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Inhibition of NF-κB Activity by Thalidomide through Suppression of IκB Kinase Activity*

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The sedative and anti-nausea drug thalidomide, which causes birth defects in humans, has been shown to have both anti-inflammatory and anti-oncogenic properties. The anti-inflammatory effect of thalidomide is associated with suppression of cytokine expression and the anti-oncogenic effect with inhibition of angiogenesis. It is presently unclear whether the teratogenic properties of thalidomide are connected in any way to the beneficial, anti-disease characteristics of this drug. The transcription factor NF-κB has been shown to be a key regulator of inflammatory genes such as tumor necrosis factor-α and interleukin-8. Inhibition of NF-κB is associated with reduced inflammation in animal models, such as those for rheumatoid arthritis. We show here that thalidomide can block NF-κB activation through a mechanism that involves the inhibition of activity of the IκB kinase. Consistent with the observed inhibition of NF-κB, thalidomide blocked the cytokine-induced expression of NF-κB-regulated genes such as those encoding *interleukin-8*, *TRAF1*, and *c-IAP2*. These data indicate that the therapeutic potential for thalidomide may be based on its ability to block NF-κB activation through suppression of IκB kinase activity.

Thalidomide was distributed in the late 1950s as a sedative and an anti-nausea medication for first trimester pregnancy (1). Prenatal use of the drug, however, produced severe developmental defects to the human fetus, including limb deformities (2). Investigation into the mechanism of action by thalidomide demonstrated that it acts as a teratogen and not as a mutagen (3, 4). The teratogenic properties of thalidomide are poorly understood but have been proposed to involve the production of reactive oxygen species leading to subsequent DNA damage (5). Although the correlation of birth defects with use of thalidomide led to its removal from the market, continued clinical use established that thalidomide possesses immunomodulatory and anti-angiogenic properties, both of which are currently under investigation (6–9). Diseases such as erythema nodosum leprosum (10–12), rheumatoid arthritis (13–16), and cancer (17–19) are currently being treated with thalidomide, although its mechanism of action remains unclear. As an immunomodulator (20, 21), thalidomide has been shown to suppress lipopolysaccharide-induced production of TNFα[1] (22, 23) and IL-12 (24, 25), two cytokines critical for the induction of cellular immune responses. The anti-angiogenic properties of thalidomide have been demonstrated by its ability to inhibit growth factor-induced neovascularization in rabbit (26) and mouse corneal assays (27). Neovascularization occurs through a process that requires the induction of a number of cellular genes including *IL-8* (28, 29). Transcriptional up-regulation of *IL-8*, as well *TNFα* and *IL-12*, can occur through activation of the transcription factor NF-κB (30).

NF-κB is a DNA-binding factor, originally identified as a regulator of immunoglobulin κ light chain gene expression (31), that functions as a dimer of subunits of a family of ubiquitously expressed transcription factors (32, 33). Five mammalian members of the family have been identified as follows: p50/NF-κB1, p65/RelA, c-Rel, RelB, and p52/NF-κB2. Although numerous homodimeric and heterodimeric forms of this factor have been identified, classic NF-κB is composed of the p50-p65 heterodimer. In unstimulated cells, the majority of NF-κB is localized to the cytoplasm where it is tightly bound to the inhibitory proteins of the IκB family. Specifically, IκBα is a key molecular target involved in the regulation of NF-κB transcription factors during inflammatory responses. Upon stimulation by extracellular inducers of NF-κB, such as TNFα or IL-1β (30), IκBα is rapidly phosphorylated by the IκB kinase (IKK) complex on serine residues 32 and 36 (34). This phosphorylation leads to the ubiquitination and subsequent degradation of IκBα by the proteasome followed by nuclear translocation of NF-κB. Once NF-κB enters the nucleus, it can positively regulate the expression of genes involved in the immune and inflammatory response, such as *IL-8*, *IL-12*, and *TNFα* (35). Additionally, NF-κB is now known to be a critical regulator of the oncogenic process through its ability to regulate genes involved in cell growth, suppression of apoptosis, and metastasis (36–38). We demonstrate here that NF-κB DNA binding is inhibited by thalidomide through a mechanism that involves the suppression of IKK activity. Consistent with its ability to block NF-κB binding, we show that thalidomide also inhibits the expression of IL-8 message as well as other NF-κB-dependent genes. Our data provide a molecular mechanism to potentially explain the anti-inflammatory and anti-oncogenic properties of thalidomide.

