Elevated Constitutive IκB Kinase Activity and IκB-α Phosphorylation in Hs294T Melanoma Cells Lead to Increased Basal MGSA/GRO-α Transcription

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Elevated Constitutive IκB Kinase Activity and IκB-α Phosphorylation in Hs294T Melanoma Cells Lead to Increased Basal MGSA/GRO-α Transcription[1]

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ABSTRACT

The basal transcription of the CXC chemokine, melanocyte growth stimulatory activity (MGSA)/growth-regulated protein (GRO)-α, is up-regulated in Hs294T melanoma cells compared with the normal retinal pigment epithelial (RPE) cells. Previous studies characterized a cytokine-inducible, functional nuclear factor (NF) -κB consensus element in the immediate 5′ regulatory region of the MGSA/GRO-α gene at −78 bp. Although the cytokine-inducible mechanisms for transcription of this gene are fairly well delineated, the mechanisms involved in its basal up-regulation of transcription in Hs294T melanoma cells are poorly understood. Recently, we demonstrated an increased rate of IκB-α degradation in Hs294T cells, which leads to an increased nuclear localization of NF-κB (R. L. Shattuck-Brandt and A. Richmond. Cancer Res., *57:* 3032–3039, 1997). Here we demonstrate that Hs294T melanoma cells have elevated basal IκB kinase (IKK) activity relative to RPE cells, causing an increased constitutive IκB-α phosphorylation and degradation. We also show here that the resultant elevated nuclear NF-κB (p50/p65) in these cells is responsible for the increased basal transcription of MGSA/GRO-α. Pretreatment of Hs294T or RPE cells with proteasome inhibitors MG115 or MG132 captures the slower migrating, constitutively phosphorylated form of IκB-α in Hs294T melanoma cells, but not in RPE cells. In addition, a phospho-specific antibody that specifically recognizes the inhibitory form of IκB that is phosphorylated at Ser-32 reacted with IκB-α in Hs294T cell, but not in unstimulated RPE cells. Although the basal level of protein expression of IKK-α or IKK-β are the same in both Hs294T and RPE cells, immunoprecipitation with IKK-α antibody combined with activity assay reveal a constitutively active IKK complex in Hs294T melanoma cells. Cotransfection of a 350-bp MGSA/GRO-α promoter-luciferase reporter construct with either the dominant negative IKK-α or the repressors of NF-κB, the IκB-α wild type or mutants lacking the inducible phosphorylation sites, demonstrates that the increased basal MGSA/GRO-α transcription in the Hs294T cells is due to the enhanced nuclear activation of NF-κB.

INTRODUCTION

Melanoma cells have been widely used to investigate changes involved in malignant transformation, including the search for genes expressed in malignant but not normal epithelial cells or melanocytes. We initially have purified and characterized MGSA/GRO[3] from the cultures of human malignant melanoma Hs294T cells (1–4). MGSA/GRO-α subsequently was demonstrated to function as a growth regulator or chemoattractant for cells that express CXCR2, such as neutrophils, epithelial cells, dendritic cells, and others (5–7). Expression of this chemokine is deregulated in several viral, inflammatory, and neoplastic conditions, including malignant melanoma (8–12). Aberrant overexpression of MGSA/GRO-α has been implicated in transformation and melanoma tumor progression both *in vitro* and *in vivo* (13, 14). Characterization of the 5′ regulatory region of MGSA/GRO-α by our group revealed a functional, cytokine-responsive NF-κB enhancer that increases MGSA/GRO-α gene transcription in concert with HMGIY and a yet-to-be identified immediate upstream region-binding factor (15–17). The constitutive interaction of NF-κB with these transcription elements leads to a transcriptional up-regulation of MGSA/GRO-α in Hs294T melanoma cells (18). However, the precise signaling mechanisms involved in the increased constitutive MGSA/GRO-α gene transcription in Hs294T cells and other transformed cells are unknown.

Rel/NF-κB proteins constitute a family of structurally related transcription factors that are regulated by interaction with a family of regulatory proteins, the IκB proteins. NF-κB is a ubiquitous transcription factor that is activated by a wide range of stimuli. NF-κB exists in the cytoplasm in the homo- or heterodimeric forms of Rel or Rel/NF-κB proteins (for reviews, see Refs. 18, 19). A conserved Rel homology domain in the N-terminal region of these proteins contains a nuclear localization signal, the domains conferring the dimerization and DNA binding. In unstimulated cells, IκB sequesters NF-κB in the cytoplasm by direct physical interactions that mask the nuclear localization signal. Stimulation of cells with multiple NF-κB inducers leads to phosphorylation of IκB-α at Ser-32 and Ser-36, ubiquitination at Lys-21 and Lys-22, and degradation of the inhibitor by 26S proteasome. Although the biochemical mechanisms involved in the processing of IκB-α and subsequent nuclear localization of NF-κB are fairly well-characterized, the kinases involved in the upstream signaling pathway of IκB-α phosphorylation have been identified only recently (20–25). These studies revealed that two IKKs, termed IKK-α and IKK-β, form homo- or heterodimeric complexes and constitute part of a large 700- to 900-kDa enzyme complex. Both of these kinases can phosphorylate IκB-α at serines 32 and 36 *in vitro* (20–25). Emerging data suggest that IKKs are substrates for members of the MAP3K family of proteins kinases, such as NIK. Consistent with these data, NIK can phosphorylate IKK-α specifically at serine 176 but cannot phosphorylate IKK-β (26). However, the complex pathway of signaling mediators involved in the NIK activation or IKK activation are poorly understood. It is now well-established that NF-κB is up-regulated in tumor cells compared with normal control cells (27–29) and this offers resistance to apoptosis induced by various cytotoxic cytokines (30, 31), viral mediators (32), or chemotherapeutic agents (33).

