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Targeting the NF-κB Pathway in Estrogen Receptor Negative MDA-MB-231 Breast Cancer Cells Using Small Inhibitory RNAs

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Abstract Cancer cells in order to survive are often mutated to block apoptosis. One chemotherapeutic option is the re-establishment of apoptosis. An example of such a therapy is the PKC inhibitor Gö6976, which activates apoptosis and shrinks in vivo tumors in estrogen receptor-negative breast cancers. We proposed as a mechanism blockage of activation of the transcription factor NF-κB, which is anti-apoptotic and often elevated in cancers. Over recent years, questions have arisen regarding the specificity of these ''small-molecule inhibitors.'' We have therefore explored the role of NF-κB inhibition in MDA-MB-231 breast cancer cells using small inhibitory RNAs (siRNA). siRNAs designed against NF-κB protein p65 (RelA) and IKKα, IKKβ, and IKKγ, strongly decreased the target proteins. But, unlike Gö6976, they did not decrease basal NF-κB or cause apoptosis. In particular, the decrease in p65 protein had no effects on apoptosis or cell proliferation, thus questioning the importance of NF-κB alone in the maintenance of these cells. Furthermore, the proteasome inhibitor MG-132 caused loss of IκBα, and an increase of it is phosphorylated form, but basal NF-κB was unchanged, whilst activation of NF-κB by TNFα was completely inhibited, suggesting that MG-132 activity is independent of constitutive NF-κB activation. We ascribe these differences to the specificity of inhibition by siRNAs as compared to the well-known non-specificity of small-molecule inhibitors. We conclude that the mutations in these cancer cells made them resistant to apoptosis, by elevating their NF-κB and activating other basal pathways that are blocked by Gö6976 but not by IKK and p65 siRNAs. J. Cell. Biochem. 98: 221–233, 2006.

Key words: nuclear factor κB (NF-κB); inhibitor of κB Kinase (IKK); siRNA; breast cancer

A variety of mutations, which prevent apoptosis and promote cell growth arise in cancers. These frequently include tumor suppressor genes p53, retinoblastoma protein (Rb) and adenomatous polyposis coli (APC), and oncogenes such as phosphoinositide-3 kinase (PI3K), AKT, and cyclin D1 [Vogelstein and Kinzler, 2004]. Defects in the apoptotic machinery in tumor cells are frequently associated with resistance to chemotherapy [Zhivotovsky and Orrenius, 2003]. The transcription factor nuclear factor-κB (NF-κB) can be constitutively highly expressed in cancer cells and promotes tumorogenicity [Lin and Karin, 2003; Shishodia and Aggarwal, 2004], both inhibiting apoptosis (e.g., Bcl-x_L, Survivin) and stimulating proliferation (e.g., cyclin D1). Constitutive NF-κB activity has been implicated in the development of estrogen receptor negative (ER−) breast cancers, which promotes an invasive and metastatic phenotype [Nakshatri and Goulet, 2002]. In particular, the p65 (RelA)/p50 heterodimer is predominant [Sovak et al., 1997]. This transcription factor thus provides a therapeutic target whose inhibition can result in the shift of balance away from growth towards apoptosis [Monks et al., 2004]. As an example, activation of apoptosis by Gö6976, an inhibitor of protein kinases C (PKC) α and β, caused dramatic shrinkage of tumors grown from ER- mouse mammary epithelial cells (CSMLO) cells implanted in A–J mice [Biswas et al., 2001]. This therapy is specific because it affects a process unique to these cancer cells, which kills

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cells rather than reversibly blocking proliferation. Gö6976 activates apoptosis of ER− and epidermal growth factor receptor-1 (EGFR-1) overexpressing breast cancer cells, but not of ER+ cells [Biswas et al., 2000] whose NF-κB activity is inhibited in the presence of estrogen [Nakshatri et al., 1997]. Subsequent dissociation and proteolysis of the inhibitor of κB (IκB) allows nuclear translocation of NF-κB and transactivation of its target genes [Karin, 1999]. The mechanism proposed was that Gö6976, through the inhibition of PKC, inhibits the signal from EGFR-1 to NF-κB. The proposed pathway utilizes the inhibitor of κB kinase (IKK) complex (IKK-α, -β, -γ) that phosphorylates IκB, the inhibitor of NF-κB. Thus inhibition of these signals culminates in the decrease of active NF-κB.

There are two main problems with this model. First, Gö6976 is not a specific inhibitor of just PKC α and β, rather it has been shown to inhibit other kinases [Davies et al., 2000]. Second, numerous other kinases and pathways other than the IKK complex have been reported to phosphorylate both IκB and NF-κB, resulting in the activation of gene transcription [Chen et al., 2002a]. Casein kinase II (CK2) which phosphorylates multiple serine/threonine sites at the C-terminus of IκBα [McElhinny et al., 1996], is associated with elevated nuclear NF-κB levels in breast cancer cell lines and primary tumors [Romieu-Mourez et al., 2001], and mediates NF-κB activation in response to UV [Kato et al., 2003]. Other kinases implicated in NF-κB activation include PI3 kinase [Kang et al., 2003], Nemo (IKKγ)-independent NF-κB activation by lymphotoxin-β receptor [Saitoh et al., 2002], and calpain in Her-2/neu overexpressing cells [Pianetti et al., 2001]. An unknown kinase alongside IKKα, IKKβ, IKKε, and TANK-binding kinase 1 (TBK1) mediates p65 serine 536 phosphorylation [Buss et al., 2004]. Acetylation of p65/RelA has also been shown to be involved in the regulation of NF-κB activity [Chen et al., 2002b].

Two approaches are used here to investigate the role of NF-κB activation in ER− MDA-MB-231 breast cancer cells in vitro. siRNAs were designed and applied to decrease production of p65 (RelA) which is a component of the predominant NF-κB heterodimer in breast cancer, and the IKK proteins -α, -β, and -γ, central components of the IKK-based process. They specifically blocked production of the target proteins; but their effects on both constitutive basal NF-κB and on its increased activation by tumor necrosis factor-α (TNFα) were minimal. The second approach was to prevent proteolysis of IκB by applying the proteasome inhibitor MG-132, which decreases NF-κB activation by inhibiting IκB degradation [Amit and Ben-Neriah, 2003]. The objectives of these studies are not to provide new insights into the regulation of NF-κB and it's function, rather we have approached this study with interest in the utility of NF-κB as a therapeutic target and to gain further understanding of it's role in the ER− MDA-MB-231 breast cancer cell line. The two approaches used to inhibit the NF-κB pathway did not mirror the effects seen with the Gö6976 kinase inhibitor. The complex nature of NF-κB signaling and activation, along with the inherent shortcomings of small-molecule kinase inhibitors are discussed.