MATERIALS AND METHODS

Cell Culture—Human Jurkat T cell lymphocytes were maintained in RPMI 1640 plus 10% fetal bovine serum and antibiotics. The human vascular endothelial cell line EA.hy926 (a gift from Cora Jean S. Edgell, University of North Carolina, Chapel Hill) was maintained in Dulbec-

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This work is dedicated to the memory of Mary Ellen Keifer.

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[1] The abbreviations used are: TNFα, tumor necrosis factor-α; IKK, IκB kinase; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MHC, major histocompatibility complex; EMSA, electrophoretic mobility shift assays.

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co's modified Eagle's medium H supplemented with 10% fetal bovine serum, 1× hypoxanthine/aminopterin/thymidine medium supplement (Sigma) and antibiotics.

Cell Treatment—Cells were treated with 10 (Jurkat) or 5 ng/ml (EA.hy926) human recombinant TNFα (Promega) diluted in phosphate-buffered saline. Thalidomide (generously provided by the Celgene Corp., Warren, NJ) was resuspended in dimethyl sulfoxide (Me_2SO) and used at a final concentration of 10 or 40 μg/ml. IL-1β (Promega) was used at a concentration of 10 ng/ml.

Nuclear and Cytoplasmic Extracts—Cells were passaged 24 h prior to treatment in 10 ml of medium in 100-mm dishes (EA.hy926) or 25-cm^2 flasks (Jurkat) at a density of 2×10^6 (EA.hy926) or 1×10^6 cells/ml (Jurkat). Post-treatment, the cells were harvested by scraping (EA.hy926) or by centrifugation (Jurkat), washed 2 times with phosphate-buffered saline, and lysed, on ice, in 3 pellet volumes of cytoplasmic extraction buffer (10 mM HEPES, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.2 (EA.hy926) or 0.075% (Jurkat) Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml each of aprotinin, leupeptin, and pepstatin). Nuclei were pelleted and washed once in 100 μl of cytoplasmic extraction buffer without Nonidet P-40 and repelleted. The supernatant was added to the cytoplasmic samples. The nuclear pellet was resuspended and lysed in 2 pellet volumes of nuclear extraction buffer (20 mM Tris, pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, 2.5 μg/ml each of aprotinin, leupeptin, and pepstatin). The final salt concentration was adjusted to 400 mM with NaCl. The cytoplasmic and nuclear extracts were cleared by centrifugation, and supernatants were transferred to new tubes. A final concentration of 20% glycerol was added to the cytoplasmic extracts. Both nuclear and cytoplasmic extracts were assayed for protein concentrations using a Bio-Rad protein assay dye that incorporates the Bradford method. All extracts were stored at −70 °C until analyzed.

Electrophoretic Mobility Shift Assays (EMSA)—Equal amounts of nuclear extract (5 μg of protein) were incubated for 15 min at room temperature with a ^{32}P-labeled DNA probe containing an NF-κB-binding site from the class I MHC promoter (5′-CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC-3′) or an Oct-1-binding site (5′-TGTCGAATGCAAATCACTAGAA-3′) in binding buffer (10 mM Tris, pH 7.7, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA) plus 2 μg of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech). The final salt concentration was adjusted to 50–100 mM using NaCl. Complexes were separated on a 5% non-denaturing polyacrylamide gel, dried, and autoradiographed. For supershift assays, nuclear extracts were preincubated for 15 min with 1–2 μl of rabbit polyclonal antibodies raised against the NF-κB family subunits p65 (Rockland Co., Gilbertsville, PA) or p50 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) prior to the addition of the DNA probe.

Reporter Gene Assay—Transient transfections were performed using Superfect (Qiagen Inc., Valencia, CA) and 5 μg of a luciferase reporter construct containing 3 tandem wild-type or mutated NF-κB-binding sites from the promoter region of the class I MHC promoter (a gift from Bill Sugden, University of Wisconsin, Madison). 24 h post-transfection, the cells were either not treated or treated with TNFα with or without thalidomide for specified times. Cells were harvested by centrifugation, washed with phosphate-buffered saline, and lysed with 1× Reporter Lysis Buffer (Promega) according to the manufacturer's instructions. Luciferase assays were performed in duplicate using equal amounts of protein with 200 μM D-luciferin (Sigma) as the substrate (39). Relative light units were measured with an AutoLumat LB953 luminometer (Berthold Analytical Instruments, Inc, Nashua, NH) and standardized to light units obtained from transfections performed with salmon sperm DNA.