We previously have shown that the increased nuclear basal NF-κB activity (p50/p65) results from an increased cytoplasmic IκB-α degradation in Hs294T melanoma cells as compared with ARPE cells, a variant of normal RPE cells (12). In an attempt to further elucidate the mechanisms involved in the constitutive activation of NF-κB and/or MGSA/GRO-α in Hs294T melanoma cells, we demonstrate here that the Hs294T cells have higher IKK activity, which culminates in the constitutive phosphorylation of IκB-α. In addition, we show that the elevated constitutive IKK activity and enhanced phosphorylation of IκB-α lead to the increased constitutive transcription of MGSA/

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[3] The abbreviations used are: MGSA, melanocyte growth stimulatory activity; GRO, growth-regulated protein; NF, nuclear factor; IKK, IκB kinase; NIK, NF-κB-inducing kinase; RPE, retinal pigment epithelial; β-gal, β-galactosidase; GST, glutathione *S*-transferase; IL, interleukin; WT, wild type.

GRO-α in these cells. This is the first study to demonstrate elevated IKK activity or enhanced basal IκB-α phosphorylation in a solid tumor-derived cell line.

MATERIALS AND METHODS

Cells. Hs294T melanoma cells are a continuous cell line established from a human melanoma metastatic to the lymph node. These cells were obtained from American Type Culture Collection (Rockville, MD). RPE cells are normal retinal pigment epithelial cells that were cultured by Dr. Glen Jaffe from the North Carolina Organ Donor and Eye Bank within 24 h of death. The purity of RPE cell cultures was confirmed by cytokeratin staining as described previously (34). RPE and Hs294T cells were cultured as described previously (35).

Reagents. GST-IκB-α full-length (1-317) fusion protein and the antibodies to human IκB-α, IκB-β, IKK-α, and IKK-β were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific IκB-α antibody kit was purchased from New England Biolabs (Beverly, MA). Proteasome inhibitors MG115 and MG132 were purchased from CalBiochem-NovaBiochem Corp. (San Diego, CA).

Reporter and Expression Vectors. MGSA/GRO-α-LUC (350 bp) was constructed by inserting the promoter region (-306 to $+45$) of MGSA/GRO-α in pGL3-LUC reporter vector. NF-κB-LUC was constructed by inserting two consensus NF-κB elements in the minimal promoter pGL3-LUC reporter vector. The previously described dominant negative IKK-α constructs were kindly provided by David Goeddel at Tularik, Inc., South San Francisco, CA (20). Expression vectors encoding dominant negative forms of IκB-α have been described (36, 37). The pRSV β-gal reporter vector was purchased from Promega (Madison, WI).

Western Blot Analysis. Whole-cell lysates were obtained according to the standard protocol in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with Complete protease inhibitors (Boehringer Mannheim), phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride) and 100 μg/ml phenylmethylsulfonyl fluoride. Whole cell lysates (50 μg) were resolved on 13% SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad Laboratories.), and probed with the appropriate antibodies; the signal was visualized by enhanced chemiluminescence assay.

Immunoprecipitation and *in Vitro* Kinase Assays. To determine IKK activity, the whole-cell lysates were obtained in immunoprecipitation buffer [50 mM HEPES, (pH 7.6), 250 mM NaCl, 10% glycerol, 1 mM EDTA] containing 0.1% NP40 with protease and phosphatase inhibitors similar to the ones used to obtain the whole-cell lysate used for Western analysis. A 500-μg sample of the lysate was used for immunoprecipitation. The cell lysate was cleared and incubated for 2 h at 4°C with IKK-α antibody, at which time the A/G agarose-conjugated beads were added to the tube and incubated overnight at 4°C. The immunoprecipitates were washed extensively with immunoprecipitation buffer. *In vitro*, the kinase assays were performed with GST-IκB-α fusion full-length (amino acids 1–317) protein in 20 μl of kinase buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM DTT, 100 μM ATP, and 5 μCi of $^{\gamma\text{-}32}$P ATP (26). A portion of the immunoprecipitated IKK-α complex was incubated in the kinase buffer with 4 μg of the substrate for 30 min at room temperature. Samples were analyzed by 10% SDS-PAGE and autoradiography. The remaining portion of the immunoprecipitated samples were run on a separate 10% SDS-PAGE and were Western blotted with the IKK-α antibody to check for equality of loading.

