Regulation of Expression of BIK Proapoptotic Protein in Human Breast Cancer Cells: p53-Dependent Induction of BIK mRNA by Fulvestrant and Proteasomal Degradation of BIK Protein


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Abstract

Induction of mRNA for BIK proapoptotic protein by doxorubicin or γ-irradiation requires the DNA-binding transcription factor activity of p53. In MCF7 cells, pure antiestrogen fulvestrant also induces BIK mRNA and apoptosis. Here, we provide evidence that, in contrast to doxorubicin or γ-irradiation, fulvestrant induction of BIK mRNA is not a direct effect of the transcriptional activity of p53, although p53 is necessary for this induction. It is known that p53 up-regulated modulator of apoptosis (PUMA) mRNA is induced directly by the transcriptional activity of p53. Whereas γ-irradiation induced both BIK and PUMA mRNA, only BIK mRNA was induced by fulvestrant. Whereas both fulvestrant and doxorubicin induced BIK mRNA, only doxorubicin enhanced the DNA-binding activity of p53 and induced PUMA mRNA. Small interfering RNA (siRNA) suppression of p53 expression as well as overexpression of dominant-negative p53 effectively inhibited the fulvestrant induction of BIK mRNA, protein, and apoptosis. Transcriptional activity of a 2-kb BIK promoter, which contained an incomplete p53-binding sequence, was not affected by fulvestrant when tested by reporter assay. Fulvestrant neither affected the stability of the BIK mRNA transcripts. Interestingly, other human breast cancer cells, such as ZR75-1, constitutively expressed BIK mRNA even without fulvestrant. In these cells, however, BIK protein seemed to be rapidly degraded by proteasome, and siRNA suppression of BIK in ZR75-1 cells inhibited apoptosis induced by MG132 proteasome inhibitor. These results suggest that expression of BIK in human breast cancer cells is regulated at the mRNA level by a mechanism involving a nontranscriptional activity of p53 and by proteasomal degradation of BIK protein. (Cancer Res 2006; 66(20): 10153-61)

Introduction

Evidence is accumulating that hormonal therapy of breast cancer is not only cytostatic but also cytotoxic (1). The pure antiestrogen fulvestrant has been shown to induce apoptosis of estrogen receptor (ER)-positive primary breast cancers in postmenopausal patients (2), ER-positive ductal carcinoma in situ tissue xenografts transplanted in nude mice (3), and human breast cancer cells maintained in nude mice (4) or cell culture (5, 6). Based on observations that expression of antiapoptotic BCL-2 in MCF7 breast cancer cells is enhanced by 17β-estradiol (E2) and suppressed by antiestrogens, several previous studies suggested that the reduction in BCL-2 expression is involved in the mechanism of antiestrogen-induced apoptosis (4, 7, 8). Other studies suggested the importance of IFN regulatory factor-1 (IRF-1) transcription factor in fulvestrant-induced MCF7 cell apoptosis (6), although the possible roles of IRF-1 in the regulation of expression of BCL-2 and other apoptosis-related genes have not been sufficiently understood.

By DNA microarray, our laboratory and others found that expression of BIK, a proapoptotic BH3-only protein (9, 10), was strongly suppressed by E2 (11, 12) in MCF7 cells. BIK binds directly to BCL-2 or BCL-XL via its BH3 domain and inactivates their antiapoptotic functions (13). BIK is inducible by doxorubicin or γ-irradiation, which activates the DNA-binding transcription factor activity of p53 tumor suppressor protein as well as adenoviral overexpression of p53 (14, 15). In our previous study, we examined the role of BIK on the mechanisms of action of hormonal agents on apoptosis of MCF7 and found that fulvestrant strongly induced expression of both BIK mRNA transcripts and protein and killed these cells by apoptosis in a BIK-dependent manner (5). In contrast, fulvestrant only moderately affected the expression of other antiapoptotic and proapoptotic members of BCL-2 family, such as BCL-2, BCL-XL, BAX, BID, BAD, BAK1, or HRK (5, 11).

To obtain further insights into mechanisms of regulation of BIK expression in breast cancer cells, we examined the effects of fulvestrant and proteasome inhibitors on the expression of the BIK mRNA transcripts and protein in several representative human breast cancer cell lines. We present evidence that, although p53 is necessary for the fulvestrant induction of the BIK mRNA transcripts in MCF7 cells, this induction is not a direct consequence of the transcription factor activity of p53. We also show that other breast cancer cells that are resistant to the cytotoxic action of fulvestrant rapidly degrade BIK protein by a proteasome-dependent mechanism.