MATERIALS AND METHODS

Materials and Cell Culture

MDA-MB-231 breast cancer cells were obtained from the American Type Culture collection (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) with Glutamax, Opti-MEM®I reduced serum medium, Trypsin-EDTA (10×) penicillin-streptomycin (100×), and phosphate buffered saline, pH 7.4 (PBS) were purchased from GIBCO (Carlsbad, CA). Fetal Bovine Serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA). siRNA smartpools and the scrambled siRNA oligo were obtained from Dharmacon Research (Lafayette, CO). Custom designed siRNA oligos were synthesized by QIAGEN (Valencia, CA). The design of the individual siRNA duplexes is summarized in Table I. Oligofectamine transfection reagent was purchased from Invitrogen

TABLE I. Summary of siRNAs Used in This Study

Gene	siRNA duplex
Scrambled	5′-AUGAACGUGAAUUGCUCAAUU UUUACUUGCACUUAACGAGUU-5′
IKKα	5′-GCAGGCUCUUUCAGGGACATT TTCGUCCGAGAAAGUCCCUGU-5′
IKKβ	5′-GGUGGAAGAGGUGGUGAGCTT TTCCACCUUCUCCACCACUCG-5′
IKKγ	Dharmacon Cat No. D-003767-01
P65	5′-GCCCUAUCCCUUUACGUCATT TTCGGGAUAGGGAAAUGCAGU-5′

(Carlsbad, CA). MG-132 and human recombinant Tumor Necrosis Factor-α (TNF-α) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

RNA Interference (siRNA)

Exponentially growing, sub-confluent, MDA-MB-231 cells were seeded in antibiotic-free DMEM + 10% FBS at a density of 5.5×10^4 cells/ml (6 well plate = 2 ml and 10 cm culture dish = 12 ml). The following methods are for the preparation of siRNA oligos in a 6-well plate (the quantities for 10 cm culture dishes were sixfold greater). All siRNA studies where performed in the absence of antibiotics. Two μl of siRNA oligo (20 nM stock) were diluted in 178 μl of Opti-mem, and 4 μl Oligofectamine was diluted in 16 μl of Opti-MEM and left to equilibrate at room temperature for 10 min. The siRNA dilution was then added to the Oligofectamine tube, mixed by gentle pipeting and complexes allowed to form for 20 min. During complex formation, the medium was aspirated from the wells and the cells washed once with 2 ml of serum free DMEM medium, followed by the addition of 800 μl of serum free DMEM. Following complex formation, the siRNA/oligofectamine complexes were added to the cells, mixed gently and incubated for 4 h at 37°C. Subsequently, 1 ml of DMEM + 20% FBS was added to each well and the cell incubated for a further 68 h, unless otherwise stated.

Immunoblotting

Cells treated with different agents were washed twice with ice-cold PBS. Cells were lysed without trypsinization for 30 min at 4°C with gentle shaking using extraction buffer containing 150 mM NaCl, 50 mM Tris.Cl, pH 8.0, 1% NP-40, 0.5% DOC, 0.1% SDS, and 0.02% sodium azide and 40 μl/ml of complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Samples were passed through a 25 gauge needle 20 times, and the lysate collected following centrifugation at 12,000*g* for 5 min at 4°C. Protein concentrations were determined using the Bio-Rad *DC* protein assay (Bio-Rad, Hercules, CA). Equal amount of protein (60 μg/lane) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to PROTRAN nitrocellulose membrane (Schleicher & Schuell Bioscience GmbH, Keene, NH). The membranes were incubated with antibodies to IKKα, IKKβ, IKKγ, phospho-IκBα (recognizing phosphorylated serines 32 and 36) and p65 (RelA) (Oncogene Research Products, Boston, MA), IκBα, (Cell Signaling Technology, Beverly, MA) in 5% non-fat milk (Bio-Rad). After washing with TBS-Tween the membranes were incubated with peroxidase-conjugated goat anti-mouse or donkey anti-rabbit antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in 5% non-fat milk, followed by visualization using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ). β-Actin (Sigma) was used to confirm equal protein loading.

Nuclear Extract Preparation and NF-κB Binding

Nuclear extracts were prepared from 10 cm culture plates using the Nuclear Extract Kit purchased from Active Motif (Carlsbad, CA) and the manufacturers' instructions. The concentration of nuclear protein was determined using the Bio-Rad Protein Assay kit. Active NF-κB was determined in 3 μg of nuclear protein using the TransAM™ NF-κB p65 Transcription Factor Assay kit (Active Motif) using the manufacturers' instructions.

Cell Growth Assay

Exponentially growing cells were seeded into 6-well plates at 1.1×10^5 cells/well in 2 ml of DMEM + 10% FBS without antibiotics. Twenty-four hours later the cells were treated with siRNA as stated above for 4 h in serum-free conditions. Cells were prepared for counting as follows. The medium was aspirated carefully from the wells and 250 μl of trypsin added, followed by inactivation using 250 μl of DMEM + 10% FBS. Cells were fixed using 500 μl of Carnoy's fixative (3:1 methanol:acetic acid). Cell aggregates were removed by repeated pipeting. 250 μl was then diluted 1:40 in Isoton (Beckman Coulter, Inc., Fullerton, CA), and 500 μl counted in triplicate using a Z1™ Series Coulter Counter (Beckman Coulter); objects below 8 μM were excluded from the analysis. Counts were made every 24 h. The time zero count was made at the time of the siRNA addition.