Transfection and Reporter Activity Assay. Hs294T and RPE cells were plated in 60-mm dishes at a density of 2×10^5 cells. The following day, the 60% confluent cells were transfected with the respective constructs by the calcium phosphate method (38). Ten μg of the MGSA/GRO-α-LUC or NF-κB-LUC and 5 μg of the pRSV β-gal were used. The quantity of the expression vectors used are indicated in the respective figures. In each of the experiments, the total DNA transfected was kept constant by supplementation with puc19 DNA. The reporter gene activity was determined with the Luciferase Assay System (Promega) reagents and the use of Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). All of the values were normalized with β-gal expression (26) to correct for differences in transfection efficiency.

RESULTS

Constitutive IκB-α Phosphorylation in Hs294T Cells. ARPE cells are immortal variants of RPE cells. In prior studies (12), we used ARPE cells as the control cells for Hs294T melanoma cells and demonstrated that Hs294T cells have lower IκB-α protein due to an increased rate of degradation relative to ARPE cells (12). To override the differences that immortality may induce on these cells, we have used RPE cells as control cells in this study. Initially, to confirm that IκB-α protein levels also are higher in RPE cells, we performed Western blot analysis for IκB-α on whole-cell lysates. Similar to ARPE cells, RPE cells have much higher IκB-α than Hs294T cells (Fig. 1*A*, *Lane 1 versus Lane 2*). To evaluate the rate of degradation of IκB-α, the cells were treated with cycloheximide (10 mg/ml) to inhibit *de novo* protein synthesis, for 0, 15, 30, 45, 90, or 120 min, were lysed in RIPA buffer, and the whole-cell lysates were analyzed for IκB-α (Fig. 1*B*). RPE cells exhibited a slower rate of IκB-α degradation (86 min) compared with Hs294T cells (38 min). The higher basal IκB-α and slower rate of IκB-α degradation in RPE cells compared with Hs294T cells are consistent with the results obtained from ARPE cells (12).

Similar experiments with IκB-β indicated lower steady-state levels compared with IκB-α in both Hs294T and RPE cells. In addition, there were no differences in the basal level of IκB-β between RPE and Hs294T cells (data not shown). In both cell types, IκB-β was barely degraded over the 120 min time point tested (Fig. 1*C*).

Upon activation, IKK phosphorylates IκB-α at serine residues 32 and 36, targeting it to the ubiquitin-proteasome degradation pathway (39, 40). The more than 2-fold higher rate of IκB-α degradation in the Hs294T melanoma cells suggests that IκB-α in these cells may be

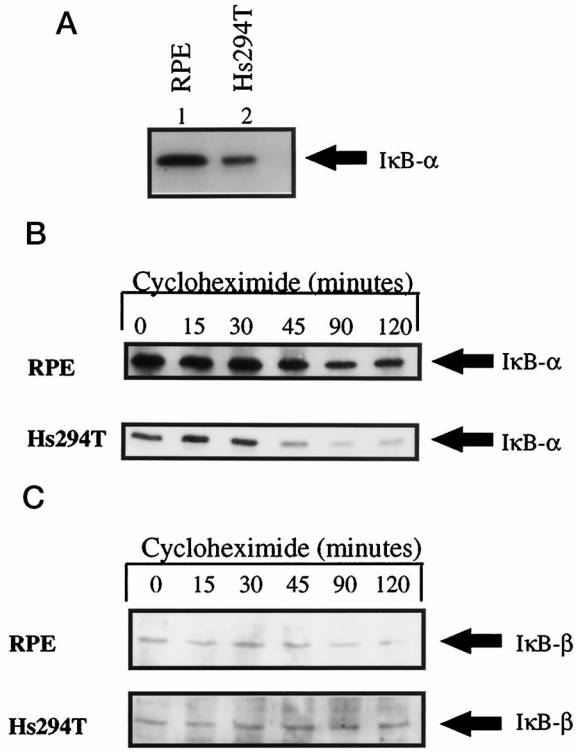

Fig. 1. Hs294T cells contain low levels of IκB-α due to an increased rate of degradation compared with RPE cells. Total cellular protein was solubilized from unstimulated Hs294T or RPE cells in RIPA buffer. Whole-cell extracts (50 μg) were analyzed for IκB-α (*A* and *B*) or IκB-β (*C*) by Western blotting using the respective antibodies. *B* and *C*, cells were treated with cycloheximide for time periods indicated to monitor half-life of the protein. There were no differences between RPE and Hs294T cells in the IκB-α protein level or its degradability. Blots for RPE and Hs294T cells were performed simultaneously and exposed to enhanced chemiluminescence for the same length of time to verify differences.