Materials and Methods

Cell culture. MCF7 cells (BUS stock; ref. 11) were obtained from A.M. Soto and C. Sommenschein (Tufts University, Boston, MA) and maintained in phenol red–free DMEM supplemented with 5% FCS (HyClone, defined grade, Logan, UT), which supported proliferation of MCF7 cells with an efficacy similar to 30 to 60 pmol/L. E2 (11). T47D, ZR75-1, SKBr3, and MDA435 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained similarly. Fulvestrant was purchased from Tocris Bioscience (Ellisville, MO). MG132, doxorubicin, paclitaxel, and z-VAD-fmk were purchased from Sigma (St. Louis, MO). Bortezomib was a gift from Millennium Pharmaceuticals (Cambridge, MA).
Cell proliferation and apoptosis. Number of cells in 96-well culture plates was determined using the CyQuant kit (Molecular Probes/Invitrogen, Carlsbad, CA; ref. 16). Apoptotic cells were identified by the sulforhodamine (SR)–conjugated fluorescence inhibitor of caspases assay using the SR-DEVD-fmk caspase-3/caspase-7 substrate (Immunochemistry Technologies, Bloomington, MN; ref. 17). SR-DEVD-fmk-positive cells were identified under an inverted phase-contrast/epi-fluorescence microscope (525–550 nm excitation/575–750 nm emission).

Real-time quantitative reverse transcription-PCR and Western blotting. Amounts of mRNA transcripts for human BIK, WISP2, p53, and β-actin were determined by real-time quantitative reverse transcription-PCR (QRT-PCR) using the ABI 7500 System (Applied Biosystems, Foster City, CA). The Taqman assay primer/probe sets were purchased from Applied Biosystems (BIK, Hs00609635_m1; WISP2, Hs00180242_m1; PUMA, Hs00248075_m1; p53, Hs00153349_m1; and β-actin, 4352933E). Relative amounts of the mRNA transcripts were calculated using the ΔΔCt method (18) with β-actin mRNA as an internal reference. Immunoblotting was done following the standard protocol, and the relative strength of protein expression was determined using the NIH Image software. Anti-BIK (N-19), anti-PUMA (N-19), and anti-actin (I-19) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p53 antibody (PB53-12) was from Abcam (Cambridge, MA).

p53 DNA-binding activity. Binding of p53 to its consensus DNA sequence was determined using kits for TransBinding p53 assay and electrophoretic mobility shift assay purchased from Panomics (Fremont, CA).

Small interfering RNA and expression plasmids. Small interfering RNA (siRNA) suppression of BIK was described previously (5). The siRNA suppression kit for p53 (siGENOME human TP53 siRNA kit) was purchased from Dharmacon (Lafayette, CO). Expression plasmids for wild-type (WT) p53 (p53wt), dominant-negative p53 (p53dd; ref. 19), and control vector were gifts from L. Ellisen and S. Maheswaran (Massachusetts General Hospital, Charlestown, MA). Transfection was done using Oligofectamine (Invitrogen).

BIK promoter activity and BIK mRNA stability. Human BIK promoter (20) was amplified by PCR from normal colon genomic DNA and inserted into the pGL3-Basic firefly luciferase reporter plasmid (Promega, Madison, WI). MCF7 cells were transfected with BIK promoter reporter plasmids and a control plasmid expressing the Renilla luciferase (pRL-tk; ref. 21) and incubated in the presence or absence of 100 nmol/L fulvestrant for 48 hours, and expression of reporter genes was determined by the Dual Luciferase assay (Promega). Stability of the BIK mRNA transcripts was examined by determining the time-dependent decrease in BIK mRNA amount after terminating cellular mRNA synthesis with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) by QRT-PCR as described previously (22).

BIK cDNA and exon sequencing. BIK cDNA was synthesized from total RNA of breast cancer cells by RT-PCR as described (5) and subjected to direct sequencing. The four protein-encoding exons and adjacent intronic sequences of human BIK gene (20) were amplified from genomic DNA of breast cancer cells and sequenced.

Statistics. When parametric variants were distributed normally, significance of difference in the means of two groups was determined by Student’s t test. When the nature of the data distribution was unknown, Ps were calculated by Student’s t test, and the statistical significance was further confirmed by Wilcoxon test.

Results

Cytocidal effect of fulvestrant on ER-positive human breast cancer cell lines. To obtain insights into mechanisms of the fulvestrant-induced apoptosis of ER-positive breast cancer cells (5), we examined effects of fulvestrant on growth of MCF7, T47D, and ZR75-1 cells (23). Cell growth curves of fulvestrant-exposed MCF7 cells showed an inverted U shape, reflecting a massive loss of live cells (Fig. 1A, left). In contrast, growth curves of fulvestrant-exposed T47D cells maintained a J shape with up to 50% reduction in cell number compared with vehicle-exposed control (Fig. 1A, right). Growth curves of ZR75-1 cells showed a similar profile as T47D cells (data not shown).

