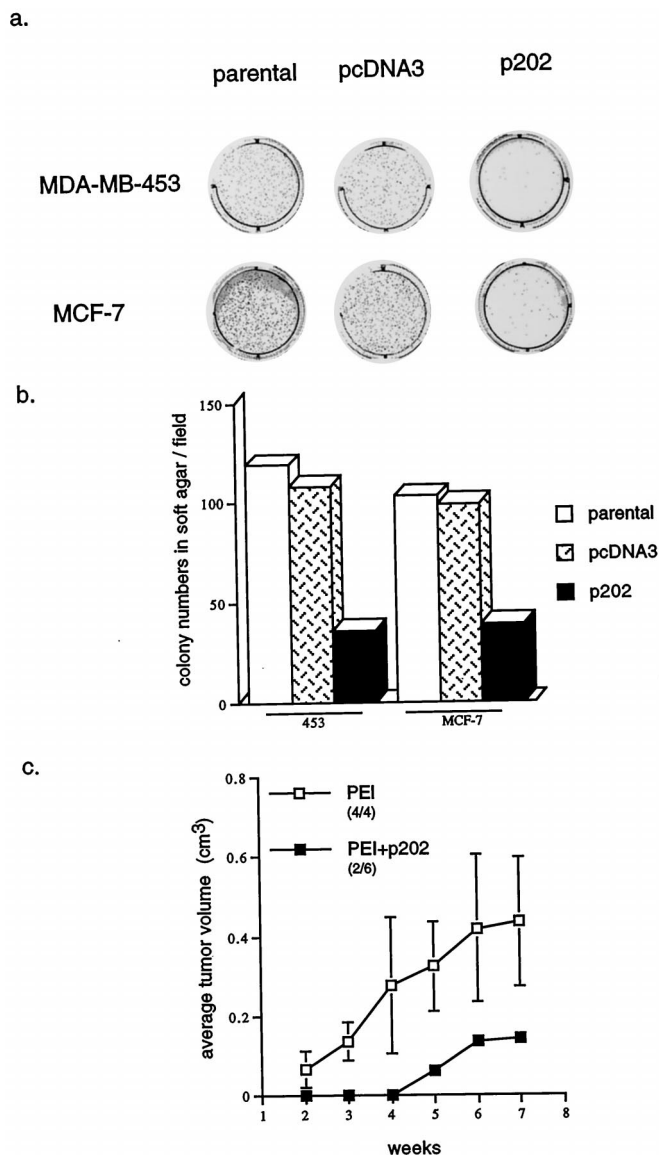


Fig. 2. p202 inhibits the transformation phenotype of breast cancer cells. *a*, colony formation in soft agar. MDA-MB-453, 453-pcDNA3, 453-p202, MCF-7, MCF-7-pcDNA3, and MCF-7-p202 cell lines were subjected to anchorage-independent growth in soft agar. *b*, number of colonies formed in soft agar as shown 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. 2c). 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-G₁ population) than the parental 453 cells and 453-pcDNA3 control cells in a dose-dependent manner (Fig. 3a). 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. 3b). 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- κ B (13–15). To test that hypothesis, we tested whether p202 expression could affect the NF- κ B-mediated transcription activation in response to TNF- α treatment. We cotransfected CMV-p202 and a NF- κ B-activatable promoter-reporter construct (κ B-luc), *i.e.*, an I κ B promoter-driven luciferase gene, into 453 cells in the presence of TNF- α (Fig. 4a). As expected, κ B-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- κ B molecule to elicit such transcription repression, we cotransfected CMV-p202 with a Rel-A (a p65 subunit of NF- κ B) cDNA expression vector and κ B-luc. As shown in Fig. 4b, whereas p202 expression alone has no effect on κ B-luc, it could greatly repress NF- κ B (Rel-A)-activated I κ B pro-