constitutively phosphorylated at Ser-32 and Ser-36. The phosphorylated form of IκB-α is a highly transient form of IκB-α and is traditionally known to be difficult to capture. The proteasome inhibitors MG115 and MG132 have been used extensively to inhibit the IκB-α degradation and to capture and visualize the phosphorylated form of IκB-α (40–42). Hence, to stabilize this phosphorylated form of IκB-α, RPE or Hs294T cells were treated with 50 μM MG115 or MG132 for 2 h, the whole-cell lysates were collected, and the IκB-α electrophoretic mobility was analyzed (Fig. 2*A*). As seen in Fig. 2*A*, Hs294T cells treated with either MG115 or MG132 contain a slower migrating form of IκB-α. However, the slower migrating form of IκB-α cannot be detected in RPE cells (Fig. 2*A*). To determine whether the slower migrating form of IκB-α represents constitutively phosphorylated IκB-α, we used a phospho-specific IκB-α antibody that can recognize the phosphorylated epitope at Ser-32 of IκB-α. RPE or Hs294T cells were pretreated with 50 μM MG115 or MG132 for 2 h, then subsequently treated with IL-1 for 30 min. The whole-cell lysates obtained from either treated or untreated control cells were Western blotted initially with phospho-specific IκB-α antibody. Consistent with our previous observations that Hs294T cells, but not RPE cells, have a constitutively phosphorylated IκB-α, the Western analysis with p-IκB-α antibody revealed that Hs294T cells have much higher quantities of IκB-α phosphorylated at Ser-32 (Fig. 2*B*, *Lane 4 versus Lane 1*). IL-1 can induce specific phosphorylations on IκB-α at Ser-32 and Ser-36 (43), and hence the IL-1-treated samples were used as positive controls (*Lanes 2*, *3*, *5*, and *6*). Also in agreement with our previous observation that IL-1 can activate NF-κB to a greater extent in RPE than in Hs294T cells (35), we noticed that IL-1-induced IκB-α phosphorylation in RPE is much higher than in Hs294T cells (Fig. 2*B*, *Lanes 2* and *3 versus Lanes 5* and *6*). The blot was normalized for loading with the use of IκB-α antibody. Longer exposure of the blot reveals the hyperphosphorylated, slow migratory IκB-α in this gel in *Lanes 2*, *3*, *4*, *5*, and *6*.

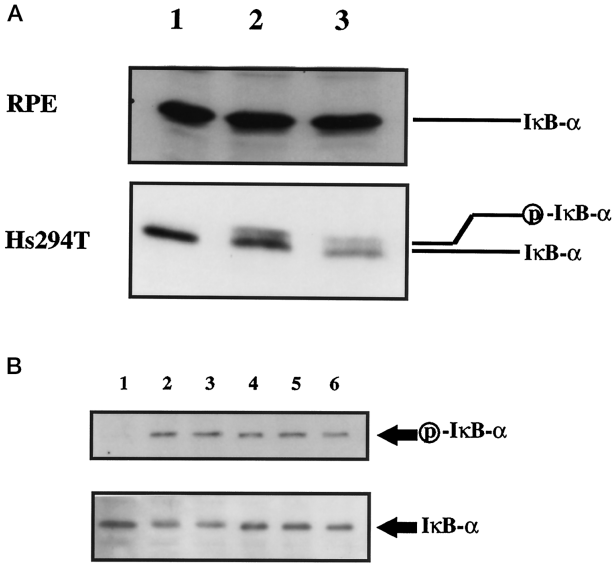

Fig. 2. Hs294T cells contain a constitutively phosphorylated IκB-α. *A*, RPE or Hs294T cells were treated with 50 μM of proteasome inhibitors MG115 or MG132 for 2 h, and whole-cell extract was collected in RIPA buffer as indicated in "Materials and Methods." Control cells contained vehicle in the media. Western blotting with IκB-α antibodies obtained from Santa-Cruz Biotechnology revealed the constitutive presence of a slow-migrating IκB-α in Hs294T cells only. *Lane 1* control; *Lane 2*, cells with MG115; *Lane 3*, cells with MG132. *B*, RPE or Hs294T cells were initially pretreated with 50 μM of proteasome inhibitors MG115 or MG132 and then were subsequently treated with IL-1. Control cells had the vehicle in the media. Whole-cell lysates in RIPA buffer were subjected to Western blotting with phospho-specific IκB-α antibody (New England Biolabs), which detected only phosphorylated IκB-α. The blot was normalized with IκB-α antibody (New England Biolabs) that recognizes both phosphorylated and nonphosphorylated forms of IκB-α. *Lane 1*, RPE control; *Lane 2*, RPE + IL-1 with MG115; *Lane 3*, RPE + IL-1 with MG132; *Lane 4*, Hs294T control; *Lane 5*, Hs294T + IL-1 with MG115; *Lane 6*, Hs294T + IL-1 with MG132.

