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Nuclear Factor-κB Is Constitutively Active in C-Cell Carcinoma and Required for RET-induced Transformation[1]

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ABSTRACT

Specific point mutations of the *RET* proto-oncogene have been demonstrated to be responsible for multiple endocrine neoplasia (MEN) types 2A and 2B, for familial medullary thyroid carcinoma (MTC) syndromes, as well as for sporadic MTC. Here we show that nuclear factor (NF)-κB is activated in RET-associated C-cell carcinoma specimens. TT cells, a human MTC cell line expressing MEN 2A type RET, display transcriptionally active RelA(p65) in the nucleus. NF-κB activity in these cells is attributable to constitutive IκB kinase (IKK) activity and high turn over of IκBα. RET harboring the mutations C634R (MEN 2A) or M918T (MEN 2B), in contrast to wild-type RET, activates a NF-κB-dependent reporter construct upon transient transfection in HeLa cells. We show that the prototype RET mutation C634R enhances phosphorylation of IκBα by IKKβ but not by IKKα. RET-induced NF-κB and IKKβ activity requires Ras function but does neither involve the classical mitogen-activated protein kinase kinase/extracellular signal-regulated kinase nor the phosphoinositide 3-kinase/Akt pathways. In contrast, RET-induced NF-κB activity is dependent on Raf and MEKK1. Inhibition of constitutive NF-κB activity results in cell death of TT cells and blocks focus formation induced by oncogenic forms of RET in NIH 3T3 cells. These results suggest that RET-mediated carcinogenesis critically depends on IKK activity and subsequent NF-κB activation.

INTRODUCTION

The *ret* gene encodes a transmembrane receptor tyrosine kinase that is involved in development and malignant transformation of tissues derived from the neural crest (Refs. 1 and 2 and reviewed in Ref. 3). Activation of the RET receptor normally arises from the formation of a complex between RET and a member of the GDNF[3] family receptor α, which is in turn activated by ligand binding. According to the current model, a glycosyl-phosphatidylinositol-anchored GDNF family receptor α surface molecule mediates high-affinity ligand binding and thereby stimulates RET-tyrosine phosphorylation. Ligands for those receptor heterodimers include the transforming growth factor-β-related neurotrophic factors GDNF and neurturin (4). Qualitative and quantitative alterations of RET signaling have been shown to be associated with a variety of human disorders.

Congenital loss of functional RET because of specific point mutations leads to Hirschsprung's disease, a malformation characterized by the absence of autonomous enteric ganglia (Ref. 5 and reviewed in Ref. 6). Thus, RET signaling induced by neurotrophins plays an important role in neuronal migration during embryonal development. This is further supported by the observation that mice homozygous for disrupted *RET* or *GDNF* alleles display the respective intestinal neuron abnormalities in addition to a defect in kidney development (7, 8). On the other hand, constitutive RET kinase activity is associated with different forms of thyroid malignancy. Germ-line activation of the *ret* gene attributable to specific point mutations causes neoplastic transformation of the calcitonin-secreting thyroidal C-cells, also referred to as MTC. These tumors characterize three dominantly inherited tumor syndromes, *i.e.*, familiar MTC and the MEN syndromes types 2A and 2B (3, 9, 10). *ret* mutations observed in familial MTC and MEN 2A most commonly involve one of five extracellularly located cysteine residues and lead to ligand-independent homodimerization mediated by intermolecular disulfide bonding (11). By contrast, the MEN 2B mutation changes the methionine codon 918 in the kinase domain, thereby altering substrate specificity (12). Interestingly, the same mutation has been described in about one-third of sporadic cases of MTC (9). Another way to confer constitutive kinase activity to the RET protein is realized by chromosomal translocations giving rise to the *RET/PTC* oncogenes, which can be found in ~30% of papillary thyroid carcinomas. In those cases, constitutive activity of the RET kinase domain is attributable to the functional properties of the 5′ fusion partners (13).

Signal transduction processes induced by activated RET include binding of adaptor molecules such as Grb2, Grb7, Grb10, and Enigma, phosphorylation of Shc, as well as activation of PI3K, phospholipase Cγ, and the nonreceptor tyrosine kinase Src (reviewed in Ref. 14). Functional analysis of diverse neuroectodermal and non-neuronal cell lines have revealed that RET MEN 2A and 2B proteins can activate c-Jun NH_2-terminal kinases via Rho/Rac (15) as well as a Ras/mitogen-activated protein kinase signaling cascade (16). This suggests that AP1 and Elk1 transcription factors may be involved in RET mitogenic signaling. To further investigate nuclear events turned on by oncogenic RET, we focused on NF-κB, for which a contribution to the neoplastic transformation as well as an antiapoptotic function has emerged from the analysis of human tumor samples and cell lines (17, 18).

The NF-κB/Rel transcription factor family comprises NF-κB1(p105/p50), NF-κB2 (p100/p52), RelA(p65), RelB, and c-Rel, all of them sharing an NH_2-terminal Rel homology domain, which serves dimerization, DNA binding, and nuclear localization properties (reviewed in Ref. 19). NF-κB proteins classically representing p50/p65 heterodimers reside in the cytoplasm complexed to specific inhibitors termed IκBs (IκBα, IκBβ, and IκBε). Activation of NF-κB is mediated by IKK, a multiprotein complex composed of IKKα, IKKβ, and a regulatory IKKγ subunit (20). IKK induces phosphorylation of IκBα at serine residues 32 and 36, which triggers IκBα degradation by the 26S proteasome and release of NF-κB to the nucleus (19, 21). Activation of the IKK complex *in vitro* has been shown to be mediated by upstream acting kinases including NIK, NAK, as well as MEKK1 (22–26). In addition, NF-κB has been shown to be activated