When exposed to fulvestrant for 96 hours, most MCF7 cells were detached from the dish (Fig. 1B, phase contrast) or showed marked plasma membrane blebs (Fig. 1B, high-magnification phase contrast), which are morphologic characteristics of apoptotic cells (24). In contrast, fulvestrant-exposed T47D or ZR75-1 cells did not show any significant morphologic signs of cell death. However, the cytostatic effect of fulvestrant on these cells was evident (Fig. 1B, phase contrast; see the lower cell density compared with vehicle-exposed control). These results indicate that fulvestrant is more cytocidal to MCF7 cells than to T47D or ZR75-1 cells, although it is still significantly cytostatic to the latter two cell lines.

To evaluate the apoptosis-inducing effect of fulvestrant, we detected activation of caspase-3/caspase-7, a late-stage event of apoptosis, by staining cells with the DEVD-fmk caspase inhibitor conjugated with SR fluorescence dye. The DEVD-fmk binds specifically to activated caspase-3 and caspase-7 as an irreversible inhibitor. Therefore, SR dye fluorescence identifies apoptotic cells in live cultures (17). This assay was developed for effective detection of apoptosis of MCF7 cells, which lack caspase-3 but activate caspase-7 during apoptosis (25). As shown in Fig. 1B, DEVD staining, the fulvestrant-exposed MCF7 culture remarkably accumulated SR-DEVD-positive cells (~30% of total cells; see Fig. 1C), confirming that the fulvestrant-induced massive MCF7 cell death was due to apoptosis. In contrast, SR-DEVD-positive cells were rarely observed in fulvestrant-exposed T47D or ZR75-1 cells or in vehicle-exposed controls of all cell lines. These observations indicate that fulvestrant is only weakly cytocidal, if at all, to T47D and ZR75-1 cells. Exposure to 1 μmol/L paclitaxel induced marked cell death in MCF7, T47D, and ZR75-1 cultures with comparable efficacies, and T47D and ZR75-1 accumulated about 10% to 15% SR-DEVD-positive cells in this condition (Fig. 1B and C). Therefore, the absence of SR-DEVD-positive cells in fulvestrant-exposed T47D and ZR75-1 cultures was not due to general resistance of these cells to apoptosis or lack of activation of caspase-3/caspase-7 during apoptosis. The apoptotic nature of the fulvestrant-induced MCF7 cell death was further confirmed using z-VAD-fmk, a pan-caspase inhibitor that prevents cells from apoptosis but not from other types of cell death (26). In the presence of z-VAD-fmk, the fulvestrant-induced MCF cell death was suppressed almost completely (Fig. 1D).

Doxorubicin and γ-irradiation induces both BIK and PUMA mRNA in MCF7 cells, whereas fulvestrant induces only BIK mRNA. To obtain insights into the mechanism of the fulvestrant-induced apoptosis, we determined the time-dependent aspects of the fulvestrant-induced expression of BIK mRNA. When MCF7 cells were exposed to fulvestrant, the amount of BIK mRNA remained low for 12 hours, increased by ~25-fold at 24 to 36 hours, and then decreased (Fig. 2A, left, solid circle). Amount of BIK mRNA in the vehicle-exposed culture slightly increased when medium was changed with 48-hour intervals (Fig. 2A, left, open circle). Because BIK mRNA is strongly induced in an estrogen-deficient culture condition (5, 11), this increase was likely due to consumption of limited amounts of estrogens in the medium by MCF7 cells. In agreement with this interpretation, amount of BIK mRNA in the control culture did not increase when medium was changed more frequently with 24-hour intervals (Fig. 2A, left, square). In contrast, expression of mRNA for estrogen-inducible gene WISP2 (11) was strongly suppressed by fulvestrant (Fig. 2A, right, solid circle).
Figure 1. Fulvestrant-induced growth suppression and apoptosis of human breast cancer cells. A, effects of fulvestrant on growth of MCF7 and T47D cells. Cells were inoculated in 96-well plates (2,000 per well) and incubated for 36 hours without fulvestrant. Then, exposure to 0, 10, or 100 nmol/L fulvestrant was initiated by medium change (arrows). Thereafter, culture medium was changed every 48 hours, maintaining the same concentrations of fulvestrant. Relative cell numbers in each well were determined by CyQuant assay. Points, mean (n = 8); bars, SE. B, breast cancer cell apoptosis induced by fulvestrant or paclitaxel. Cells were exposed to 100 nmol/L fulvestrant for 96 hours, 1 μmol/L paclitaxel for 48 hours, or 0.1% vehicle (ethanol) for 96 hours. Apoptotic cells were identified by red fluorescence of SR-DEVD staining. The phase-contrast image shows the plasma membrane blebbing of fulvestrant-exposed apoptotic MCF7 cells. C, quantitative representation of apoptotic cells shown in (B). Ratios of the apoptotic cells were determined by counting 300 cells per field and calculating mean (columns) and SE (bars) of six fields. *, P = 1 × 10^{-13}; **, P = 8 × 10^{-17}; and #, P = 0.013. D, z-VAD-fmk suppression of fulvestrant-induced MCF7 cell apoptosis. MCF7 cells were exposed to 100 nmol/L fulvestrant for 96 hours in the presence or absence of 50 μmol/L z-VAD-fmk. The phase-contrast image shows massive round-shaped dead cells exposed to fulvestrant in the absence of z-VAD-fmk.
BIK mRNA is inducible by γ-irradiation and doxorubicin, both of which activate the transcription factor activity of p53 (15). To obtain insights into whether fulvestrant induces BIK mRNA in MCF7 cells through the same mechanism as the γ-irradiation induction of BIK mRNA, we compared the time-dependent aspects of induction of the mRNA transcripts for BIK and PUMA after exposure to fulvestrant and γ-irradiation (Fig. 2B). PUMA is another BH3-only protein and its mRNA is inducible by the transcription factor activity of p53 (27, 28). In MCF7 cells, amounts of the mRNA transcripts for both BIK and PUMA increased by ~7-fold at about 6 hours after γ-irradiation and then decreased, implying that γ-irradiation induces both BIK and PUMA mRNA through a common mechanism. On the other hand, fulvestrant induction of BIK mRNA was weak at 6 hours but remarkably strong at 24 hours. However, in contrast to γ-irradiation, fulvestrant did not significantly induce PUMA mRNA at either time point. Therefore, the mechanism of the fulvestrant induction of BIK mRNA does not simultaneously induce PUMA mRNA. These results suggest that fulvestrant induces BIK mRNA through a mechanism different from that of γ-irradiation induction of BIK mRNA. Doxorubicin also induced BIK mRNA in MCF7 cells (Fig. 2C). Fulvestrant induced BIK mRNA far more strongly than doxorubicin after 42-hour exposure. In contrast, paclitaxel, a chemotherapeutic agent that induces apoptosis of breast cancer cells in a manner independent of their p53 status (29), also induced MCF7 cell apoptosis but without inducing BIK mRNA expression. Therefore, the induction of the BIK mRNA is not a nonspecific consequence of activation of the apoptotic pathway.