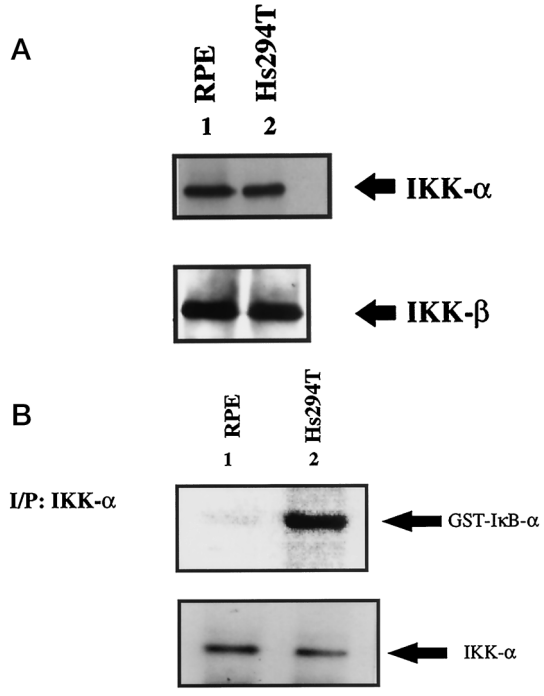

Fig. 3. Hs294T and RPE cells contain similar levels of IKK-α or IKK-β, but the activity of IKK is significantly higher in Hs294T cells. *A*, whole-cell lysates (50 μg) obtained from unstimulated RPE or Hs294T cells were Western blotted with IKK-α or IKK-β antibody; *top panel*, IKK-α ; *bottom panel*, IKK-β. *B*, 500 μg of whole-cell lysate obtained in nondenaturing immunoprecipitation buffer was immunoprecipitated with IKK-α antibody as outlined in "Materials and Methods." *In vitro* IKK kinase activity was measured with the immunoprecipitate using the full-length GST-IκB-α as the substrate according to the protocol described in "Materials and Methods." The *top panel* shows phosphorylated GST-IκB-α on an autoradiograph and represents the IKK activity. The blot was normalized by Western blotting with IKK-α antibody.

Elevated IKK Activity in Hs294T Cells. The activation of NF-κB by cytokines involves the successive action of NIK and IKK-α and IKK-β. Recently, it was shown that NIK can phosphorylate IKK-α specifically at Ser-176 in response to IL-1 or TNF, but the phosphorylation status of IKK-β is unaffected (26). Generally, these intermediates function in a transient manner. However, recent evidence that HTLV-1-infected T lymphocytes express constitutively active IKK suggested the possibility that these kinases potentially also could be expressed in a stable manner. To examine the alterations in the NF-κB-activating pathway in Hs294T cells that lead to increased phosphorylation and degradation of IκB-α, we initially assessed the level of steady-state IKK-α and IKK-β protein expression (Fig. 3*A*). In both RPE and Hs294T cells, the levels of IKK-α, or -β are almost equal. To evaluate any differences in the activity of this enzyme complex in Hs294T and RPE cells, we immunoprecipitated the IKK complex using a polyclonal IKK-α antibody. Immunoprecipitation with IKK-α antibody in the native conditions precipitated a large ~700-kDa complex (44). An IKK kinase assay was performed using full-length IκB-α GST fusion protein as the substrate. The kinase assay performed on these immunoprecipitates indicated that the activity of the IKK complex in Hs294T cells is much higher than in RPE cells (Fig. 3*B*). The observation that the activity of IKK is higher in Hs294T cells is parallel with our findings that IκB-α is constitutively hyperphosphorylated and is degraded more rapidly.

Dominant Negative IKK-α or Constitutive Inhibition of NF-κB Differentially Reduces the Basal MGSA/GRO-α Expression. We now know that Hs294T melanoma cells have higher nuclear levels of

NF-κB p65 as a result of elevated IKK activity that results in enhanced constitutive phosphorylation and subsequent rapid degradation of IκB-α compared with RPE cells. To determine whether this increased IKK activity and the resultant enhanced nuclear NF-κB is responsible for the increased MGSA/GRO-α transcription in Hs294T cells, we transiently cotransfected the dominant negative IKK-α mutants [IKK-α(KA)] or the repressors of NF-κB, the IκB-α WT or mutants (S/A, ΔN), along with 350-bp MGSA/GRO-α-LUC and β-Gal reporter constructs into Hs294T or RPE cells. The IKK-α(KA) dominant negative mutant contained alanine replacement at Lys-44 and has been shown to inhibit the basal or cytokine-induced NF-κB (20). The IκB-α S/A mutant has alanine inserted at Ser-32 and Ser-36 (36), and the ΔN IκB-α construct is a deletion mutant lacking amino acids 1–36 (36). The IκB-α WT, S/A, or ΔN constructs previously have been shown to inhibit the cytokine activation of NF-κB (36, 37). Cotransfection with the IKK-α dominant negative construct reduces the basal MGSA/GRO-α-LUC expression in a concentration-dependent manner in both Hs294T cells and RPE cells (Fig. 4), demonstrating that NF-κB is essential for its basal expression in these cells. At IKK-α(KA) plasmid concentrations of 0.5, 1, 5, or 10 μg in the transfection reaction, the basal MGSA/GRO-α-LUC was decreased to 25, 24, 17.5, and 11% of the control in RPE cells, whereas it was decreased to 48, 53, 38.5, and 38.5% of control in Hs294T cells, respectively. These results are consistent with our previous observations illustrating that mutation in the NF-κB element resulted in reduced expression of MGSA/GRO-α (17). However, the decrease in MGSA/GRO-α expression was always higher in RPE cells at any given concentration of IKK-α(KA) construct, suggesting that the higher IKK activity in Hs294T cells requires higher concentrations of IKK-α(KA) to elicit the same response as in RPE cells, where the activity of IKK is much lower. The difference in the decrease between RPE and Hs294T cells was always ~2-fold, which corresponds well with an ~2-fold greater rate of IκB-α degradation in Hs294T cells. These results provide a strong correlation between the activity of IKK, NF-κB, and the increased MGSA/GRO-α gene transcription in Hs294T melanoma cells.