Northern Analysis—RNA was isolated using the RNeasy Total RNA Kit as recommended by the manufacturer (Qiagen). Samples were run on a formaldehyde-agarose gel and transferred overnight to nylon filter. Cross-linking of the RNA to the nylon filter was done using a UV cross-linker (Stratagene, La Jolla, CA). Membranes were probed for IL-8 mRNA expression and GAPDH mRNA (loading control) using randomly labeled probe (Amersham Pharmacia Biotech) at 68 °C in Quickhyb (Stratagene) solution as recommended by the manufacturer. Washes were performed twice in 2× SSC, 0.1% SDS for 15 min at 42 °C, and once with 0.1× SSC, 0.1% SDS for 5 min at 60 °C. Filters were exposed to film overnight.

Ribonuclease Protection Assay—EA.hy926 endothelial cells were treated for 60 min with TNFα (5 ng/ml) alone or in the presence of thalidomide (40 μg/ml) or Me_2SO (40 μl). Cells were scraped, and total RNA was harvested using Trizol solution (Life Technologies, Inc.). Using a custom made Riboquant™ Multiprobe RNase Protection Assay System (Pharmingen) containing a template of the NF-κB-responsive genes as follows: *TRAF1, TRAF2, A1/Bfl-1, c-IAP2*, *IL-8*, and *IL-2Rα*, RNAs were hybridized overnight and subjected to RNase treatment as recommended by the manufacturer. Annealed protected RNA products were fractionated by SDS-PAGE and analyzed by autoradiography.

Western Blot Analysis—50 μg of cytoplasmic extracts were fractionated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked and probed for IκBα degradation using an antibody specific for IκBα (Santa Cruz Biotechnology). To control for loading, the membranes were stripped and reprobed using an antibody for α-tubulin (Sigma).

Kinase Assay—Cells were either not treated or treated with TNFα or TNFα plus thalidomide for specified times and harvested by centrifugation in ice-cold phosphate-buffered saline containing phosphatase inhibitors (40). Equal amounts (500 μg) of whole cell extracts were immunoprecipitated using an antibody against the β subunit of IKK (a gift of Dr. F. Mercurio, Signal Pharmaceuticals, San Diego, CA). Kinase activity was determined by incubating the immunoprecipitates with 4 μg of GST-IκBα (amino acids 1–54) wild-type substrate or a mutated form of IκBα (S32T,S36T) in the presence of [γ-^{32}P]ATP, as described previously (40). The immunoprecipitates were subjected to SDS-PAGE, dried, and visualized by autoradiography.

Quantitative Analysis—Autoradiographs were captured and stored for analysis using a gel capturing system that utilizes the NIH Image 1.61 software. Gel captures were quantified using volume quantitation and local median background correction using Molecular Dynamics ImagequaNT™ software program.

RESULTS AND DISCUSSION

Thalidomide Blocks NF-κB Induction by the Inflammatory Cytokines TNFα and IL-1β—Previous reports have provided evidence that thalidomide can inhibit the DNA binding activity of NF-κB (41, 42). However, these reports did not address whether the observed inhibition of DNA binding affected NF-κB transcriptional activity. Furthermore, the mechanism by which thalidomide suppresses NF-κB remains unknown. To gain insight into these unanswered questions, we first assayed NF-κB DNA binding activity in Jurkat T cells treated with both an inflammatory cytokine and thalidomide. We examined the potential effects of thalidomide treatment on NF-κB activation by stimulating cells with 10 ng/ml TNFα for 10 min in the presence or absence of thalidomide (Fig. 1*A*). As expected, NF-κB was induced within 10 min after TNFα treatment as seen by increased DNA binding activity (Fig. 1*A*, compare *lanes 1* and *2*). Based on the average of five independent experiments, TNFα-induced binding of NF-κB was inhibited by 62% when thalidomide was added to the cells simultaneously with cytokine treatment (Fig. 1*A, lanes 6* and *7*) as measured by volume quantitation (see "Materials and Methods"). Treatment of cells with thalidomide (Fig. 1*A, lanes 4* and *5*) or Me_2SO alone (*lane 3*) had no effect on basal NF-κB DNA binding activity. Thalidomide treatment also had no inhibitory effect on the binding of a second transcription factor, Oct-1 (Fig. 1*B*). Furthermore, direct addition of thalidomide to TNFα-treated nuclear extracts did not affect the binding of NF-κB to the DNA (data not shown). These data indicate that suppression of NF-κB binding by thalidomide is not due to interference between the transcription factor and the DNA, but rather that thalidomide acts specifically to inhibit TNFα-induced NF-κB activity and not as a general transcription factor inhibitor. Supershift assays indicate that the major NF-κB complex induced by TNFα stimulation was the p50-p65 heterodimer (Fig. 1*C*).