To examine whether fulvestrant induces BIK mRNA through activating the transcriptional function of p53, we determined effects of fulvestrant on the DNA-binding activity of p53 in MCF7 cells. As shown in Fig. 2D, nuclear extract prepared from MCF7 cells exposed to doxorubicin contained significantly greater amount of transcriptionally active p53 than vehicle-exposed control, agreeing with previous reports (27, 30). In contrast, nuclear extract of fulvestrant-exposed MCF7 cells did not show any evidence of increase in the transcriptionally active p53. The absence of enhancement of the DNA-binding activity of p53 by fulvestrant was also confirmed by gel shift assay using an oligonucleotide probe for p53 binding (data not shown).

We confirmed that the BIK mRNA induction by doxorubicin or γ-irradiation was dependent on p53 by siRNA suppression of p53 expression (Fig. 2E). When expression of p53 was specifically and effectively suppressed by siRNA, doxorubicin or γ-irradiation did not induce PUMA mRNA. Control siRNA did not affect the PUMA mRNA induction (compare Fig. 2E with Fig. 2B and C). In the presence of p53 siRNA, γ-irradiation did not induce BIK mRNA at

Figure 2. Expression of the BIK, PUMA, and WISP2 mRNA transcripts in MCF7 cells. A, time course of fulvestrant effects on expression of the mRNA transcripts for BIK (left) and WISP2 (right). Subconfluent cells were exposure to 100 nmol/L, fulvestrant (●) or 0.1% vehicle (ethanol; ○ and □). Medium was changed every 24 (●) or 48 (○ and □) hours, maintaining the fulvestrant concentrations. At the indicated time points, amounts of the BIK and WISP2 mRNA transcripts were determined by real-time quantitative reverse transcription PCR (QRT-PCR). Results of a typical experiment. Points, mean of three measurements; bars, SE. Reproducibility was confirmed by three independent experiments. Note that amount of the WISP2 mRNA is shown in log-scale. B, induction of the BIK and PUMA mRNA transcripts by γ-irradiation or fulvestrant. Cells were exposed to γ-ray (1 Gy), fulvestrant (100 nmol/L), or vehicle. After the indicated periods, amounts of the BIK and PUMA mRNA transcripts were determined by Taqman QRT-PCR. Columns, mean of three independent experiments; bars, SE. C, effects of doxorubicin, paclitaxel, and fulvestrant on expression of the BIK mRNA transcripts. Cells were exposure to doxorubicin (1 μmol/L), paclitaxel (1 μmol/L), fulvestrant (100 nmol/L), or vehicle. Culture medium was changed every 24 hours, maintaining the fulvestrant concentrations. After the indicated periods, amounts of the BIK mRNA transcripts were determined by Taqman QRT-PCR. Columns, mean of three independent experiments; bars, SE. D, effects of fulvestrant and doxorubicin on the DNA-binding activity of p53 in MCF7 cells. Nuclear extracts were prepared from cells exposed to fulvestrant (100 nmol/L) or doxorubicin (1 μmol/L) for indicated periods. p53 protein binding to the consensus binding sequence of oligonucleotide DNA was determined by TransBinding assay. Columns, mean of two separate determinations; bars, range. E, effects of siRNA suppression of p53 on induction of the BIK and PUMA mRNA transcripts by γ-irradiation or doxorubicin. Cells were exposed to γ-ray (12 Gy), doxorubicin (1 μmol/L), or vehicle. Amounts of the BIK and PUMA mRNA transcripts were determined 48 hours after exposure by Taqman QRT-PCR. Results of a typical experiment. Reproducibility was confirmed by repeated experiments.
all, and doxorubicin induction of BIK mRNA was also strongly diminished. The suppressive effects of p53 siRNA knockdown on mRNA expression were specific to p53, BIK, and PUMA because expression of β-actin mRNA was not affected (data not shown).