Growth Inhibition Assay

MDA-MB-231 cells were seeded into 96-well culture plates at 1×10^3 cells/well in 100 μl

of medium. After 24 h, 100 μl of medium containing serial dilutions of MG-132 was added to the cells in triplicate. The plates were incubated with drug for a further 72 h, after which cellular growth was determined using the sulphorhodamine B (SRB) protein dye assay [Skehan et al., 1990]. In short, cells were fixed with 50% trichloroacetic acid (TCA) w/v (50 μl/well) for 1 h at 4°C. Following fixation, the plates were washed 5–6 times in water and stained with SRB (0.4% SRB (w/v) in 1% (v/v) acetic acid) for 30 min at 37°C. Excess stain was removed by washing five times in 1% (v/v) acetic acid. The plates were subsequently air-dried and the protein-bound SRB re-solubilized by the addition of 10 mM Trizma Base, pH 10.5. Colorimetric readings were made at 570 nm. The results are presented as the percent of untreated control absorbance.

Cell-Cycle Analysis

Cells were harvested 72 h after siRNA transfection, washed twice in ice-cold PBS and resuspended in 1 ml of PBS. Three milliliters of ice cold absolute ethanol was added to the cells while vortexing and the cells fixed for at least 1 h at 4°C. Cells were subsequently washed twice with PBS and stained for 3 h at 4°C in 1 ml of propidium iodide (PI) staining solution (50 μg/ml PI in PBS containing 0.5 μg/ml of RNase A). Cell-cycle distribution was analyzed using a FACScan flow cytometer using the CellQuest acquisition software (BD Biosciences, San Jose, CA.). Propidium iodide fluorescence was determined through a 585/42-nm band pass filter. Data analysis was performed with the use of ModFit LT software (Verity Software House, Topsham, ME).

Apoptosis Assay

Cell death was measured by flow cytometry (FACScan) using the Annexin V-FITC Apoptosis detection Kit 1 (BD Pharmingen™). Cells were treated with siRNA in 6-well plates as described previously and analyzed at 24, 48, and 72 h post-siRNA transfection as described in the manufacturers' instructions. As a positive control for apoptosis, MDA-MB-231 cells were treated with 10 μM Doxorubicin for 24 h and analyzed alongside the siRNA-treated cells. The data were analyzed using CellQuest software.

Quantitative RT-PCR

To confirm the effects of siRNA and proteasome inhibitors on the expression of NF-κB target genes, real-time quantitative reverse transcription-PCR was used. mRNA was isolated from cells using the RNeasy kit (QIAGEN) with an on-column DNase 1 digestion to remove DNA contamination as described in the manufacturers' instructions. RT-PCR was performed following a one-step RT-PCR protocol using the QuantiTect™ Gene Expression Assay and QuantiTect™ Probes purchased from QIAGEN. Three genes were analyzed: β-Actin (control), IκBα and A20. PCR was performed using 10 ng of RNA/reaction in a total reaction volume of 50 μl. Reverse transcription and DNA amplification were carried out using the iCycler iQ® Multicolor Real-Time PCR Detection System (Bio-Rad). β-Actin was used as an endogenous reference gene using a probe set pre-designed and validated by QIAGEN. The initial cycles of the PCR, where little variation in fluorescence was observed, were used as the baseline. An increase in fluorescent signal above the baseline indicted PCR amplification. The fluorescence threshold was set above the baseline in the exponential phase of the PCR and the Ct (threshold cycle) calculated for each reaction. Amplification efficiencies for each of the genes tested were calculated from standard curves of serial (10-fold) dilutions of the RNA template. The PCR efficiencies were found to differ between each of the genes and therefore the relative expression levels of the target genes were determined by calculating the relative amounts of RNA from the standard curves followed by normalization to the endogenous reference gene β-Actin. The data are expressed as the ratio to an untreated control sample.

Statistical Analysis

All statistical analyses were performed using Instat software, Version 2.0 (Graphpad Software, Inc., San Diego, CA). Data were analyzed using the paired Tukey multiple comparison test.

RESULTS

siRNA Inhibition

The siRNA treatments, assessed by immunoblot 72 h after transfection, effectively reduced the protein level for each of the genes targeted (Fig. 1A). The 72 h time-point was chosen as the