To determine whether thalidomide could inhibit the induction of NF-κB by other stimuli, we tested its effect on NF-κB activation by the inflammatory cytokine IL-1β. Jurkat cells were treated with IL-1β in the absence or presence of thalidomide, and nuclear extracts were prepared from cells harvested at the specified time points (Fig. 1*D*). As seen with TNFα-thalidomide co-treatment, the presence of thalidomide at the time of IL-1β induction suppressed activation of NF-κB. We

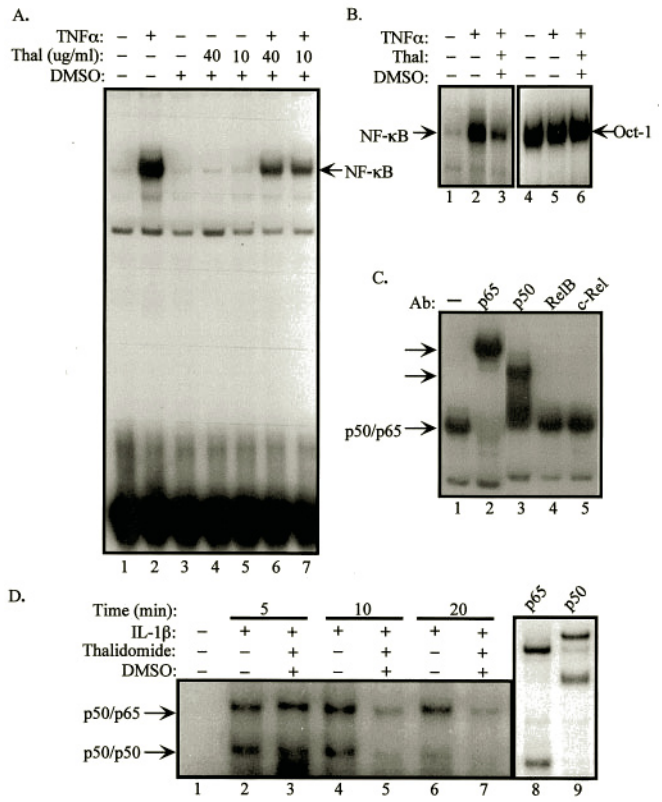

FIG. 1. **Thalidomide inhibits DNA binding activity of the p50/p65 NF-κB heterodimer in the presence of the inducers TNFα and IL-1β.** *A*, Jurkat T lymphocytes were either not treated (*lane 1*) or treated with 10 ng/ml TNFα (*lane 2*), Me_2SO (*DMSO*) (*lane 3*), thalidomide (*Thal*) alone at 40 μg/ml (*lane 4*), or 10 μg/ml (*lane 5*), or thalidomide plus TNFα for 10 min (*lanes 6* and *7*). Cells were harvested, and nuclear extracts were prepared and analyzed by EMSA. The data shown are representative of five independent experiments. *B,* cells were treated as in *A,* and EMSA was repeated with nuclear extracts incubated with a DNA probe containing either an NF-κB-binding site (*lanes 1–3*) or an Oct-1-binding site (*lanes 4–6*). *C,* nuclear extracts were preincubated with antibodies (*Ab*) raised against various NF-κB subunits and analyzed by EMSA. *D,* cells were either untreated (*lane 1*) or treated with IL-1β (10 ng/ml) in the presence (*lanes 3, 5,* and *7*) or absence (*lanes 2, 4,* and *6*) of thalidomide at the indicated time points and analyzed by EMSA. Supershift analysis was performed using the p65 (*lane 8*) or the p50 (*lane 9*) antibodies.

found both heterodimer and homodimer forms of NF-κB were inhibited at 10 and 20 min (Fig. 1*D, lanes 5* and *7*) post-induction but not at 5 min (Fig. 1*D*, compare *lanes 2* and *3*). Supershift analysis exhibits authentic NF-κB complexes being induced following IL-1β treatment (Fig. 1*D*, *lanes 8* and *9*). Overall, these data indicate that thalidomide can block NF-κB activation by cytokines that utilize distinct upstream signaling pathways, suggesting that the mechanism of suppression by thalidomide acts at a downstream site that is common to both the IL-1β and TNFα signaling cascades.