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[3] The abbreviations used are: GDNF, glial cell line-derived neurotropic factor; MTC, medullary thyroid carcinoma; MEN, multiple endocrine neoplasia; Grb, growth factor receptor binding protein; PI3K, phosphoinositide 3-kinase; NF-κB, nuclear factor-κB; IKK, IκB kinase; NIK, NF-κB-inducing kinase; NAK, NF-κB-activating kinase; ERK, extracellular signal-regulated kinase; MEKK, mitogen-activated protein kinase/ERK kinase kinase 1; TNF, tumor necrosis factor; EMSA, electrophoretic mobility shift assay; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PVDF, polyvinylidene difluoride; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; β-Gal, β-galactosidase; GST, glutathione *S*-transferase; HA, hemagglutinin antigen.

through phosphorylation of serines located in the transactivating domain of the RelA(p65) subunit (27, 28).

Here we show that NF-κB represents a downstream target of oncogenic RET. RET-induced NF-κB activation depends on IKK-mediated IκBα degradation and requires functional Ras, Raf, as well as MEKK1. RET induced NF-κB activation is not accomplished by MEK/ERK proteins belonging to the classical mitogenic kinase cascade (29) nor by PI3K/Akt or p38, which are known to be involved in NF-κB activation (30, 31). The signal cascade described here suggests an IKK activation directly induced by Ras, Raf, and MEKK1. Consistent with the role of NF-κB in neoplastic transformation, we found that RET-induced transformation of fibroblasts can be ablated by inhibition of NF-κB. Furthermore, inhibition of NF-κB in MTC cells resulted in an apoptotic response. Thus, our data suggest that NF-κB-dependent transcription plays an essential role in the development of MTC induced by oncogenic RET.

MATERIALS AND METHODS

Thyroid Tissues. Thyroid tissue was obtained from 15 patients undergoing surgery for thyroid carcinoma at University Hospital (Halle, Germany). Histopathological analysis was performed by two independent pathologists. Studies involving the use of human tissue were approved by the Ethical Committee of the University of Halle, and all patients gave written consent.

Cell Culture and Treatments. TT MTC and HeLa cells were obtained from the American Type Culture Collection. Jurkat T cells were a generous gift from G. J. Nabel (Howard Hughes Medical Institute, University of Michigan, Ann Arbor, MI). TT cells and Jurkat T cells were grown in RPMI 1640, and HeLa cells were grown in DMEM. Medium was supplemented with 10% heat-inactivated FCS and 1% (w/v) penicillin/streptomycin. NIH 3T3 fibroblasts derived from a single clone ($B_2$5) were kindly provided by J. W. G. Janssen (32). These cells were maintained in DMEM, supplemented with 10% newborn calf serum and 1% (w/v) penicillin/streptomycin.

Recombinant human TNF-α and LY294002 were obtained from Sigma Chemical Co., Lactacystin and SB203580 were from Calbiochem, and PD98059 was from New England Biolabs. PD98059, LY294002, as well as Lactacystin were dissolved in DMSO, TNF-α was dissolved in medium, and SB203580 was dissolved in H_2O.

Plasmid Constructs. Generation of the NF-κB reporter gene (*3xκBIFNβLuc*) as well as the control plasmid (3xIFNβLuc) was described previously (33). Expression vectors for HA-IKKα and HA-IKKβ and dominant-negative mutants of IKKα(K44M), IKKβ(K44M), MEKK1Δ(K432M), GST-IκBα(1–54), and GST-IκBα(1–54AA) were generously provided by M. Karin (34). Expression vectors for N17 RAS and for a kinase-defective Raf-1 mutant (Raf-1 C4) were a kind gift of U. Rapp (35). The dominant-negative MEK1, MEK(S217A), was obtained from S. Cowley (36). The full-length human RET cDNA, kindly provided by M. Takahashi (Nagoya University School of Medicine, Nagoya, Japan), was subcloned into the *Hin*dIII and *Xba*I sites of pcDNA3 (Invitrogen) to generate pcDNA-RET. Point mutations C634R (MEN 2A) and M918T (MEN 2B) as well as Akt(K179A) and IκBα S32AS36A were introduced by primer-mediated, site-directed mutagenesis using the Quick Change Mutagenesis kit (Stratagene) to generate pcDNA-RET C634R, pcDNA-RET M918T pcDNA-Akt(K179A), and pcDNA-IκBα S32AS36A. Sequence confirmation of the respective point mutations was performed on both strands. Plasmid DNA was purified from bacterial cultures using the Endo Free Plasmid Extraction kit (Qiagen).

EMSAs. Preparation of nuclear and cytoplasmic protein extracts as well as EMSAs were performed essentially as described (37). Briefly, 5 μg of nuclear proteins were incubated with 5 μg of poly(deoxyinosinic-deoxycytidylic acid) and 40,000–80,000 cpm of labeled oligonucleotides for 30 min at 4°C. Samples were analyzed by PAGE on 5% native gels. Supershift assays were performed with polyclonal antibodies against NF-κB1(p50), NF-κB2(p52), RelA(p65), RelB, and c-Rel (Santa Cruz Biotechnology). Antibodies were added to the reaction mixtures at a concentration of 1 μg/20 μl. Incubation was performed at 4°C for 30 min.