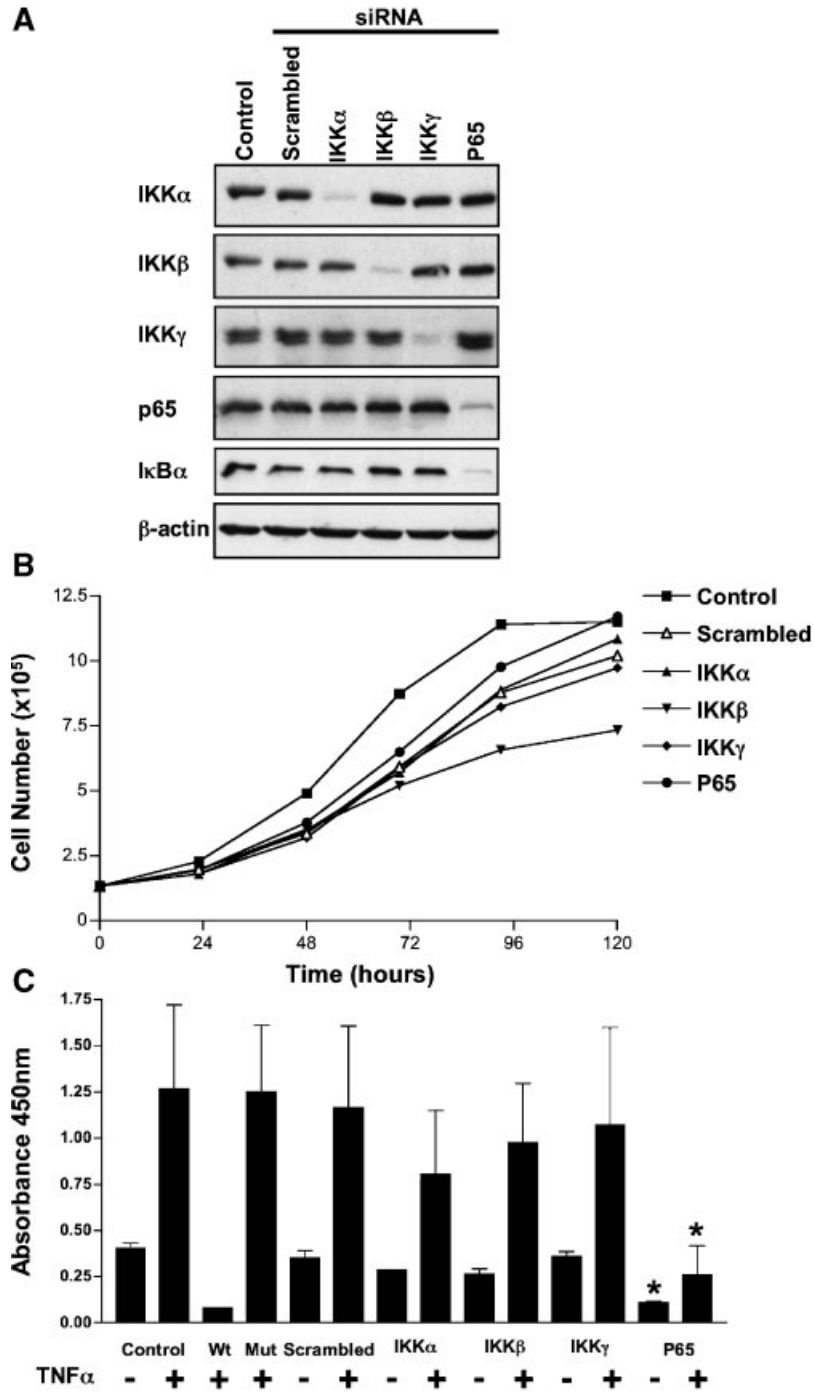

Fig. 1. Effects of siRNA on cellular protein levels and on cell growth. MDA-MB-231 cells were transfected with siRNA oligos in serum free conditions for 4 h. **A**: Whole-cell lysates were taken at 72 h post-transfection and equal amounts (60 μg/lane) of cellular protein were separated on 10% SDS–PAGE gel and transferred to nitrocellulose. The levels of IKKα, IKKβ, IKKγ, p65 and IκBα were determined by Western blot analysis as described under Materials and Methods. β-actin was used as a loading control. **B**: Cell number was determined at 24-h intervals following siRNA transfection (■-Control, △-Scrambled, ▲-IKKα, ▼-IKKβ, ◆-IKKγ and ●-p65) using a Z1 Coulter counter. **C**: Nuclear levels of active NF-κB 72 h post-siRNA transfection. MDA-MB-231 cells were transfected in serum-free medium with siRNAs for 4 h. Nuclear extracts were isolated at 72 h as described in the Materials and Methods. Samples were also exposed to TNFα (5 ng/ml) for 30 min prior to the end of the experiment. Equal amounts of nuclear extracts (3 μg) were analyzed for active NF-κB using an oligonucleotide-specific p65 ELISA assay. Specificity was checked by competition analysis using soluble wild-type (Wt) and mutated (Mut) NF-κB consensus oligonucleotides. The amount of active NF-κB was determined by colorimetric analysis at 450 nm. The data shown are means ± SD of n = 3 experiments. The asterisks indicate data that are significantly different from control (paired Tukey test): $^*P < 0.05$.

reduction in protein expression, determined by immunoblots analysis, was found to be maximal at that time (data not shown). IκBα remained unaffected following each treatment, with the exception of the decrease seen in the p65 siRNA-treated cells that mirrored the reduction in p65 due to the autoregulatory loop that promotes transcription of IκBα and terminates NF-κB activity.

Cell Survival and Growth

Constitutive activation of NF-κB is generally considered to be an element in the promotion of ER-negative breast cancer growth. Therefore, following siRNA transfection net cell growth of MDA-MB-231 was determined using a Coulter Counter (Fig. 1B). p65, IKKα, and IKKγ siRNAs were not important determinants of proliferation; only small differences from the growth of control cells were observed, as with the scrambled non-specific oligo and so are considered to be an effect of transfection rather than of a specific siRNA. IKKβ siRNA treatment slightly decreased growth, and by 120 h the number of cells was about 30% lower than with the other treatments.

Cell-Cycle Analysis Following siRNA Treatment

MDA-MB-231 cells collected 72 h following exposure to different siRNAs were fixed in ethanol, stained with propidium iodide and analyzed by flow cytometry. As shown in Table II, the cell-cycle distribution for most samples did not differ from that of the control or cells treated with the scrambled siRNA. An increase of 25% in G_0/G_1 was seen in cells treated with IKKβ with a concomitant reduction in both S and G_2/M. The data support the observations of growth analysis, where the IKKβ-treated cells demonstrated slower growth compared with the other siRNA treatments.

Apoptosis Following siRNA Treatment

NF-κB activation is considered to play a central role in the balance between cell proliferation and death, promoting upregulation of genes involved in both cell division and anti-apoptosis. One of the characteristic features of early apoptosis (programmed cell death) is the loss of membrane asymmetry, which can be identified by the translocation of the phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane. Annexin V has a high affinity for phosphatidylserine and binds to cells with exposed phosphatidylserine. This morphological characteristic of apoptosis occurs prior to the loss of membrane integrity; therefore cells in early apoptosis can be identified by the ability to bind Annexin V but selectively exclude the vital dye propidium iodide (AnnexinV +ve/PI −ve). Apoptosis was measured at 24, 48, and 72 h following siRNA transfection using the Annexin V-FITC/PI method. A 24-h exposure to 10 μM Doxorubicin was used as a positive control (Table III). The levels of apoptosis were not greatly elevated from that of the control or scrambled oligo-treated cells. These data further support the growth analysis and suggest that the difference seen in the IKKβ-treated cells is related to a growth arrest in G_0/G_1 rather than loss of cells via apoptosis.

Effects of siRNA on NF-κB Activation

The effects of siRNA treatments on constitutive and induced levels of nuclear NF-κB were studied using a p65 transcription factor ELISA-based assay (Fig. 1C). Assay specificity was

TABLE II. Cell-Cycle Distribution of MDA-MB-231 Cells Treated With siRNA

	Cell-cycle phases		
	G_0/G_1	S	G_2/M
Control	59.5	24.7	15.8
Scrambled	65.2	21.9	12.9
IKKα	60.3	24.3	15.4
IKKβ	84.4	6.2	9.4
IKKγ	59.6	26.7	13.8
P65	64.3	21.5	14.2

Results of duplicate analysis are presented.