Suppression of NF-κB Transcriptional Activity by Thalidomide—To determine if thalidomide inhibited NF-κB transcriptional activity, we performed transient assays using a reporter plasmid containing multiple wild-type or mutated NF-κB-binding sites upstream of a luciferase gene. Jurkat cells were transfected with the reporter and were then treated with TNFα in the presence or absence of thalidomide for the times indicated (Fig. 2). Treatment of cells with thalidomide at the time of TNFα induction led to a 1.7-fold increase in transcriptional activity of the wild-type reporter as compared with a 6-fold increase with TNFα alone (Fig. 2*A*). The ability of thalidomide to inhibit the transcriptional induction by TNFα was transient, however, as suppression was partially lost at 9 h. This can be

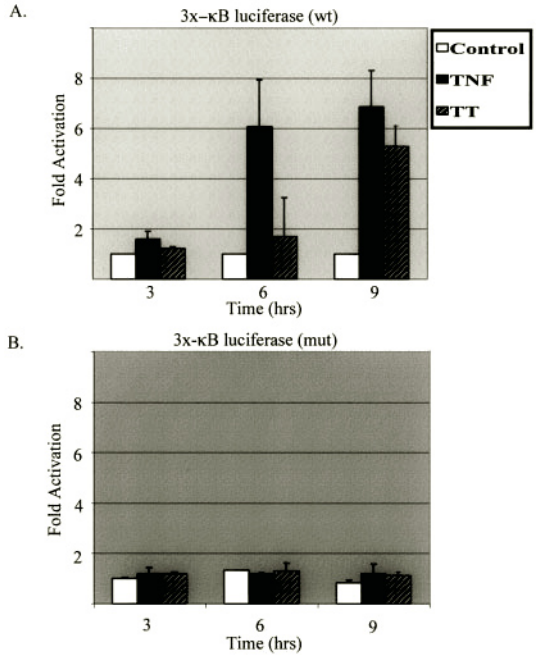

FIG. 2. **Thalidomide inhibits TNFα-induced NF-κB transcriptional activation.** Jurkat cells were transiently transfected using a reporter plasmid containing three wild-type (*A*) or mutant (*B*) NF-κB-binding sites from the class I MHC promoter upstream of a luciferase gene. 24 h post-transfection, the cells were either not treated or treated with TNFα alone or in the presence of thalidomide for the indicated times. Whole cell lysates were prepared as described under "Materials and Methods" and analyzed for luciferase activity. The data are representative of three independent experiments performed in duplicate.

explained by the fact that thalidomide has a 4.5-h half-life in aqueous solutions (42). Thalidomide did not block the activity of a reporter containing mutated NF-κB sites, indicating that the inhibition does not block general transcriptional responses (Fig. 2*B*). These results demonstrate that thalidomide not only acts to inhibit NF-κB DNA binding activity but also inhibits the ability of NF-κB to activate gene expression.

Inhibition of IL-8 Gene Expression in Endothelial Cells by Thalidomide—Thalidomide is being used as a cancer therapy partly based on its ability to inhibit neovascularization (26, 27). Angiogenic factors such as IL-8 are transcriptionally regulated by NF-κB, and TNFα-dependent angiogenesis requires the activation of NF-κB for the expression of *IL-8* (28). We were interested in evaluating the effect of thalidomide on NF-κB in endothelial cells. To do this we assayed binding activity by utilizing EA.hy926 endothelial cells, a clonal cell line derived from the human umbilical vein endothelial cells (43). We treated these cells with TNFα (5 ng/ml) in the absence or presence of thalidomide and analyzed nuclear extracts by EMSA (Fig. 3*A*). As expected, TNFα treatment led to an increase in NF-κB DNA binding activity at 10 min (Fig. 3*A, lane 2*) as compared with the untreated control (Fig. 3*A, lane 1*). As seen previously with Jurkat T cells, thalidomide treatment of EA.hy926 cells at the time of induction inhibited this increase (Fig. 3*A, lane 3*). The major complex observed in TNFα activation of endothelial cells is the p50-p65 heterodimer as observed by the supershifted complexes (Fig. 3*A, lanes 4* and *5*). These results demonstrate that thalidomide is an effective inhibitor of inducible NF-κB activity in endothelial cells. The ability of thalidomide to inhibit NF-κB activation in Jurkat cells as well as endothelial cells indicates that the inhibitory action is not cell type-specific.