Transfections and Luciferase Assays. Transfection of HeLa cells was performed by the calcium phosphate precipitation method (38). NIH 3T3 cells were transfected using FUGENE (Boehringer), unless indicated. TT cells were transfected using Lipofectamine reagent (Life Technologies, Inc.). Total amounts of transfected DNA were equalized with empty expression vector pcDNA3 where needed. Luciferase assays were performed with the Luciferase Assay System (Promega Corp.) according to the manufacturer's instructions. Relative light units were measured using a Lumat 9105 luminometer (Berthold Analytical Instruments).

X-Gal Stain of Transfected Cells. Seventy-two h after transfection, cells were washed with PBS, fixed with 1.25% glutaraldehyde for 5 min at room temperature, and washed again. Subsequently, cells were incubated with staining solution (50 mM Tris-HCl, 15 mM NaCl, 1 mM $MgCl_2$, 2.5 mM freshly prepared potassium ferricyanide/potassium ferrocyanide, and 5 mg/ml X-Gal) for 4–8 h at 37°C, and β-galactosidase-positive cells were counted in each well.

Western Blotting. Cytoplasmic protein extracts or total cell lysates were boiled in 2× SDS sample buffer (100 mM Tris-HCl, 4% SDS, 0.2% bromphenol blue, 20% glycerol, and 10% DTT) and separated by SDS-PAGE in 6 or 10% polyacrylamide gels. Proteins were transferred onto 0.2 μm PVDF membranes (Millipore) by semidry blotting. Membranes were blocked with PBS/0.1% Tween 20 (PST) containing nonfat dry milk or BSA and incubated with primary antibodies either at room temperature for 2 h or overnight at 4°C. After washing in PST for 45 min, blots were incubated with horseradish peroxidase conjugates of the appropriate secondary antibodies (Amersham) for 45 min at room temperature and washed again. Subsequently, complexes were visualized using enhanced chemiluminescence reagents (ECL; Amersham). Antibodies against IκBα(C-21), IκBβ(N-20), IKKα(B-8), IKKβ(C-20), RET(C-19), ERK1/2, Akt (Santa Cruz Biotechnology), p38, phospho-p38, phospho-ERK1/2, phospho-Akt, phospho-IκBα (New England Biolabs), and anti-HA (Berkeley Antibody Corporation) were used in this study.

Immunofluorescence. Preparation of tissue sections was performed as described previously (39). Frozen sections or cells seeded in chamber slides were air dried and fixed with 4% methanol-free formalin at room temperature for 15 min. After washing with PST, samples were permeabilized with 0.1% Triton X-100 in PBS for 5 min, rinsed twice with PST, and incubated in blocking solution (PBS containing 5% BSA and 3% normal goat serum) for 30 min at room temperature. Primary antibodies anti-p65 (Boehringer) and anti-chromogranin A (DAKO) were diluted in blocking solution 1:200 and 1:1000, respectively. Incubation was performed overnight at 4°C in a humidified chamber. After washing five times for 5 min in PST, sections were incubated with FITC-conjugated (Alexa 488; Molecular Probes) or Cy3-conjugated (Dianova) secondary antibodies, diluted 1:1500 for 1 h in a dark humidified chamber, followed by five washes for 5 min. Thereafter, sections were mounted and kept dark until analysis with a confocal microscope (Leica, TCS 4D, Germany).

TUNEL Assay. Detection of apoptotic cells by fluorescein-dUTP mediated nick end labeling of fragmented DNA was performed using the TUNEL kit (Boehringer) according to the manufacturer's instructions.

IκB Kinase Assays. NIH 3T3 cells were plated at a density of 2.5×10^5 per 35-mm well. Forty-eight h after transfection, cells were lysed for 10 min at 4°C in a buffer containing 150 mM NaCl, 25 mM HEPES (pH 8.0), 25 mM sodium PP_i, 50 mM β-glycerophosphate, 10% glycerin, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin. After centrifugation at 14,000 × *g* for 5 min, supernatants were incubated with anti-HA monoclonal antibody at 4°C for 1 h and for another 45 min after addition of 20 μl of protein A/G-agarose. The immunoprecipitates were washed two times with lysis buffer and twice with a kinase buffer containing 150 mM NaCl, 25 mM HEPES (pH 7.5), 25 mM β-glycerophosphate, and 10 mM $MgCl_2$. Immunocomplex kinase assay was performed using GST-IκBα(1–54) or GST-IκBα(1–54AA) as substrate, which were prepared as described by Baumann *et al.* (40). Immunoprecipitates were reacted with 1 μg of the respective substrate in kinase buffer containing 200 μM ATP and 5–10 μCi of [γ-^{32}P]ATP at 30°C for 20 min. Reactions were stopped by addition of SDS-PAGE sample buffer, and products were resolved on 12% gels, transferred onto PVDF membranes (Millipore), and subsequently quantified using a PhosphorImager (Molecular Dynamics) or autoradiographed.

Focus-forming Assays. NIH 3T3 cells were seeded at a density of 1.5×10^5 per 100-mm dish 12 h before transfection. Per plate 250 ng to 1 μg of the respective mammalian expression constructs were stably transfected by the calcium phosphate precipitation method using 20 μg of high molecular

Table 1 *RET mutation status of 16 C-cell carcinoma specimens examined in this study*

RET mutation analysis as well as genetic data concerning the 16 C-cell carcinoma probes are presented in correlation to the results of nuclear p65 staining (see Fig. 1).

	RET C634R	RET M918T	No RET mutation	Nuclear Rel(p65)
MEN 2A	2			2/2
MEN 2B		3		3/3
FMTC[a]	1			1/1
Sporadic		3		3/3
			7	2[b]/7

[a] FMTC, familial MTC.
[b] Metastasis.

genomic DNA derived from NIH 3T3 cells as carrier (32). Twelve h after transfection, the medium was changed to remove the precipitate, and after an additional 24 h, medium was replaced by DMEM supplemented with 5% newborn calf serum. Medium was changed every 5 days, and transformed foci were counted by phase contrast microscopy 2–3 weeks after transfection.