TABLE III. Induction of Apoptosis by siRNAs in MDA-MB-231 Cells

	Apoptosis (% of total cells) (Annexin V +ve/PI −ve)		
	24 h	48 h	72 h
Control	12.7	6.0	6.7
Scrambled	5.6	13.1	8.4
IKKα	6.2	9.3	10.5
IKKβ	14.6	7.3	10.0
IKKγ	6.1	11.5	8.9
P65	6.7	8.7	8.5
Doxorubicin	71.0		

The data presented are the means of duplicate analyses.

validated by using both non-bound wild-type and mutated NF-κB consensus oligonucleotides. Incubation of the control sample with wild-type oligonucleotide greatly reduced the final absorbance, whilst the mutated oligonucleotide had no effect. Transfections with IKKα, IKKβ, and IKKγ siRNA did not significantly decrease basal nuclear NF-κB. The exception was the p65 siRNA ($P < 0.05$), as the ELISA assay is p65-based. TNFα exposure 30 min prior to isolation of the nuclear extracts more than doubled nuclear NF-κB. siRNA treatments slightly reduced this increase as compared with TNFα-treated control cells although the change was found to be non-significant. Again, the p65 siRNA-treated cells showed little NF-κB stimulation by TNFα. According to these results, siRNA inhibition of IKKα, β, and γ did not greatly affect the basal level of NF-κB, with only modest inhibition of NF-κB activation seen following TNFα exposure.

siRNA Inhibits NF-κB-Induced Gene Expression

Analysis of down stream gene targets of NF-κB is often used alongside NF-κB binding analysis as a test for functional NF-κB activity. Both IκBα and A20 (a known TNFα-activated gene) expression were analyzed 72 h after siRNA transfection, minus or plus a 30 min exposure to TNFα (Fig. 2). p65 siRNA treatment decreased the expression of both genes, demonstrating the involvement of NF-κB. IKKα, IKKβ, and IKKγ siRNAs caused little reduction in the basal levels of expression of both IκBα and A20. The effects of the siRNA treatments were more evident following TNFα stimulation, in

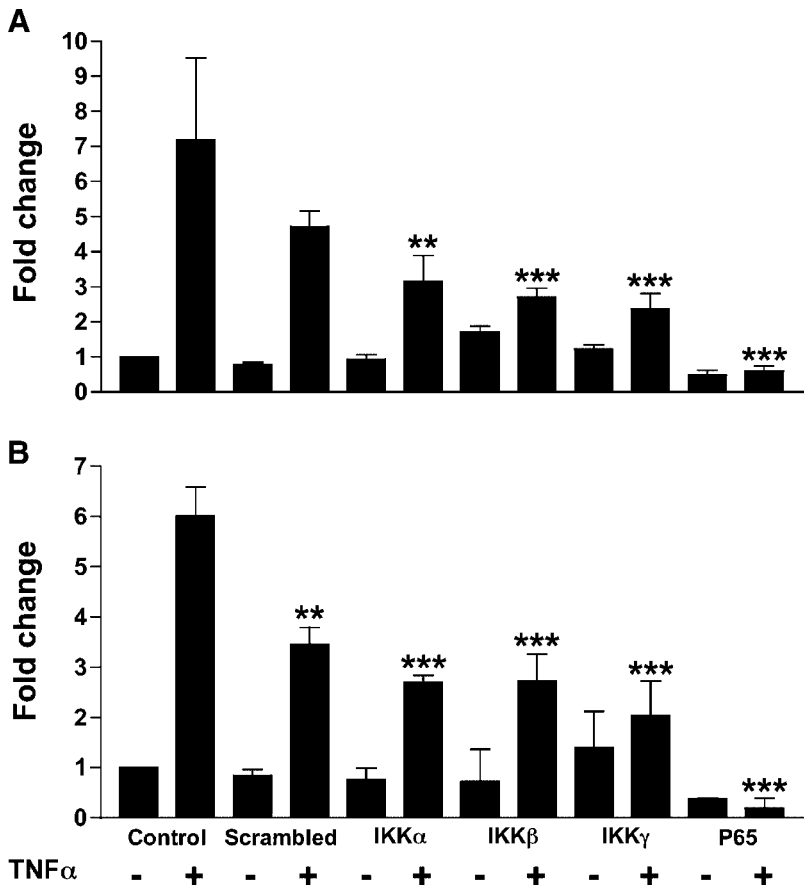

Fig. 2. Effects of siRNA treatment on the NF-κB target genes IκBα and A20. MDA-MB-231 cells were transfected in serum free medium with siRNAs for 4 h. Seventy-two hours later some cells were exposed to TNFα (5 ng/ml) for 30 min following which the cells were collected and RNA isolated as described in the Materials and Methods. The expression levels of both (**A**) IκBα and (**B**) A20, with or without TNFα exposure, were analyzed using quantitative real-time RT-PCR. β-actin levels were used to normalize the expression. The data are presented as the fold difference relative to the untreated (TNFα−) control. The data shown are the means ± SD of duplicate analyses. The asterisks indicate data that are significantly different from control (paired Tukey test): $^{**}P < 0.01$, $^{***}P < 0.001$.

which the levels of gene expression were significantly reduced ($P < 0.01$) in comparison to the TNFα-treated control, although only by about 50%, slightly more than by the scrambled oligo. p65 siRNA was able to completely inhibit TNFα stimulation of both IκBα and A20.