Since thalidomide can inhibit activation of an NF-κB reporter gene, we sought to determine if it could inhibit the

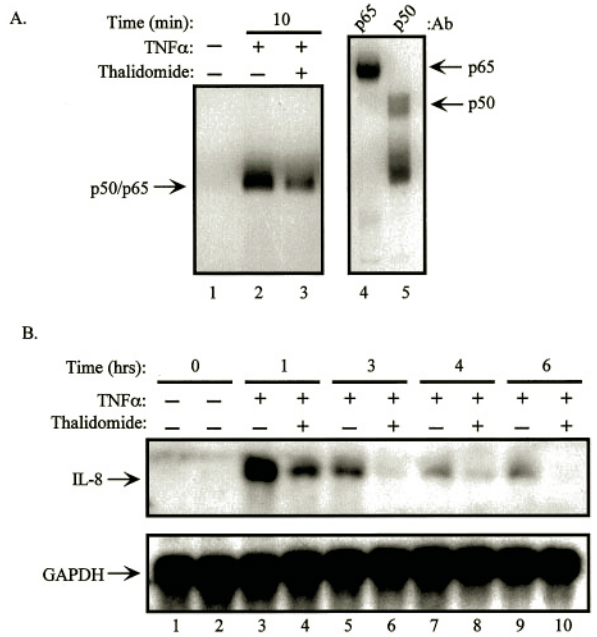

FIG. 3. **TNFα-induced transcriptional up-regulation of *IL-8* is suppressed by thalidomide treatment in endothelial cells.** EA.hy926 endothelial cells were not treated or treated with TNFα alone (5 ng/ml) or simultaneously with thalidomide (40 μg/ml) for the specified times. *A,* cells were harvested at 10 min, and nuclear extracts were prepared and analyzed by EMSA for activation of NF-κB. *B,* cells were harvested 1 h post-treatment, and total RNA was isolated and analyzed by Northern analysis for *IL-8* expression (*upper panel*). The data shown are representative of two independent experiments. GAPDH was used as a loading control (*lower panel*). *Ab*, antibody.

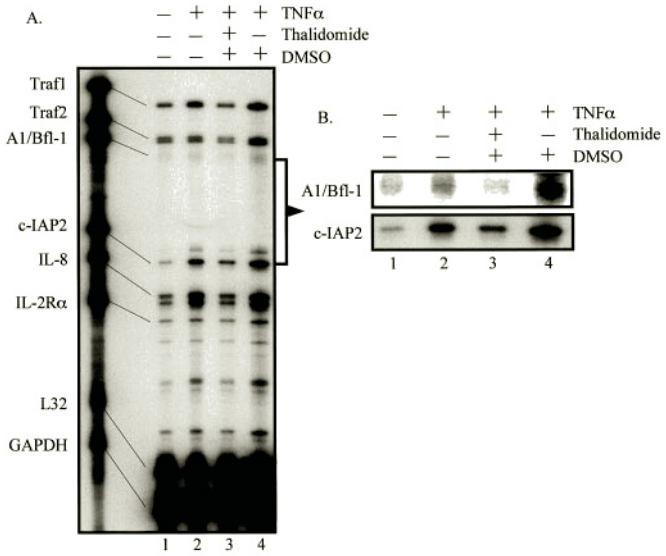

FIG. 4. **Thalidomide suppresses the activation of many NF-κB-regulated genes in the presence of TNFα.** EA.hy926 cells were treated for 60 min with TNFα (5 ng/ml) alone or in the presence of thalidomide (40 μg/ml) or Me_2SO (*DMSO*) (40 μl). The cells were harvested by scraping, and total RNA was isolated as described under "Materials and Methods." *A,* the RNA was incubated with the *in vitro* transcribed DNA template and analyzed by autoradiography. Loading control genes are represented as *L32* and *GAPDH*. *B,* autoradiography at a longer exposure time. A representative sample of NF-κB-regulated genes reveal 42% inhibition of gene expression in the presence of thalidomide for c-IAP2 and 66% inhibition for A1/Bfl-1.

transcriptional activation of an endogenous NF-κB-regulated gene. Endothelial cells were treated with TNFα, with or without thalidomide exposure, and RNA was isolated for Northern blot analysis (Fig. 3*B*). TNFα treatment alone (*lanes 3, 5, 7,* and *9*) increased the expression of IL-8 mRNA as compared with untreated cells (Fig. 3*B, lanes 1* and *2*). However, thalidomide treatment inhibited the expression of IL-8 mRNA by 56% (as measured by volume quantitation) at 1 h (Fig. 3*B*, *lane 4*). Treatment of the cells for longer time points inhibited the TNFα-induced activation of gene expression by ~90% (*lanes 6, 8* and *10*) as compared with TNFα alone. Therefore, the ability of thalidomide to inhibit the binding activity of NF-κB, as seen by EMSA, correlates well with the inhibition of NF-κB-regulated endogenous gene expression at the 1-h time point but results in an even greater inhibition of gene expression at the 3–6-h time points. Development of angiogenesis in certain models is dependent on the expression of *IL-8* (28, 44). Since *IL-8* is transcriptionally regulated by NF-κB and inhibited by thalidomide, we propose that one mechanism whereby thalidomide may function as an anti-angiogenic factor is through the suppression of NF-κB-regulated expression of *IL-8* and potentially other angiogenic factors.