RESULTS

RelA(p65) Is Localized in the Nucleus of C-Cell Carcinoma Cells. C-cell carcinoma samples (presented in Table 1), including adjacent normal thyroid tissue as well as TT-human MTC cells, were stained with anti-RelA(p65). Because this antibody is directed against the nuclear localization signal that is unmasked upon dissociation of p65 from IκBα, immunofluorescence is specific for activated NF-κB. Eleven of 16 C-cell carcinomas displayed nuclear p65, whereas no p65 staining was seen in normal thyroid tissue (Fig. 1*A*). All tumors for which a RET mutation had been documented as well as the TT cell line which bears a MEN 2A type RET mutation (41) showed p65 localized to the nucleus (Fig. 1, *C–F*). By contrast, no p65 signal was obtained in nuclei from tumor specimens lacking RET mutations (Fig. 1*B*), except for two samples representing metastases of sporadic C-cell carcinomas. All of the carcinoma samples as well as the TT cell line strongly stained positive for chromogranin A, serving as an endocrine differentiation marker.

Nuclear NF-κB Is Composed of p50/p65 Heterodimers in TT Cells. TT cells display functional and morphological characteristics of C-cell carcinoma, including the expression of chromogranin as well as a continuous secretion of calcitonin under cell culture conditions (42). Therefore, we used this cell line as an *in vitro* model system to investigate the mechanism of NF-κB activation in RET-induced MTC. We analyzed these cells by EMSA using an oligonucleotide containing the NF-κB consensus recognition site. As seen in Fig. 2*A*, TT cells displayed high levels of constitutive NF-κB DNA binding activity, which was further increased in response to TNF-α. By contrast, NF-κB binding activity in Jurkat T cells was detected only upon treatment with TNF-α. The subunit composition of the NF-κB complexes was analyzed by supershift assays (Fig. 2*B*). Addition of anti-p50 as well as anti-p65 to nuclear extracts of unstimulated or TNF-α-treated TT cells retarded the NF-κB-DNA complex, indicating that NF-κB is composed predominantly of p50 and p65 subunits. Transcriptional activity of these complexes was determined using the NF-κB-dependent reporter construct 3xκBIFNβLuc and yielded an up to 200-fold stimulation as compared with the vector without κB sites (not shown).

Constitutive NF-κB Activity in TT Cells Is Attributable to Lack of IκBα and Can Be Inhibited by Introduction of IκBα S32AS36A. Because activation of NF-κB directly depends on proper function of IκBs, we asked whether the high amounts of nuclear NF-κB seen in TT cells were the result of abnormalities in expression