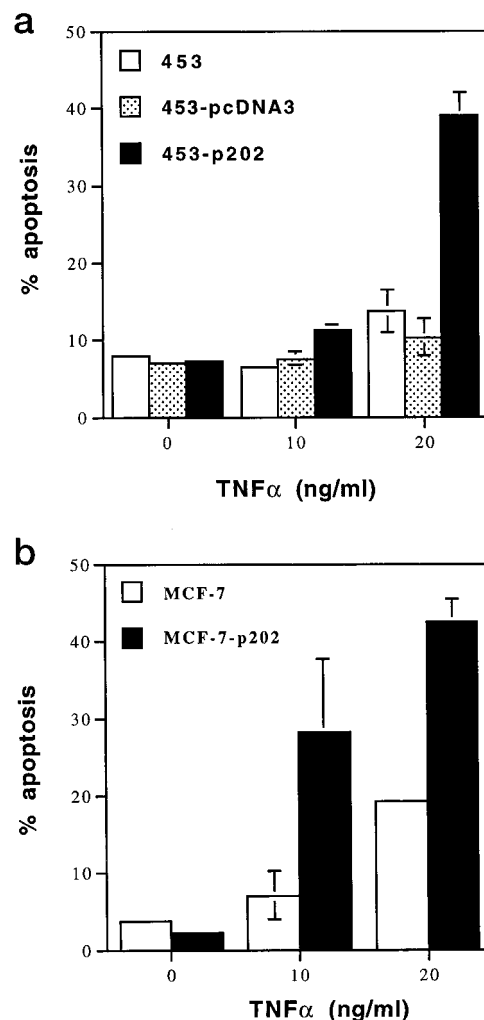
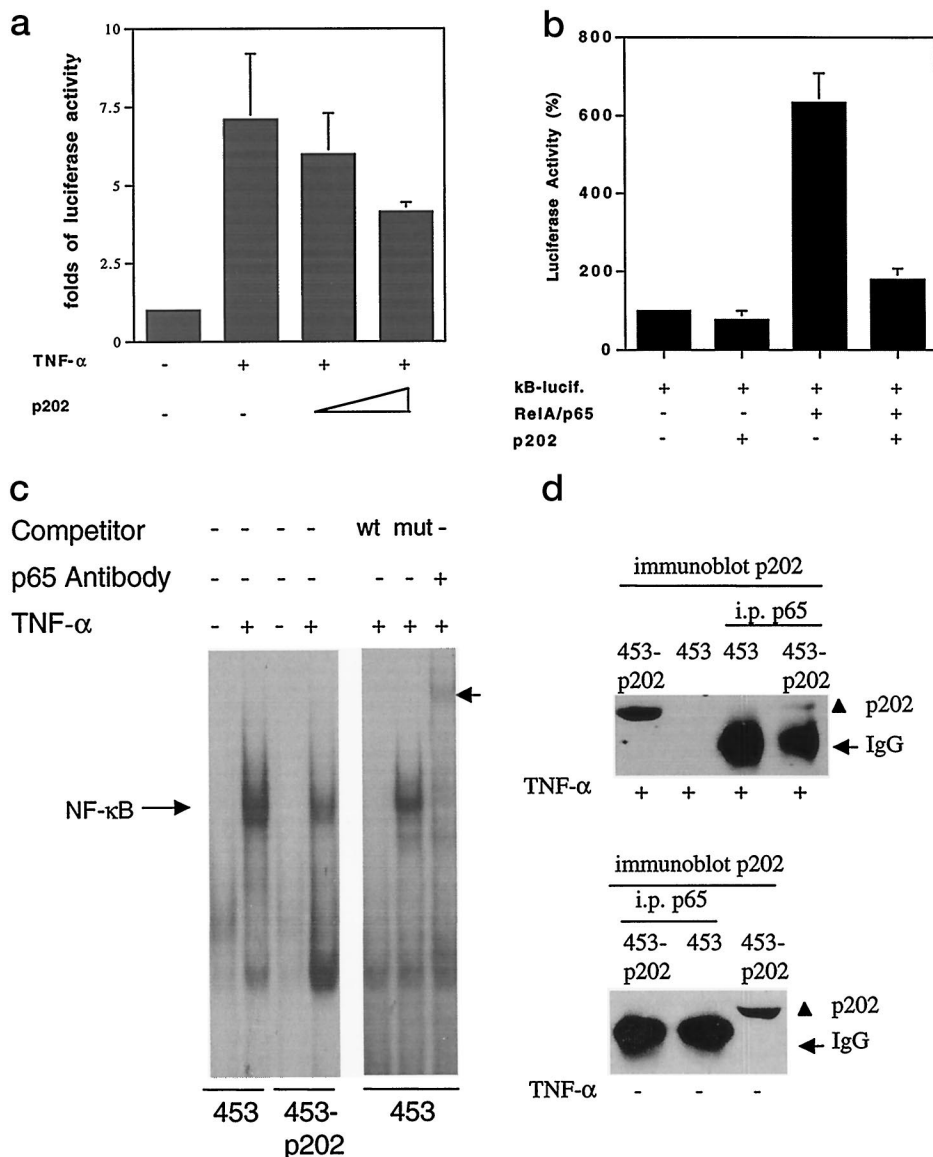


Fig. 3. p202 sensitizes breast cancer cells to apoptosis induced by TNF- α in a dose-dependent manner. *a*, 453, 453-pcDNA3, and 453-p202 cell lines were treated with TNF- α (0, 10, and 20 ng/ml) for 48 h. Bars, SE. *b*, MCF-7 and MCF-7-p202 were treated with TNF- α (0, 10, and 20 ng/ml) for 48 h. Cells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

Fig. 4. The interaction and inactivation of NF- κ B by p202 is responsible for the p202-mediated sensitization to TNF- α -induced apoptosis. *a*, p202 expression represses NF- κ B-mediated transcription activation in response to TNF- α . I κ B-Luciferase reporter gene (0.2 μ g) and CMV-p202 (0, 0.8, or 2 μ g) 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 I κ B-Luciferase expression was calculated with respect to I κ B-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 κ B-luc and \pm NF- κ B (p65) expression vector. The inhibitory activity of p202 on the induction of I κ B 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 for 30 min). *Left panel*, the activated NF- κ B (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- κ B binding site. A polyclonal Rel-A antibody supershifted the NF- κ B 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. \blacktriangle , 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- κ B by p202.

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

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. 4d, *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. 4d, *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- κ B 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- κ B. 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- κ B 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- κ B 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.

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