NF-κB regulates genes involved in inflammatory responses as well as genes associated with the inhibition of apoptosis. The ability of thalidomide to function as an anti-inflammatory agent is likely due, in part, to its ability to block the induction of inflammatory gene expression through the inhibition of NF-κB. To test the effect thalidomide exposure has on the regulation of other NF-κB-dependent genes, we implemented a ribonuclease protection assay. By using an ribonuclease protection assay template specific for several NF-κB-regulated genes (Fig. 4), we examined the effect of thalidomide treatment on NF-κB-dependent gene expression. RNA was harvested from untreated EA.hy926 endothelial cells, cells treated with TNFα, and cells co-treated with TNFα and thalidomide or Me_2SO. The RNA was hybridized to the radioactively labeled, *in vitro* transcribed DNA template. As expected, TNFα induced the expression of several NF-κB-regulated genes (Fig. 4*A, lane 2*). Consistent with the ability of thalidomide to inhibit NF-κB DNA binding activity, thalidomide not only has the ability to inhibit TNFα-induced expression of *IL-8* but can also inhibit the inducible expression of other NF-κB-regulated genes such as *TRAF1, TRAF2, A1/Bfl-1, c-IAP2,* and *IL-2Rα* (Fig. 4*A*, *lane 3*). Treatment of cells with Me_2SO in the presence of TNFα has no effect on gene expression (Fig. 4*A, lane 4*). These data indicate that thalidomide blocks the TNFα-induced expression of several NF-κB-regulated genes.

Thalidomide Inhibits IKK Activity—We have demonstrated that thalidomide can inhibit NF-κB activation in response to different cytokines that utilize distinct upstream pathways. This suggests that the inhibitory action of thalidomide on NF-κB binding lies downstream of the cytokine-receptor interaction and recruitment of associated factors but upstream of the induction of NF-κB nuclear translocation. Both TNFα and IL-1β signal NF-κB activation through the induction of IKK (30, 45–48). IKK activation results in the phosphorylation of IκBα on serine residues 32 and 36, which ultimately leads to the degradation of this inhibitor (49–52). Since thalidomide is capable of inhibiting DNA binding activity, as well as inhibiting the transcription potential of NF-κB, we performed Western blot analysis to determine what effect thalidomide has on the regulation of the NF-κB inhibitory protein, IκBα. Cytoplasmic extracts from cells treated with TNFα alone or in combination with thalidomide were examined for the presence of IκBα (Fig. 5). Although untreated cells contain high levels of IκBα (*lane 1*), TNFα treatment resulted in the degradation of 85% (measured by volume quantitation) of the protein by 30 min following cytokine exposure (Fig. 5, *lanes 2–4*). This degradation was followed by an almost complete resynthesis of IκBα by 60 min (Fig. 5, *lane 5*). However, when thalidomide

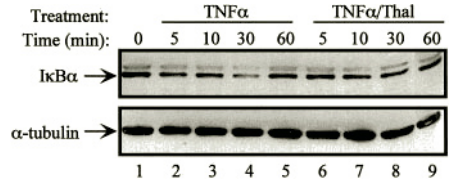

FIG. 5. **Degradation of IκBα is inhibited by thalidomide.** Jurkat cells were either not treated or treated with TNFα in the presence or absence of thalidomide (*Thal*) for the indicated time points. Cells were harvested by centrifugation, and cytoplasmic extract proteins were separated a 10% SDS-PAGE gel and transferred to nitrocellulose. The membrane was analyzed for degradation of IκBα with an anti-IκBα antibody (*upper panel*). The membrane was stripped and reprobed for expression of α-tubulin to control for loading (*lower panel*). The data presented are representative of two independent experiments.