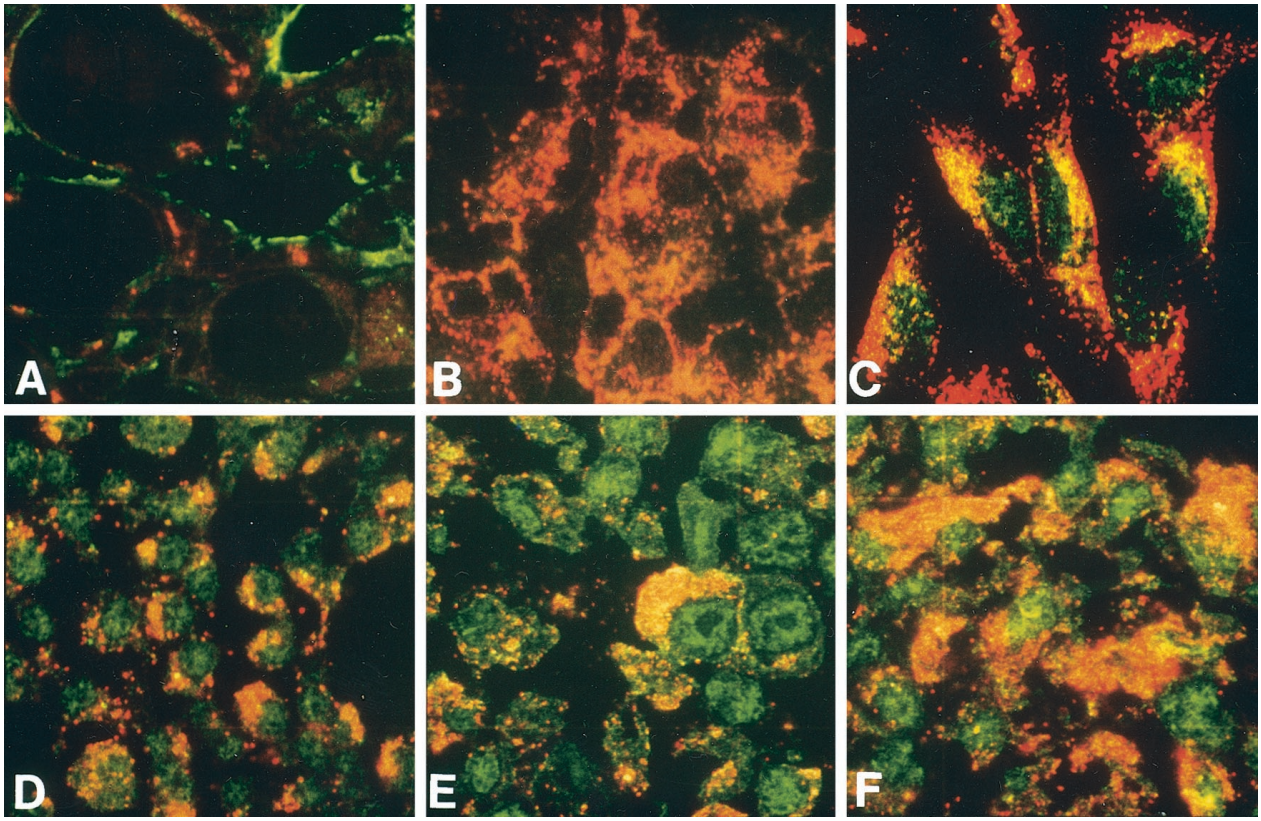

Fig. 1. RelA(p65) is localized in the nucleus of C-cell carcinoma cells. Sporadic and MEN 2-associated C-cell carcinoma as well as adjacent normal thyroid tissue samples were stained with an anti-p65 antibody that only recognizes active RelA(p65; *green* fluorescence). In addition, sections were stained with an antibody directed against chromogranin A, an endocrine differentiation marker (*red* fluorescence). *A*, normal thyroid tissue. *B*, C-cell carcinoma negative for nuclear RelA(p65). *C*, TT medullary carcinoma cell line. *D–F*, C-cell carcinoma specimens displaying nuclear RelA(p65).

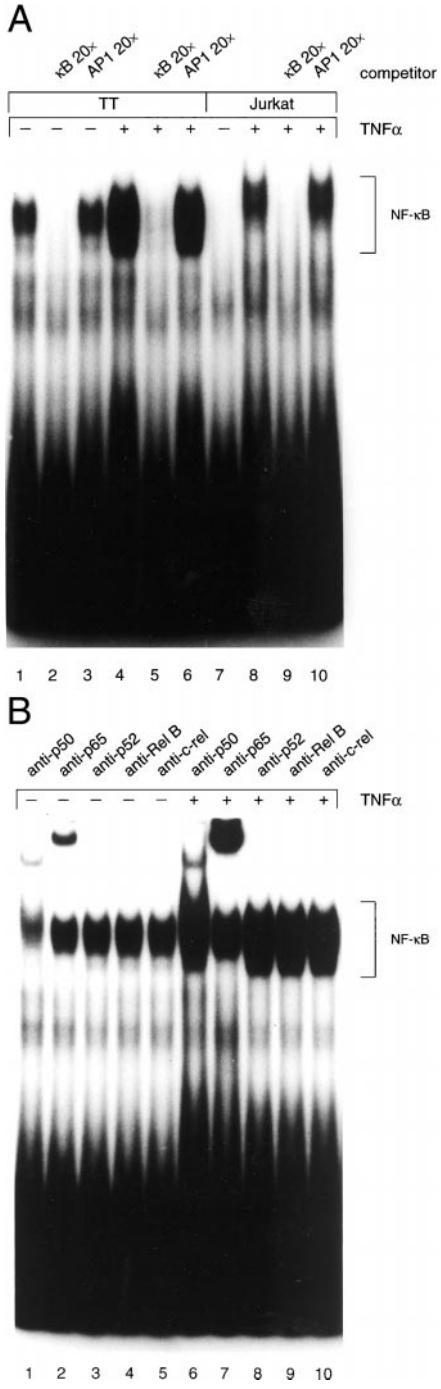

Fig. 2. NF-κB is constitutively active in TT MTC cells. *A*, electromobility shift assay showing constitutive NF-κB binding activity in TT cells. Nuclear extracts derived from untreated as well as TNF-α-stimulated (150 units/ml) TT and Jurkat T cells were reacted with a γ-^{32}P-labeled DNA probe encompassing the κB motif of the mouse κ light chain enhancer. Specificity of NF-κB binding activity was confirmed by competition experiments using 20-fold excess of the unlabeled NF-κB probe (*Lanes 2*, *5*, and *9*) or an oligonucleotide spanning the consensus AP1 binding site (*Lanes 3*, *6*, and *10*). *B*, supershift assay identifying the subunit composition of NF-κB complexes. Antibodies against NFκB1(p50), RelA(p65), NF-κB2(p52), RelB, and c-Rel were added to nuclear extracts of untreated and TNF-α-stimulated (150 units/ml) TT cells as indicated.

of IκB proteins. Immunoblotting of whole-cell extracts derived from TT cells before and after stimulation with TNF-α revealed an IκBα band of the expected size, albeit barely visible. In contrast, untreated Jurkat T cells displayed readily detectable levels of IκBα protein (Fig. 3*A*). One possibility to explain the relative lack of IκBα, despite abundant NF-κB, is a disturbed interaction between these two proteins. To clarify whether functional IκBα was able to inhibit NF-κB in TT cells, cotransfections with the NF-κB reporter and an IκBα S32AS36A expression construct were performed. This IκBα mutant failed to undergo signal-induced degradation because of substitution of serines 32 and 36 with alanines (21). As shown in Fig. 3*B*, expression of IκBα S32AS36A nearly ablated the κB-dependent reporter activity, suggesting a functional relationship between IκBα and NF-κB in TT cells.

Lactacystin Restores IκBα Function and Inhibits Endogenous NF-κB Activity in TT Cells. To further rule out the possibility that enhanced IκBα degradation was attributable to alterations of IκBα protein, we incubated TT cells with the proteasome inhibitor Lactacystin. Although untreated TT cells displayed an IκBα band on immunoblots only after long exposure times, IκBα became clearly visible in these cells upon treatment with Lactacystin. Furthermore, stimulation with TNF-α led to the appearance of an additional band representing serine-phosphorylated IκBα (Fig. 4*A*), suggesting that proteasomal degradation is the reason for the lack of IκBα in TT cells. In contrast, IκBβ was not affected by neither treatment (not shown). The functional relevance of this IκBα stabilization was confirmed by EMSA (Fig. 4*B*).