Effects of Proteasome Inhibition by MG-132

IκBα is ubiquitinated following its phosphorylation, which marks it for degradation by the proteasome. Inhibition by compounds such as MG-132 and Bortezomib/Velcade (formerly PS-341) has been shown to block NF-κB activation through the inhibition of IκBα degradation. MDA-MB-231 cells were treated with three concentrations of MG-132 (50, 100, and 200 nM), around the 50% growth inhibitory concentration (IC_{50}) of 100 nM (Fig. 3A) for 24 h, without and with 5 ng/ml of TNFα for 30 min. Immunoblot analysis identified a high level of IκBα at the expected molecular weight of 42 kD in uninhibited and unstimulated cells. This disappeared following TNFα treatment (Fig. 3B). MG-132 at 50 nM did not affect the levels of IκBα, whereas 100 nM and 200 nM strongly decreased IκBα, and lower mobility bands were seen, signifying accumulation of ubiquitinated phospho-IκBα due to the blocked proteasome activity, both with or without TNFα stimulation. The growth inhibitory effects of MG-132 in the MDA-MB-231 cells cannot be attributed to inhibition of NF-κB activation because no decrease in basal active nuclear NF-κB was detected with high MG-132 concentrations. Cells treated with 50 nM MG-132 had a similar level of active basal NF-κB as control cells, but 100 and 200 nM caused loss of IκBα and build-up of phospho-IκBα yet did not reduce active nuclear NF-κB. Following TNFα stimulation, the lowest concentration of the proteasome inhibitor did not affect NF-κB levels, but the higher concentrations significantly ($P < 0.05$) inhibited stimulation. Treatments with 100 and 200 nM MG-132 strongly elevated the expression of the downstream targets IκBα and A20 (6- and 20-fold, respectively), although both 100 and 200 nM MG-132 inhibited further stimulation by TNFα of both IκBα and A20 gene expression (Fig. 4).

DISCUSSION

Activated NF-κB has been suggested to be an important factor in the early stages of tumorigenic transformation, essential for supporting cell survival by decreasing apoptosis, promoting cell-cycle progression, and which later remains elevated but is no longer required to support tumor survival [Lin and Karin, 2003]. In this study we have applied RNA interference, a powerful technique that utilizes endogenous intracellular mechanisms to specifically target and degrade mRNA, to thereby remove an unwanted protein [Hannon, 2002]. IKKα, IKKγ, and p65 siRNAs did not modify the basal (minus TNFα) level of active nuclear NF-κB, cell proliferation, cycle distribution or apoptosis; whilst the siRNA against IKKβ, although not inducing apoptosis, did modify the growth characteristics of the cells. Similar siRNA inhibition and growth effects have been observed in HeLa cells following IKKβ siRNA, but not siRNAs against p65 or IKKα, suggesting another activity of IKKβ [Takaesu et al., 2003] (Takaesu, G personal communication). In studies reported by Buss et al. [2004], IKKα and β siRNA, the novel IKKβ inhibitor SC-514, and dominant-negative IKKα and IKKβ did not suppress constitutive p65 ser-536 phosphorylation, which is postulated to be important in assembly of the transcription factor machinery. Only SC-514 was able to partially block p65 ser-536 phosphorylation induced by interleukin-1 (IL-1). The authors also demonstrated the existence of another p65-ser 536 kinase, adding further complexity to regulation of the NF-κB pathway. The result expected from previous studies with the small-molecule PKC inhibitor Gö6976 on MDA-MB-231 cells was that siRNAs would decrease NF-κB activity and thereby increase apoptosis. On this, the scientific literature is confusing and contradictory. Selective inhibitors of IKKβ blocked NF-κB-dependent gene expression in IL-1β stimulated synovial fibroblasts [Kishore et al., 2003], whilst studies using a dominant-negative IKKβ in human breast cancer cells showed reduced NF-κB activity and growth rate, although no apoptosis was reported [Romieu-Mourez et al., 2001]. Paradoxically, activation of NF-κB has been demonstrated in mouse embryo fibroblast IKKα/β double knockouts [Tergaonkar et al., 2003]. Also, the Nemo binding domain peptide related to IKKβ binds to IKKα; its limited action blocks IκB kinase activity, and it decreases excess but not basal NF-κB [Biswas et al., 2004]. This peptide did not alone greatly affect growth or apoptosis of the ER−/human epidermal