**p53 is required for the fulvestrant induction of BIK expression and apoptosis in MCF7 cells.** We next determined whether siRNA suppression of p53 expression affects the fulvestrant induction of BIK mRNA (Fig. 3A). Expression of p53 mRNA in MCF7 cells was effectively suppressed by siRNA in the presence or absence of fulvestrant (Fig. 3A, left, black columns). Fulvestrant did not significantly affect p53 mRNA expression in cells transfected with sequence-scrambled control siRNA (Fig. 3A, left, white columns). BIK mRNA was strongly induced by fulvestrant in cells transfected with control siRNA (Fig. 3A, right, white columns). Interestingly, in the presence of p53 siRNA, fulvestrant induction of BIK mRNA was dramatically reduced (Fig. 3A, right, black columns). Because expression of β-actin mRNA was not affected by p53 siRNA in the presence or absence of fulvestrant (data not shown), this effect was not due to nonspecific suppression of mRNA synthesis. As expected from the absence of BIK mRNA induction, siRNA suppression of p53 also effectively inhibited fulvestrant-induced MCF7 cell death (Fig. 3B). This observation eliminated a possibility that nonspecific toxicity of siRNA suppression of p53

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**Figure 3.** Effects of p53 suppression on the fulvestrant induction of BIK and apoptosis in MCF7 cells. A, effects of siRNA suppression of p53 expression on the fulvestrant induction of the BIK mRNA transcripts. Cells were transfected with control or p53 siRNA for 24 hours and then exposed to 100 nmol/L fulvestrant or vehicle for additional 24 hours. Amounts of the mRNA transcripts for p53 (left) and BIK (right) were determined by real-time QRT-PCR. Columns, mean (n = 3); bars, SE. B, effects of siRNA suppression of p53 expression on the fulvestrant-induced MCF7 cell death. Cells were transfected with control or p53 siRNA and then exposed to 100 nmol/L fulvestrant or vehicle for 72 hours. Dead cells, which were identified by their round morphology and detachment from the plastic culture dish, and live cells were counted manually using a phase-contrast microscope to calculate the percentage of live cells. Columns, mean (n = 3); bars, SE. C, effects of overexpression of p53wt and p53dd on the fulvestrant induction of the BIK mRNA transcripts. Cells were transfected with expression plasmids for p53wt, p53dd, or control vector and then exposed to 100 nmol/L fulvestrant or vehicle for 48 hours. Amounts of the BIK mRNA transcripts were determined by real-time QRT-PCR. Columns, mean (n = 3); bars, SD. D, suppression of fulvestrant induction of BIK protein by p53 siRNA. Cells were transfected with control (con) or p53 siRNA and then exposed to 100 nmol/L fulvestrant for 48 hours. Expression of p53, BIK, PUMA, and actin was evaluated by immunoblotting.

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**Figure 4.** Absence of fulvestrant effects on the transcriptional activity of the proximal human BIK gene promoter and the stability of the BIK mRNA transcripts in MCF7 cells. A, genomic DNA sequence around BIK transcription initiation site. Numbers are nucleotide positions from the transcriptional initiation site (+1 transcription initiation site). Capitalized and underlined, sequence-scrambled control siRNA (Fig. 3, left, white columns). BIK mRNA was dramatically reduced (Fig. 3, right, black columns). Because expression of β-actin mRNA was not affected by p53 siRNA in the presence or absence of fulvestrant (data not shown), this effect was not due to nonspecific suppression of mRNA synthesis. As expected from the absence of BIK mRNA induction, siRNA suppression of p53 also effectively inhibited fulvestrant-induced MCF7 cell death (Fig. 3B). This observation eliminated a possibility that nonspecific toxicity of siRNA suppression of p53...
reduced cell viability and mRNA synthesis. These results suggest that the fulvestrant induction of BIK expression and apoptosis in MCF7 cells require p53.