TT Cells Display Constitutive IκB Kinase Activity. IκBα has been shown to be directly phosphorylated by catalytically active IKKα and IKKβ subunits of the IKK complex (20). Endogenous IKKα/IKKβ activity in TT cells was measured by immunocomplex kinase assays using an antibody directed against IKKα. Precipitates from unstimulated TT cells revealed strong IκB kinase activity comparable with those of TNF-α-stimulated Jurkat T cells (Fig. 5). The differences observed were not attributable to unequal precipitation efficiency, because the same membranes were immunoblotted with anti-IKKα. The integrity of the precipitated complex was confirmed by subsequent incubation with an IKKβ-specific antibody (not shown).

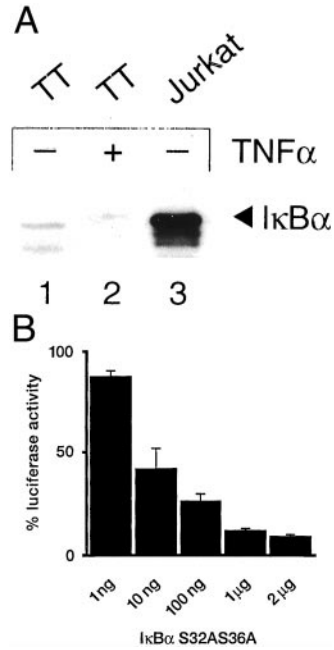

Fig. 3. NF-κB activation in TT medullary carcinoma cells is attributable to low levels of IκBα and can be inhibited by degradation-resistant mutant IκBα S32AS36A. *A*, 15 μg of cellular extracts derived from untreated or TNF-α-stimulated TT cells were analyzed by immunoblotting using an anti-IκBα monoclonal antibody. Cellular extract of unstimulated Jurkat T cells served as positive control. *B*, NF-κB activity in TT cells can be inhibited by IκBα S32AS36A. TT cells were transiently cotransfected with 3xκBIFNβLuc and increasing amounts of IκBα S32AS36A as indicated. Total DNA was adjusted to 7 μg with empty pcDNA3. Seventy-two h after transfection, cells were lysed for luciferase assay. Values represent means of three independent experiments; *bars*, SE.

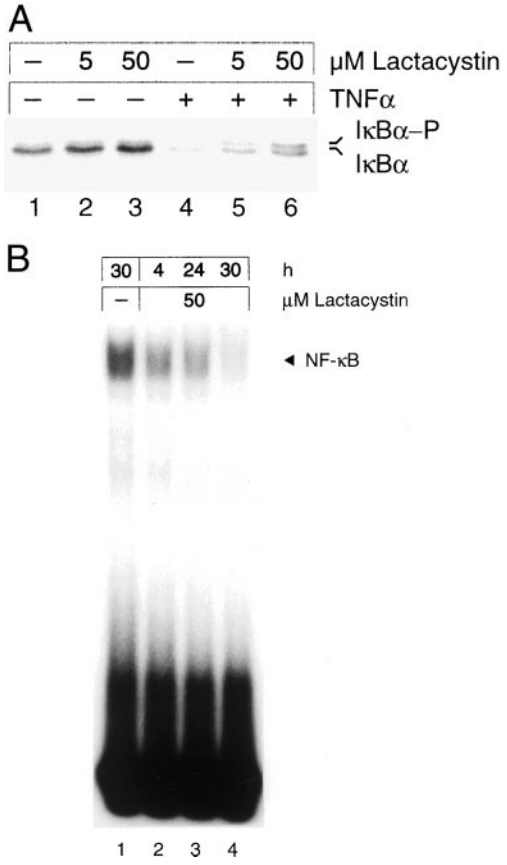

Fig. 4. NF-κB activation in TT medullary carcinoma cells is attributable to high turnover of IκBα. TT cells were incubated for 30 h with or without TNF-α (150 units/ml) and the proteasome inhibitor Lactacystin at the indicated doses. Equal amounts of cytoplasmatic extracts were assayed for IκBα (*A*) by immunoblotting. Nuclear extracts derived from Lactacystin-treated TT cells were assayed for NF-κB DNA binding activity by EMSA (*B*).

Oncogenic RET Activates NF-κB-dependent Transcription. Because TT cells display constitutive NF-κB activity without exogenous stimulation, NF-κB activity in these cells is supposed to be a consequence of alterations in intracellular signal transduction. TT cells express a constitutively activated RET receptor bearing a MEN 2A-type RET mutation at cysteine 634 (41). Therefore, we investigated the possible relevance of this mutation with respect to the observed NF-κB activation. For that purpose, we cotransfected expression vectors encoding MEN types 2A and 2B mutant RET versions as well as wild-type RET along with the 3xκBIFNβLuc reporter in HeLa cells. Although MEN 2A RET (C634R) and MEN 2B RET (M918T) vectors were able to stimulate NF-κB-dependent luciferase activity 19- and 14-fold, respectively, the wild-type RET construct exerted no effect (Fig. 6*A*). Cotransfection of IκBα S32AS36A with the oncogenic RET versions abolished RET-induced κB-dependent reporter activity. This suggests that in HeLa as well as in TT cells, oncogenic RET proteins induce NF-κB transcriptional activity through stimulating nuclear translocation of NF-κB.