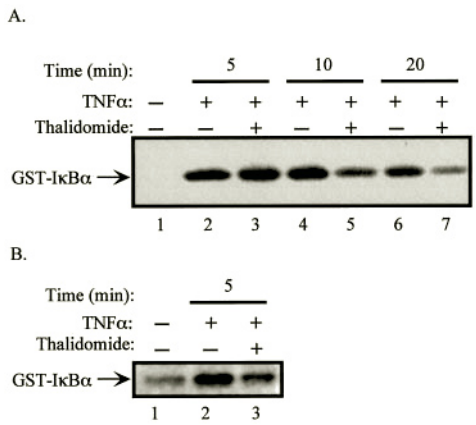

FIG. 6. **Thalidomide inhibits IKK activity.** Cells were either not treated or treated with TNFα alone or simultaneously with thalidomide at 40 μg/ml for the specified times. Whole cell extracts were prepared, and 500 μg of protein was immunoprecipitated with an anti-IKKβ antibody. *A,* immunoprecipitates were incubated with wild-type GST-IκBα fusion protein in the presence of [γ-^{32}P]ATP. The immunoprecipitates were run on a 10% SDS-PAGE gel, dried, and exposed to film. The data are representative of two independent experiments. *B,* cells were treated with thalidomide for 30 min prior to TNFα stimulation. Whole cell extracts were harvested and analyzed by *in vitro* kinase assay as stated in *A*.

was present at the time of induction, IκBα degradation is suppressed to 45% by 30 min, leaving 65% of the protein intact even in the presence of a potent NF-κB activator (Fig. 5, *lanes 6–8*). These data are consistent with the previous data on the ability of thalidomide to inhibit NF-κB binding as measured by EMSA. Moreover, no resynthesis of IκBα occurs at 60 min in the presence of thalidomide (Fig. 5, *lane 9*), unlike TNFα treatment alone. Since IκBα is transcriptionally regulated by NF-κB, these data support the finding that thalidomide inhibits the transcriptional activity of NF-κB.

To investigate the loss of IκBα degradation in the presence of thalidomide, we examined IKK activity following drug treatment. By using antibodies against the β subunit of the IKK complex, IKK was immunoprecipitated from extracts of cells that had been untreated or treated with TNFα or co-treated with thalidomide and TNFα. Immunoprecipitates were assayed for kinase activity by incubating with a GST-IκBα (amino acids 1–54) fusion protein in the presence of [γ-^{32}P]ATP, electrophoresed on a polyacrylamide gel, and analyzed by autoradiography. TNFα treatment of cells leads to an increase in kinase activity by 5 min that continued through the 20-min time period. Quantitative analysis of TNFα-induced IKK activity demonstrated that thalidomide inhibited this increase in activity by 53% at the 10- (Fig. 6*A, lane 5*) and 20-min time points (*lane 7*) as compared with TNFα alone (*lanes 4* and *6*). However, simultaneous treatment of cells with TNFα and thalidomide did not block the induction of IKK activity at 5 min (Fig. 6*A, lane 3*). In contrast, addition of thalidomide to cells for 30 min prior to TNFα stimulation inhibited IKK activity at 5 min following cytokine exposure (Fig. 6*B, lane 3*). This indicates that the lack of inhibition at the 5-min time point under simultaneous treatment conditions likely represents a lag period for the inhibitory activity of thalidomide. The lack of phosphorylation seen on a mutant GST-IκBα (S32T,S36T) substrate indicated that the phosphorylation activity of the kinase is specific for serine residues 32 and 36 (data not shown).

The ability of thalidomide to inhibit IKK activity likely explains the suppression of NF-κB DNA binding and transcriptional activity. We propose that the inhibition of NF-κB by thalidomide also explains the anti-inflammatory and anti-oncogenic properties of thalidomide. It is possible there may be other mechanisms associated with thalidomide action that can block NF-κB activity on other levels. Thalidomide may inhibit another factor or signaling pathway that may be involved in inflammatory or oncogenic potential. For example, thalidomide has also been reported to block Sp1 DNA binding activity (41), and it has been proposed that Sp1 and NF-κB can synergistically regulate transcription of certain genes (53). Thus, thalidomide may inhibit the expression of genes that are regulated by both Sp1 and NF-κB. It is certain that thalidomide can inhibit the ability of NF-κB to bind to the DNA. Our data demonstrate that thalidomide also functions to block the transcriptional activity of NF-κB. Additionally, we show that thalidomide inhibits the phosphorylation of IκBα by altering IKK activity. In summary, the data indicate that NF-κB is a molecular target for thalidomide action, potentially serving as a unifying theme to explain the ability of thalidomide to suppress inflammatory responses as well as inhibit angiogenesis.

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