Similar experiments with IκB-α WT (Fig. 5*A*) or IκB-α ΔN or S/A mutants (Fig. 5, *B* and *C*, respectively), demonstrated that the basal MGSA/GRO-α-LUC expression was dependent on NF-κB in both RPE and Hs294T cells. All three IκB-α constructs exhibited a concentration-dependent decrease in MGSA/GRO-α-LUC activity in both cell lines. However, the suppression of this MGSA/GRO-α expression was consistently greater in RPE cells relative to Hs294T cells. These results, taken in parallel with the IKK-α dominant negative cotransfection experiments, support our observations that the increased NF-κB in Hs294T cells leads to increased MGSA/GRO-α gene transcription.

DISCUSSION

It is well recognized that melanoma cells express elevated levels of MGSA/GRO-α; however, the underlying molecular mechanisms are poorly understood. Here we demonstrate that Hs294T melanoma cells, compared with RPE cells, exhibit an increased degradation rate of IκB-α due to an enhanced phosphorylation of the molecule by a constitutively active IKK. Furthermore, we show that this increased IKK activity leads to an enhanced basal MGSA/GRO-α transcription in Hs294T cells. The *rel* family of transcription factors traditionally has been known to be oncogenic (45–49), and it was recently demonstrated that tumor cells have increased NF-κB, thus protecting the cells from apoptosis (30–33). However, this is the first report to demonstrate that nonlymphoid tumor cells exhibit hyperphosphorylation of IκB-α with delineation of the signaling intermediates responsible for the constitutively elevated NF-κB in association with transformation.

IκB-α phosphorylation is a prerequisite for its ubiquitination (40). Although a hyperphosphorylated form of IκB-α was seen in Hs294T cells (Fig. 2*A*) in the Western blot with IκB-α antibody, it initially could not be confirmed whether the phosphorylations were in the N-terminal or PEST domain. The Western blotting with phospho-Ser-32 epitope-specific IκB-α antibody detected phosphorylated IκB-α in Hs294T cells but not in RPE cells (Fig. 2*B*). These data demonstrate that a significant portion of the IκB-α pool is phosphorylated at Ser-32. However, it is not clear whether only Ser-32 or both Ser-32 and Ser-36 are phosphorylated. IKK-α and IKK-β can phosphorylate both Ser-32 and Ser-36 with equal preference (20–25). The elevated IKK activity in these cells (Fig. 3*B*) suggests that the IκB-α may be phosphorylated at both serines. In addition, because phosphorylation at both serines is a prerequisite for the subsequent degradation, we predict that IκB-α is phosphorylated at both serines and not just Ser-32. However, this does not rule out the possibility of constitutive phosphorylations in the PEST domain in the IκB-α protein.

Although elevated NF-κB was reported earlier in tumor cells (27–29), it had not been shown that IκB-α is constitutively phosphorylated, which leads to its rapid degradation in any nonlymphoid system. However, Giri and Aggarwal (27) noticed a similar phenomenon in the human T-cell lymphoma HuT-78 cells, where NF-κB is constitutively up-regulated in these cells compared with Jurkat T-cell leukemia cells. The increase in NF-κB in HuT-78 cells was associated with a high basal level of IκB-α and a slower rate of basal IκB-α degradation rate in these cells. In addition, TNF was able to induce the IκB-α degradation in Jurkat T cells but not in HuT-78 cells, and antibodies to TNF reduced the basal NF-κB in HuT-78 cells. It seems that in HuT-78 cells, a continuous autocrine action of TNF up-regulated the NF-κB, which in turn may have increased the synthesis of IκB-α, which may have masked the degradation. At extended time points, blocking the NF-κB activation with cycloheximide treatment may have resulted in the decreased transcription of the IκB-α gene. Cycloheximide treatment blocks the *de novo* synthesis of the protein but not the post-translation modifications of MAD-3. Alternatively,