RET C634R Activates Kinase Activity of IKKβ but not IKKα. To further elucidate the NF-κB activation induced by oncogenic RET, we performed IκB kinase assays in NIH 3T3 cells. Therefore, cells were transiently cotransfected with RET C634R or empty vector along with HA-tagged expression constructs bearing either IKKα or IKKβ. Although RET C634R was able to markedly stimulate IKKβ activity, we failed to detect any effect on IKKα, as determined by phosphorylation of GST-IκBα(1–54). The specificity of kinase activity was demonstrated using GST-IκBα(1–54AA) as substrate, which is mutated at serines 32 and 36 and cannot be phosphorylated by IKK. Thus, *in vitro* RET C634R exclusively increases activity of IKKβ but not IKKα (Fig. 7).

RET Signals via Ras and Raf to Activate IKKβ and NF-κB. Because Ras and Raf were reported to be downstream targets of RET, we investigated whether these proteins would provide the link between RET and IKKβ. To address this question, immunocomplex kinase as well as luciferase assays using dominant-negative mutants of Ras (N17 Ras) or Raf (Raf-C4) were performed in NIH 3T3 cells. Both of these mutants were able to inhibit RET C634R-induced IKKβ activity in a dose-dependent manner (Fig. 8*A*). Similarly, cotransfection of RET C634R with N17 Ras or Raf-C4 led to a significant inhibition of NF-κB-dependent reporter activity (Fig. 8*B*). Next, we tried to determine whether constitutive NF-κB activity in TT cells, endogenously expressing activated RET, would depend on a similar signaling pathway. As seen in Fig. 8*C*, these two constructs inhibited NF-κB-dependent luciferase activity in TT cells, suggesting that Ras as well as Raf is involved in constitutive NF-κB activation in this MTC cell line.

p38-Kinase, MEK1/ERK1/2-Kinase, and PI3K/Akt-Kinase Cascades Do Not Contribute to Constitutive NF-κB DNA Binding and Transcriptional Activity in TT Cells. p38-kinase, MEK1/ERK1/2, and PI3K/Akt are known to be involved in NF-κB activation under certain conditions. Furthermore, PI3K/Akt-kinase as well as MEK1/ERK1/2-kinase cascades are activated by Ras and Raf, respectively. To determine whether constitutive NF-κB activity was mediated by one of these signal cascades, TT cells were incubated with the specific p38 inhibitor SB203580, the MEK1 inhibitor PD98059, and the PI3K inhibitor LY294002. Although immunoblots performed with phospho-specific antibodies confirmed activity as well as dose-dependent inhibition of the respective signaling cascades (not shown), corresponding nuclear extracts failed to demonstrate a decrease in NF-κB DNA binding activity (Fig. 9). A possible influence on NF-κB transcriptional activity exerted by these inhibitors was ruled out by performing transfection assays with the 3xκBIFNβLuc reporter (not shown). Thus, constitutive NF-κB activity is not a consequence of p38, MEK1/ERK1/2, or PI3K/Akt activities in TT cells.

Dominant-Negative Mutants of MEKK1Δ, IKKα, and IKKβ Inhibited NF-κB-dependent Transcription in TT Cells. Neither Ras nor Raf proteins thus far have been demonstrated to directly

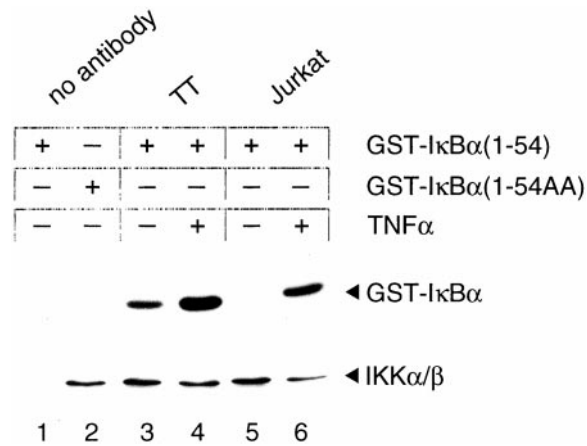

Fig. 5. TT cells display constitutive IKK activity. Endogenous IKK activity was precipitated with an anti-IKK monoclonal antibody. Immunocomplexes were assayed for kinase activity by incubation with 0.5 μg of GST-IκBα(1–54) in the presence of [γ-^{32}P]ATP, resolved on 12% gels, and transferred onto PVDF membranes. After autoradiography (*top* part), membranes were immunoblotted to determine the content of precipitated IKK (*bottom* part). Lysate without antibody (*Lane 1*) and GST-IκBα(1–54AA), which cannot be phosphorylated by IKK (*Lane 2*), served as controls.

induce IKK phosphorylation. To further characterize signals responsible for constitutive NF-κB activity in TT cells, we investigated the role of MEKK1, which has been shown recently to be required for IKKβ activation induced by oncogenic Raf (40). Transfection of a