To obtain further evidence of the importance of p53 in the fulvestrant induction of BIK, MCF7 cells were transfected with p53wt or p53dd and then exposed to fulvestrant. The p53dd mutant is defective of DNA-binding activity (19). In cells transfected with control vector or p53wt, fulvestrant increased amount of BIK mRNA by 4.5-fold. However, in cells transfected with p53dd, the fulvestrant induction of BIK mRNA was significantly suppressed, resulting in only 2.2-fold increase. The relatively modest induction of BIK mRNA by fulvestrant in this experiment was related to the transfection procedure. These results supported the notion that p53 plays significant roles in the fulvestrant induction of BIK mRNA.

To obtain additional evidence of the importance of p53 in the fulvestrant induction of BIK, we determined effects of siRNA suppression of p53 on expression of BIK protein in MCF7 cells (Fig. 3D). In cells transfected with control siRNA, fulvestrant strongly induced BIK protein. Expression of PUMA was not affected by fulvestrant, as expected from the absence of PUMA mRNA induction (see Fig. 2B). siRNA suppression of p53 resulted in a remarkable reduction in PUMA protein expression without affecting expression of β-actin, confirming the specific and efficient suppression of p53. Under these conditions, induction of BIK protein by fulvestrant was abolished almost completely by siRNA suppression of p53. Taken together, these results indicate that p53 is required for the fulvestrant induction of BIK mRNA and protein as well as apoptosis in MCF7 cells.

Fulvestrant did not affect transcriptional activity of a proximal BIK promoter or BIK mRNA stability in MCF7 cells. We next attempted to determine whether fulvestrant activates transcriptional activity of the human BIK promoter by reporter assay. To our surprise, a firefly luciferase reporter plasmid for a 2.0-kb BIK promoter ending at the TATA-less transcription initiation site reported by Verma et al. (Fig. 4A, dashed arrow; ref. 20) was totally inactive in MCF7 cells in the presence or absence of fulvestrant (Fig. 4B, ATATA). In contrast, when this BIK promoter was extended downstream by 150 nucleotides to include the TATA-controlled transcriptional initiation site that was deduced from the RefSeq sequence of human BIK mRNA (NM_001197; Fig. 4A, solid arrow), this extended promoter showed very strong transcriptional activity, although no fulvestrant effect was observed (Fig. 4B, +TATA).

We next attempted to determine effects of fulvestrant on the intracellular stability of BIK mRNA in MCF7 cells. After exposing cells to fulvestrant, E2, or vehicle, mRNA synthesis was blocked by DRB, an inhibitor of RNA polymerase II-dependent transcription, and time-dependent decrease in the amount of BIK mRNA was determined by QRT-PCR (22). As shown in Fig. 4C, the log-scale plotted mRNA decay showed comparable slopes in the presence of estradiol or fulvestrant, indicating that estrogen does not affect stability of the BIK mRNA transcript. Taken together, these results suggested that fulvestrant induces BIK mRNA in MCF7 cells primarily enhancing transcription by affecting cis-elements located outside the 2.0-kb proximal promoter.

WT BIK mRNA is constitutively expressed in ZR75-1 cells. We then attempted to determine whether the resistance of T47D and ZR75-1 cells to the cytocidal action of fulvestrant is related to different strength of BIK expression in these cells. Expression of BIK mRNA in T47D cells was weak and unaffected by fulvestrant (Fig. 5A). BIK mRNA expression in SKBr3 ER-negative human breast cancer cells was also weak and unaffected by fulvestrant. MB435, which had been considered previously as a highly aggressive breast cancer cell line but revealed recently to be a
melanoma cell line (31), did not express BIK mRNA. However, ZR75-1 cells expressed BIK mRNA very strongly and constitutively even in the absence of fulvestrant (Fig. 5A). Among these cell lines examined, only MCF7 and ZR75-1 cells express p53wt (32). Therefore, these observations may imply that p53 is necessary for strong expression of BIK mRNA in breast cancer cells, although the reason of the strong expression of BIK mRNA in ZR75-1 cells even in the absence of antiestrogen is unknown.

Nucleotide sequencing of BIK cDNA prepared from MCF7, T47D, ZR75-1, and SKBr3 cells did not show any mutations or polymorphisms within the 483-nucleotide open reading frame. Genomic DNA sequences of all four protein-coding exons and adjacent intronic sequences of BIK gene (20) in 11 human breast cancer cell lines (MCF7, BT-483, HS467, HS578, MDA157, MDA175, MDA231, MDA415, MDA436, MDA453, and MDA468) did not show mutations either.