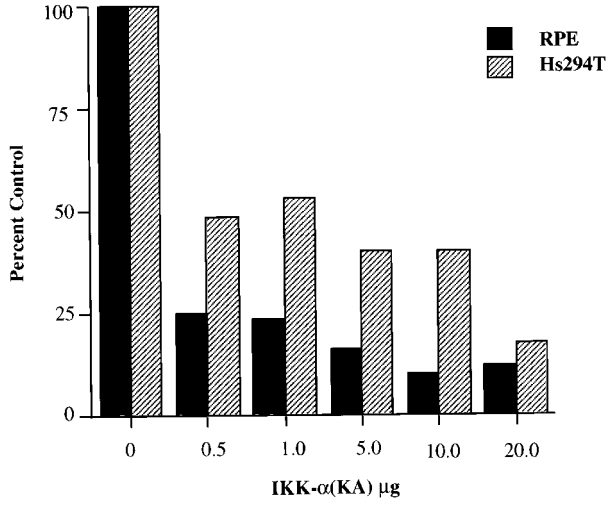

Fig. 4. Elevated IKK activity in Hs294T cells leads to increased MGSA transcription, and the dominant negative IKK-α vector differentially reduces basal MGSA/GRO-α-LUC transcription in RPE and Hs294T cells. Cells were cotransfected with increasing amounts (0–20 μg) of IKK-α(KA), a dominant negative mutant of IKK-α, and constant amounts of MGSA/GRO-α-LUC (10 μg) and β-gal (5 μg). Forty-eight h after transfection, the unstimulated cells were assayed for basal luciferase activity. Luciferase values were normalized with β-gal values, and the values are depicted as percent control, the control being the 0 μg of IKK-α(KA). The total quantity of the DNA was kept constant by adding puc19 DNA. The transfections with RPE and Hs294T cells were conducted simultaneously to avoid experimental variations. A representative of three independent experiments is shown. The qualitative results of all three experiments were similar.