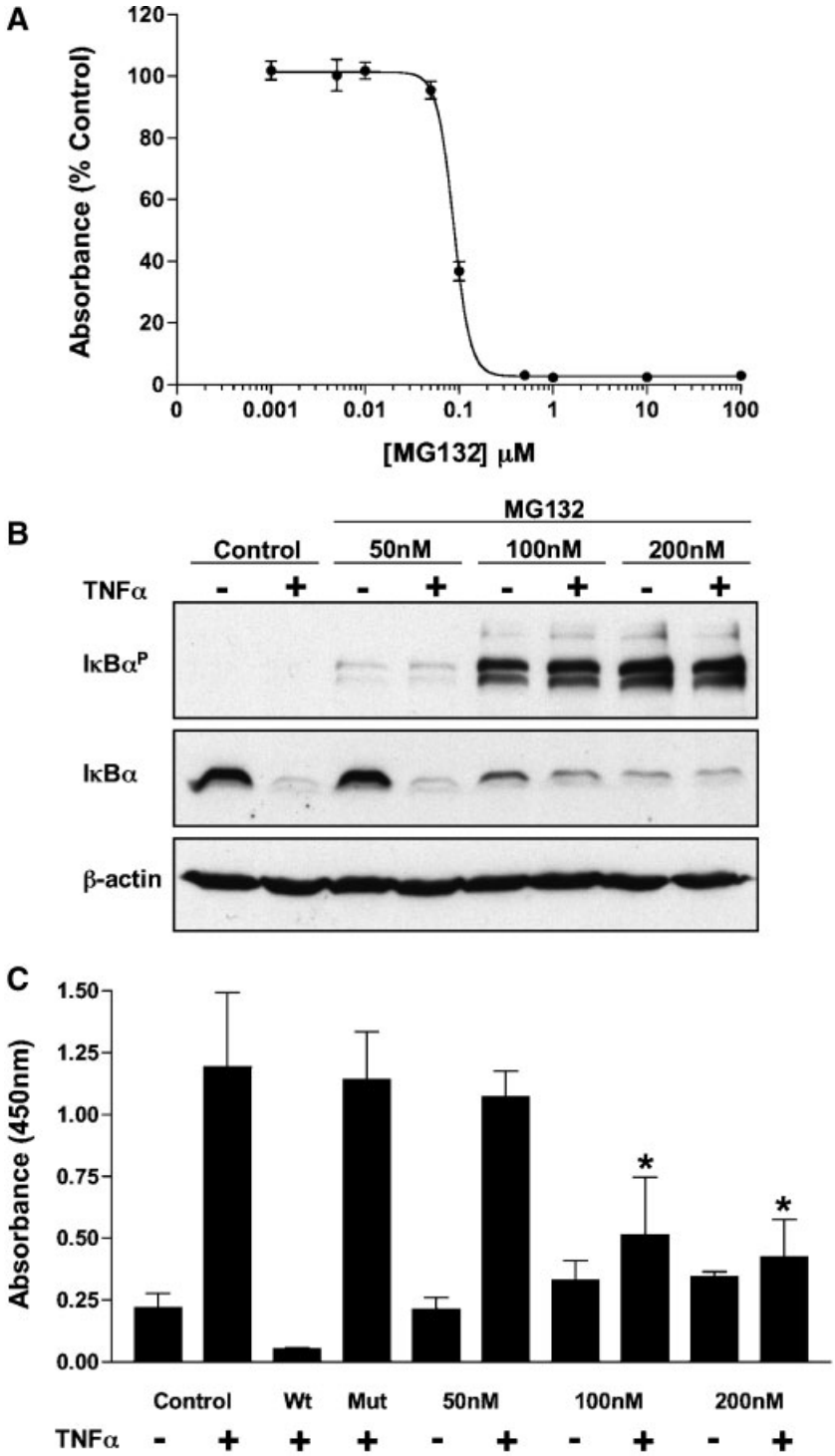

Fig. 3. Effects of the proteasome inhibitor MG-132 on MDA-MB-231 cells. **A**: Cells were seeded in 96-well plates and treated with a range of MG-132 concentrations and growth inhibition analyzed using the SRB assay. **B**: MDA-MB-231 were exposed to MG-132 for 24 h followed by a 30 min TNFα (5 ng/ml) treatment. Whole-cell lysates were subsequently isolated and equal amounts (60 μg/lane) of cellular protein were separated on a 10% SDS–PAGE gel and transferred to nitrocellulose. The levels of and IκBα and phospho-IκBα were determined by western blot analysis as described under Materials and Methods. β-actin was used as a loading control. **C**: Nuclear levels of activated NF-κB following a 24 h exposure to MG-132, followed by 30 min TNFα (5 ng/ml). Equal amounts of nuclear extracts (3 μg) were analyzed for active NF-κB using an oligonucleotide-specific p65 ELISA assay. Specificity was checked by competition analysis using soluble wild-type (Wt) and mutated (Mut) NF-κB consensus oligonucleotides. The amount of active NF-κB was determined by colorimetric analysis at 450 nm. The data shown are representative means ± SD of $n = 3$ experiments. The asterisks indicate data that are significantly different from control (paired Tukey test): $^*P < 0.05$.

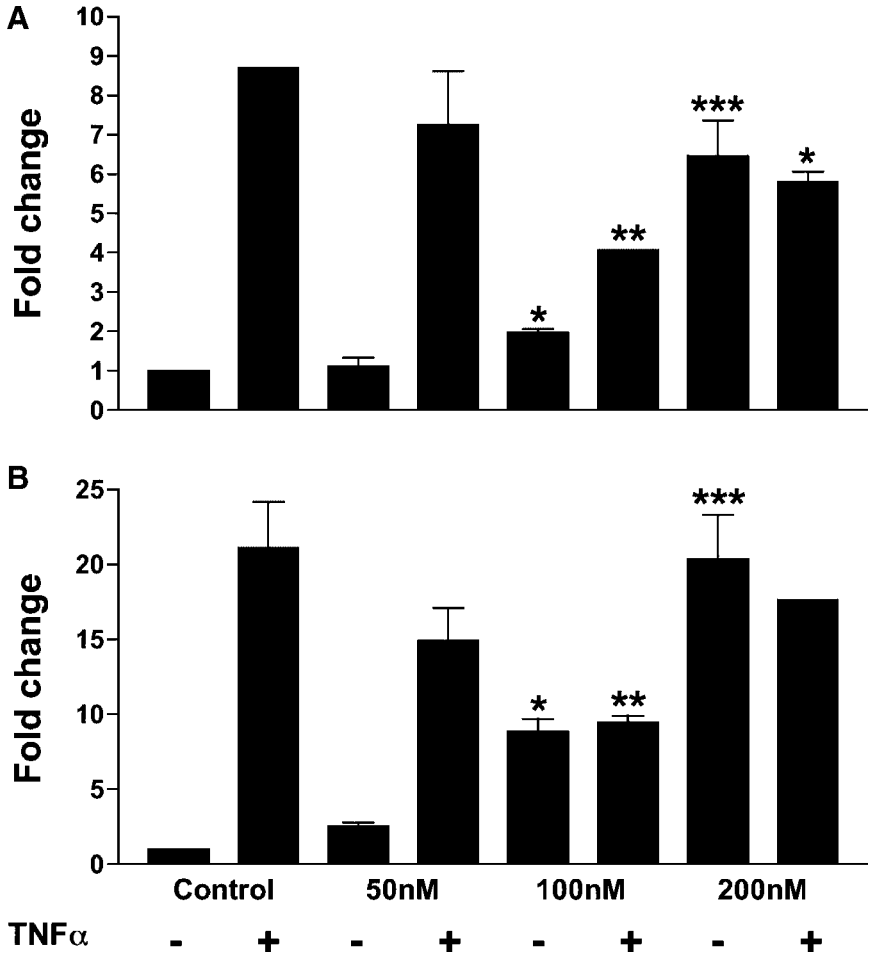

Fig. 4. Effects of MG-132 treatment on the NF-κB target genes IκBα and A20. MDA-MB-231 were exposed to MG-132 for 24 h followed by a 30 min TNFα (5 ng/ml) treatment, the cells were collected and RNA isolated as described in the Materials and Methods. The expression levels of both (**A**) IκBα and (**B**) A20, with or without TNFα exposure, were analyzed using quantitative real-time RT-PCR. β-actin levels were used to normalize the expression. The data are presented as the fold difference relative to the untreated (TNFα−) control, and are the means ± SD of duplicate analysis. The asterisks indicate data that are significantly different from control (paired Tukey test): $*P \leq 0.05$, $**P < 0.01$, $***P < 0.001$.

growth factor receptor 2 + (HER2) SKBR3 cell line, but together with heregulin it induced apoptosis. These data, together with the work presented here, suggest that the IκB kinase complex alone does not regulate NF-κB activation, and the lack of an apoptotic effect following p65 siRNA suggests that NF-κB is not the sole determinant of death in MDA-MB-231 cancer cells.