**BIK protein is rapidly degraded in breast cancer cells by a proteasome-dependent mechanism.** We next determined amounts of BIK protein in breast cancer cell lines. Despite their strong expression of BIK mRNA, ZR75-1 cells expressed only minimal amounts of BIK protein in the presence or absence of fulvestrant (Fig. 5B). T47D or SKBr3 did not express significant amounts of BIK protein either. Thus, the fact that only MCF7 cells strongly express BIK protein after exposure to fulvestrant provides an explanation for the exceptionally high sensitivity of this cell line to the cytocidal action of fulvestrant.

Because it has been reported that BIK is degraded by proteasome-dependent mechanism (33–35), we attempted to determine whether proteasomal degradation of BIK prevents ZR75-1 cells from BIK-dependent apoptosis. When breast cancer cells were exposed to MG132, a wide-spectrum proteasome inhibitor (36), significant accumulation of BIK protein was observed in all cells (Fig. 5B). In MCF7 cells, exposure to MG132 alone led to a modest accumulation of BIK protein, and this induction was dramatically augmented by coexposure to fulvestrant. In T47D, ZR75-1, and SKBr3 cells, exposure to MG132 alone resulted in significant accumulation of BIK protein, but in these cells, coexposure to fulvestrant showed no effect. The actin-normalized amount of BIK protein expressed in the presence of MG132 was greatest in ZR75-1 cells and smallest in T47D cells, agreeing with the amounts of BIK mRNA shown in Fig. 5A. These results strongly suggest that BIK protein is actively synthesized in breast cancer cells but rapidly degraded by a proteasome-dependent mechanism, thus preventing cells from BIK-induced apoptosis.

Interestingly, whereas the fulvestrant-induced BIK protein expressed in MCF7 cells in the absence of MG132 always appeared as a single band on immunoblotting (Fig. 5D, lanes 2 and 7), BIK induced in the presence of both fulvestrant and MG132 always appeared as 23/24-kDa double bands (Fig. 5D, lanes 6 and 9). The MG132-induced BIK protein in ZR75-1 and SKBr3 cells also appeared as double bands (Fig. 5B). Bortezomib, another potent proteasome inhibitor (37–39), induced BIK protein in the same manner as MG132 (Fig. 5D, lanes 3 and 4). These observations may suggest that BIK is converted from the 24-kDa form to the 23-kDa form before its degradation by the proteasome, implying the possible importance of post-translational protein modifications in the regulation of BIK protein degradation.

**BIK plays a significant role in proteasome inhibitor-induced apoptosis of ZR75-1 cells.** The possible application of proteasome inhibitors for the treatment of breast cancers has been explored recently (39). Because MG132 caused massive death of ZR75-1 cells, we attempted to determine whether BIK plays a significant role in this phenomenon. As shown in Fig. 6A, MG132-induced expression of BIK protein in ZR75-1 cells was effectively and specifically suppressed by siRNA. In this experimental condition involving
transient transfection procedure, BIK protein induced by MG132 often appeared as a single band for unknown reasons. After transfection of siRNA, cells were exposed to MG132 or vehicle for 48 hours, and their viability was determined by phase-contrast microscopy (Fig. 6B). ZR75-1 cells transfected with control siRNA were killed by MG132 very effectively. In contrast, cells transfected with BIK siRNA were significantly resistant to the cytoidal action of MG132. These results indicate that BIK protein plays a significant role in the cytoidal action of proteasome inhibitors in ZR75-1 cells.

Discussion

The pure antiestrogen fulvestrant is significantly cytoidal to ER-positive human breast cancers (1–6). Whereas antiestrogen induction of apoptosis has been extensively characterized with MCF7 cells, reports on other cell lines have been few. Our present study showed that MCF7 cells were highly sensitive to the cytoidal action of fulvestrant, whereas T47D and ZR75-1 cells were resistant to it (Fig. 1). Importantly, however, T47D and ZR75-1 cells were still sensitive to the cytoidal action of fulvestrant (Fig. 1B). A few previous studies reported apoptosis of T47D and ZR75-1 cells in the presence of 100 nmol/L to 10 μmol/L fulvestrant (i.e., 10–1,000 times higher concentrations than our experimental condition) and charcoal/dextran-stripped serum (40, 41). Because these cells are highly sensitive to serum deprivation (41), it is conceivable that they might become sensitive to the cytoidal action of high concentrations fulvestrant when growth factors in the stripped serum is limited. Although Bouker et al. (6) reported fulvestrant-induced apoptosis of MCF7, T47D, and ZR75-1 cells, the relative sensitivities of these three cell lines were not directly compared. Because expression of IRF-1, a transcription factor that plays critical roles in the fulvestrant-induced breast cancer cell apoptosis, was at least 8-fold lower in fulvestrant-exposed T47D cells than MCF7 cells, the cytoidal action of fulvestrant could be far weaker in T47D cells than in MCF7 cells. Methods of detecting apoptotic cells might also have contributed to the apparent difference between our and previous studies. Because MCF7 cells lack caspase-3 (25), their apoptosis-induced DNA fragmentation is not robust, making it difficult to precisely evaluate MCF7 cell apoptosis by TUNEL assay (42). In our hands, the sensitivity and credibility of detecting apoptosis by counting cells with subnormal amounts of genomic DNA content by fluorescence-activated cell sorting was also limited. The Annexin V staining detects one of the earliest apoptotic events (43, 44), whereas the SR-DEVD-fmk staining (Fig. 1B) detects late-stage events (17). Our present study used the SR-DEVMD method, which may have lower sensitivity than Annexin V staining but have better specificity through directly detecting activation of the executor caspases (17).