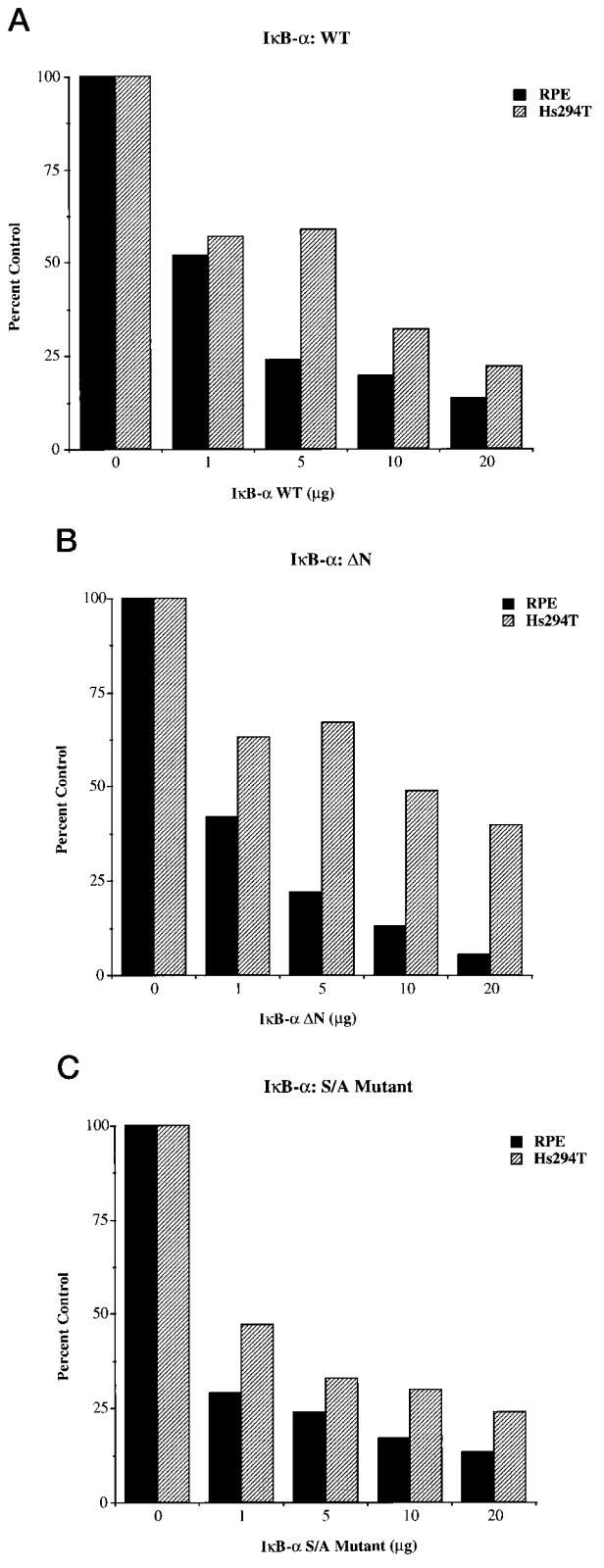

Fig. 5. Enhanced NF-κB activity in Hs294T cells leads to increased MGSA/GRO-α transcription, and the repressors of NF-κB differentially reduce basal MGSA/GRO-α-LUC transcription in RPE and Hs294T cells. Cells were cotransfected with increasing amounts (0–20 μg) of either puc19 or IκB-α WT (*A*), or IκB-α ΔN (*B*) or IκB-α S/A (*C*) constructs along with 10 μg of MGSA/GRO-α-LUC and 5 μg of β-gal plasmids. Cells were harvested 48 h after transfection and assayed for basal MGSA/GRO-α-LUC activity. Values were normalized for transfection efficiency with β-gal. Values are indicated as percent control, the control being the cells transfected with an equivalent amount of puc19 plasmid. The transfections with RPE and Hs294T cells were conducted simultaneously to avoid experimental variations. A representative of three independent experiments is shown. The qualitative results of all of the experiments were similar.

the differences in the mechanism for constitutive nuclear NF-κB activity in Hs294T melanoma cells *versus* HuT-78 lymphoid cells may be in the subunit composition of NF-κB, with consequent differences in the regulation of expression of various genes differentially (50). However, the HTLV-1-infected Jurkat T cells behave similarly to Hs294T melanoma cells in the mechanism of activation of the basal NF-κB. The Tax oncoprotein of HTLV-1 associates with IKK complex (29, 51, 52) and persistently activates IKK-α and IKK-β, leading to phosphorylation of IκB-α at Ser-32 and Ser-36, thus enabling a rapid degradation of IκB-α in these cells. Our findings here correspond well with the HTLV-1-infected Jurkat system and may underline a common phenomenon involved in the transformed cells.

The reduction in the basal MGSA/GRO-α-LUC activity by cotransfection with IKK-α(KA) or the IκB-α WT, S/A, or ΔN constructs (Figs. 4 and 5) was more significant in RPE than in Hs294T cells. This finding is likely explained by the fact that Hs294T cells have higher IKK activity and elevated NF-κB, and hence, more IKK-α(KA) or IκB-α is required to produce an equivalent decrease in the basal MGSA/GROα-LUC activity. Our observation that transfection with dominant negative IKK-α constructs or mutant IκB-α constructs reduces the basal MGSA/GRO-α-LUC activity indicates a direct role for elevated NF-κB in the enhanced basal transcription of MGSA/GRO-α. The differential reduction in the basal MGSA/GRO-α-LUC expression in Hs294T *versus* RPE cells upon cotransfection with IKK-α(KA) supports our observations of IKK-α activity differences between these two cell types. It also indicates that the increased constitutive MGSA/GRO-α in Hs294T cells is due to the increased IKK activity, leading to increased nuclear NF-κB activity.

In our present study, although we confirmed the activation of the IKK complex in Hs294T cells (Fig. 3*B*), we could not specifically conclude whether the increase in activity is a result of IKK-α or IKK-β activation or both. IKK-α and -β form homo- and heterodimers and then phosphorylate IκB-α. In our IKK-α immunoprecipitation experiments, we have effectively coprecipitated IKK-β (data not shown), which suggests the presence of IKK-α homodimers and IKK-α/IKK-β heterodimers. This complex shows higher endogenous activity in Hs294T cells compared with RPE cells. Cotransfection of Hs294T cells with IKK-α (KA) with MGSA/GRO-α-LUC blocks ~90% of the luciferase activity in RPE cells and ~80% of the luciferase activity in Hs294T cells at the highest concentration tested (Fig. 4). These data imply that, in theory, ~10% of the IKK activity in RPE cells and ~20% of the activity in Hs294T cells could be derived from IKK-β homodimers.

NF-κB is an important transcription factor that controls the expression of several inflammatory cytokines (53), chemokines (16), and enzymes that protect the cells from cell death (54). In addition, NF-κB plays a key role in the transcriptional regulation of several tumorigenic and angiogenic factors (19). More than 60% of the melanoma cells had increased mRNA of MGSA/GRO-α, IL-1β, IL-6, IL-7, and IL-8 mRNA (13, 14). Most of these genes contain a functional, cytokine-inducible NF-κB element in their 5′ regulatory regions and are up-regulated with proinflammatory cytokines. The increased IκB-α phosphorylation and degradation, due to the increased IKK activity in these cells, might be responsible for the increased expression of these genes in melanoma cells. Our experiments with MGSA/GRO-α reporter constructs indicate that, in fact, the elevated NF-κB in the Hs294T melanoma cells is responsible for the increased MGSA/GRO-α transcription. Although it is not known how the NF-κB is activated in the melanoma cells, we predict that one or more of the several cytokines activated in these cells act in an autocrine fashion and thus might cause the constitutive activation of NF-κB. Interestingly, with the mouse melanocyte model, we have observed that stable overexpression of MGSA/GRO-α results in constitutive activation of

NF-κB.[4] These findings raise the intriguing possibility that MGSA/GRO-α may regulate its own expression via an NF-κB-dependent autocrine loop.

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