How important NF-κB is for the survival of cells not exposed to an apoptotic stress, such as caused by drugs, is put into question by the negative result when the NF-κB subunit p65 was eliminated by siRNA. This strongly decreased IκBα protein levels, A20 and IκBα gene transcription, and stimulation of NF-κB by TNFα, but apoptosis was not increased and growth was unaltered. siRNAs inhibited the TNFα induction of NF-κB via IKK, which therefore must be by a different mechanism than the basal pathway, and it probably involves a kinase because the kinase inhibitor Gö6976 effectively lowers its activity. We conclude that absence of NF-κB does not by itself activate apoptosis.

The ability of siRNAs to block the IKK complex but not to influence NF-κB or apoptosis suggests possibilities that include ineffective inhibitions allowing leakage of upstream signals, or other pathways that activate NF-κB. Contradictions with siRNA silencing suggest that Gö6976 affects a number of pathways and multiple targets. The primary target of Gö6976 is upstream of the NF-κB complex, at PKC

which itself is involved in a number of intracellular signaling pathways. Indeed, Gö6976 has been identified to inhibit not only PKCα and β but a number of other protein kinases to a similar and in some cases greater extent. These included MAPK-activated protein kinase-1b (MAPKAP-K1b), Mitogen- and stress-activated protein kinase 1(MSK1), 3-phosphoinositide-dependent protein kinase 1 (PDK1), p70 ribosomal protein S6 kinase (S6K1), phosphorylase kinase (PHK) [Davies et al., 2000], and also Chk1 and Chk2 (check point kinase) cyclin-dependent kinases [Kohn et al., 2003]. An effect of siRNA that could be critical to explaining the differences observed in comparison to other techniques is the depletion of a protein, rather than replacement/competition with the endogenous protein. Dominant-negative IKKβ produces a protein that binds to form an IKK complex, which cannot further activate NF-κB via IκBα phosphorylation. Removal of the IKK complex components by siRNA could cause a stress, which activates compensatory pathways. The differences between RNAi and small-molecule inhibitors are reviewed in detail by Fitzgerald [2005], and support the conclusions of this study. The author discusses the phenotypic discrepancies that can result from the actions of RNAi versus small-molecule inhibitors and how the disruption of a protein complex, via loss of a component, can have a profoundly different outcome than a small-molecule inhibitor, which simply inactivates an internal enzymatic domain.

NF-κB is only one factor implicated in the maintenance of the anti-apoptotic phenotype of ER− breast cancer cells, and activity of the IKK complex is not solely focused on regulating NF-κB activity. The Forkhead transcription factor FOXO3a also causes growth arrest and apoptosis [Burgering and Kops, 2002]. Active IKKβ phosphorylates FOXO3a, marking it for ubiquitination and then degradation by the proteosome [Hu et al., 2004]. Thus, IKKβ inactivation of FOXO3a should decrease apoptosis independently of its activating NF-κB. The activity seen with the IKKβ siRNA could thus mainly be through FOXO3a rather than NF-κB, if NF-κB activity is maintained via other compensating NF-κB activation pathways.

To confirm that basal NF-κB levels can be independent of the removal of IκBα, the proteasome inhibitor MG-132, an established NF-κB inhibitor, was applied [Grisham et al., 1999; Goldberg and Rock, 2002]. Similar to prior observations [Palombella et al., 1998; Grisham et al., 1999], MG-132 depleted non-phosphorylated IκBα and, with or with-out TNFα, resulted in the build-up of (ubiquitinated) phospho-IκBα. The basal level of NF-κB was not reduced, but rather increased slightly. MG-132 blocked stimulation of NF-κB by TNFα. Its failure to strongly decrease basal levels of NF-κB supports a non-IκB pathway that controls constitutive NF-κB activation. Analysis of the down stream NF-κB gene targets showed that MG-132 stimulated expressions of both IκBα and A20, although further stimulation by TNFα treatment was inhibited at higher concentrations. MG-132 has recently been shown [Buss et al., 2004] to enhance basal p65 ser-536 phosphorylation but not affect inducible ser-536 phosphorylation and also to activate endogenous IKK. These results suggest that the effects of the proteasome inhibitor are more at the level of NF-κB than on the expression of its transcripts. Collectively these data suggest that proteasome inhibition effects on NF-κB activity might be primarily on the stimulated rather than the basal level.

In conclusion, many kinases other than IKK have been shown to phosphorylate both IκB and NF-κB (see the Introduction). Is another kinase in part responsible for the elevated constitutive NF-κB in cancer cells? Specific targeting of the NF-κB pathway in MDA-MB-231 using siRNAs has demonstrated that the constitutive, basal level is independent of IKK signaling and insensitive to the prevention of IκBα degradation by proteasome inhibition. Thus the survival of these cells is not solely dependent on NF-κB activity but more likely a combination of NF-κB and other signaling pathways. This would explain Gö6976 effects of both blocking NF-κB and causing apoptosis in vitro and the tumor regression and inhibition seen in vivo. This study has helped to clarify a growing concern surrounding the effects of small-molecule inhibitors. It is important to recognized that the lack of specificity seen with small-molecule inhibitors can been misleading in identifying single, linear pathways as the cause of tumor promotion. It maybe the multiple and often unknown activities of these drugs that synergize and produce the desired anti-tumor activity. The activity of small-molecule inhibitors cannot be defined through a single target and although specificity can be defined in vitro, this specificity

cannot necessarily be translated to the biological system, as a plethora of targets are more than likely affected. The advent of target-specific agents such as siRNA has revealed the extent and complexity of intra-cellular signaling pathways and pitfalls that can be encountered in deciphering such pathways using small-molecule inhibitors. NF-κB exemplifies such as problem, where multiple pathways and post-translational modifications control normal NF-κB function, and this complexity may be further convoluted in cancer. The intricacy of the NF-κB pathway should be in no way be considered a surprise, considering the number of gene targets for this transcription factor.

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