It has been reported previously that BIK mRNA was inducible in human KB epithelial cells by doxorubicin or γ-irradiation as well as by overexpression of p53 (15). Although these results suggested that induction of BIK mRNA by doxorubicin or γ-irradiation may be dependent on p53, this has been remained to be proven. Our present study showed that specific suppression of p53 expression by siRNA resulted in remarkable loss of BIK mRNA induction by these stimuli in MCF7 cells (Fig. 2E), providing strong support for this notion. Our study also showed that p53 is required for the fulvestrant induction of BIK mRNA in MCF7 cells and subsequent apoptosis (Fig. 3). Importantly, however, the p53-dependent mechanism of the fulvestrant induction of BIK mRNA in MCF7 cells appears different from another p53-dependent, BIK-inducing mechanism, which is activated by doxorubicin or γ-irradiation and involves the DNA-binding transcription factor activity of p53. The following results support this interpretation. First, siRNA suppression of p53 expression abolished the fulvestrant induction of BIK mRNA and protein as well as apoptosis (Fig. 3A, B, and D). Second, transfection of p53dd significantly suppressed the fulvestrant induction of BIK mRNA (Fig. 3C). Third, whereas both BIK and PUMA mRNA transcripts were quickly induced by γ-irradiation with the same time course, fulvestrant induced BIK mRNA but not PUMA mRNA (Fig. 2B), which is inducible by doxorubicin or γ-irradiation through a mechanism dependent on the transcription factor activity of p53 (27). Fourth, whereas doxorubicin induced BIK mRNA (Fig. 2C) and concomitantly activated the DNA-binding activity of p53 (Fig. 2D), fulvestrant did not activate this p53 function (Fig. 2D). We also showed that the intracellular stability of the BIK mRNA transcripts in MCF7 cells was not affected by estrogen or antiestrogen (Fig. 4C) and that a 2.0-kb proximal promoter of human BIK gene was not activated by fulvestrant in MCF7 cells (Fig. 4B). These results suggest that fulvestrant induces BIK mRNA in MCF7 cells by enhancing transcription through cis-elements located outside the 2.0-kb proximal promoter without changing the BIK mRNA stability. The precise mechanism that p53 enhances BIK gene transcription without acting as a DNA-binding transcription factor remains to be elucidated by future studies.

The importance of the BH3-only proapoptotic proteins in cancer therapy has become increasingly evident, and a wide variety of transcriptional and post-translational mechanisms that regulate their expression and activity have been reported (10, 45). Proteasomal protein degradation plays a critical role in the regulation of BIM (33, 46–48), NOXA (49, 50), and BIK (33–35). Our present study showed that proteasome inhibitors caused rapid accumulation of BIK protein in human breast cancer cell lines and subsequently induced apoptosis (Fig. 5). Importantly, by specifically suppressing BIK protein accumulation by siRNA in proteasome-exposed cells, we showed that the proteasome-induced death of ZR75-1 cells was largely dependent on BIK (Fig. 6). Future studies involving in vivo models, such as tumor xenografts in nude mice, are desired to explore the potential usefulness of manipulating BIK expression in human breast cancer as a therapeutic strategy.

In summary, we have shown that p53 is necessary for the fulvestrant induction of BIK mRNA, protein, and apoptosis in MCF7 cells. However, whereas doxorubicin or γ-irradiation induces BIK mRNA through activating the DNA-binding transcription factor activity of p53, fulvestrant induction of BIK mRNA seems independent of this p53 function. In ZR75-1 cells, BIK mRNA is constitutively expressed, but cells evade apoptosis by effectively degrading BIK protein through a proteasome-dependent mechanism. The cytoidal action of proteasome inhibitors on ZR75-1 cells was largely dependent on accumulation of BIK protein. Thus, expression of BIK proapoptotic protein in human breast cancer cells is regulated at both transcriptional and post-translational levels. Mechanisms involved in these regulations may be important drug targets that could increase cancer cell apoptosis and/or sensitivities to other types of therapies through induction of BIK.

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