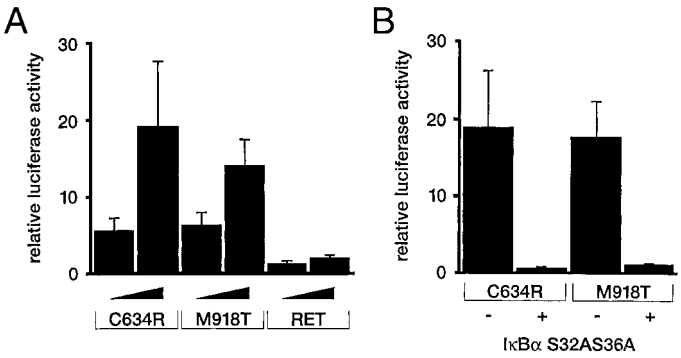

Fig. 6. Oncogenic RET induces NF-κB-dependent transcription. *A*, HeLa cells were transiently transfected with the 3xκBIFNβLuc reporter (5 μg/35-mm well) together with expression vectors encoding oncogenic RET mutants C634R and M918T or wild-type RET (1 or 3 μg, respectively). Forty-eight h after transfection, cells were lysed for luciferase assay. *Columns*, fold induction of luciferase activity relative to the reporter alone and represent means of four independent experiments performed in triplicate; *bars*, SE. *B*, cotransfection of IκBα S32AS36A abolishes NF-κB activation induced by oncogenic RET in HeLa cells. Cells were treated as described above. Three μg of the respective RET mutants were cotransfected with 3 μg of IκBα S32AS36A. Values represent means of two independent experiments performed in triplicate; *bars*, SE.

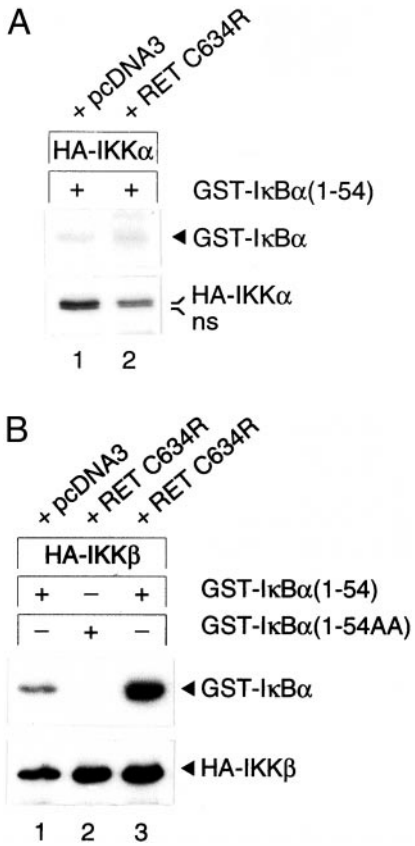

Fig. 7. Activation of IKKβ but not IKKα is induced by oncogenic RET. *A*, NIH 3T3 cells were transiently transfected with 3 μg of HA-IKKα along with an expression vector of RET C634R or empty pcDNA3 (3 μg each). Immunocomplexes were precipitated with anti-HA monoclonal antibody and assayed as described in "Materials and Methods." Kinase activity as determined by autoradiography of radiolabeled GST-IκBα is shown in the *top* part; amounts of precipitated IKKα were verified with an anti-HA monoclonal antibody on the same membrane (*bottom* part). *B*, cotransfection of NIH 3T3 cells with HA-IKKβ along with RET C634R or empty pcDNA3. Kinase assay and visualization of bands were performed as described above.

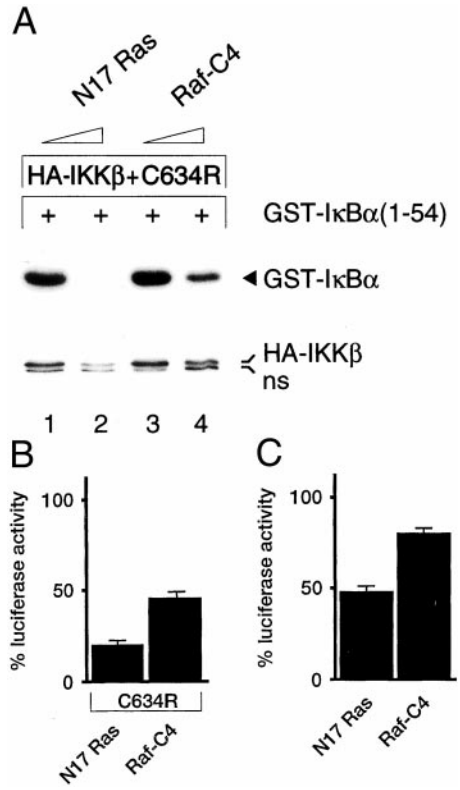

Fig. 8. Effect of dominant-negative mutants of Ras or Raf on RET C634R-induced NF-κB activation and IKKβ activity. *A*, dose-dependent inhibition of RET C634R-induced IKKβ activity by dominant-negative Ras or Raf. Dominant-negative mutants of Ras or Raf (1 or 5 μg, respectively) were transiently cotransfected with 3 μg of RET C634R in NIH 3T3 cells as indicated. Kinase assay was performed as denoted above. *B*, NIH 3T3 cells were transfected with 1 μg of 3xκBIFNβLuc along with 3 μg of RET C634R and the indicated expression constructs (4 μg each). Values relative to RET C634R (as described for Fig. 6) represent means of three independent transfections performed in triplicate; *bars*, SE. *C*, TT cells were transfected with 3 μg of 3xκBIFNβLuc along with the indicated expression constructs (4 μg each). Values relative to pcDNA3 represent means of three independent transfections performed in triplicate; *bars*, SE.

kinase-inactive MEKK1Δ(K432M) almost completely blocked NF-κB-dependent transcription in TT cells. Consistent with this, dominant-negative IKKβ(K44M) significantly inhibited NF-κB activity, whereas dominant-negative IKKα(K44M) had only little effect on NF-κB-dependent transcription in this assay (Fig. 10). This suggests that MEKK1Δ directly or indirectly transmits an activating signal from Ras/Raf to IKK. Furthermore, IKK activity in TT cells seems to depend largely on IKKβ function.

Inhibition of IκBα Degradation with Lactacystin Induces Apoptosis in TT Cells. Inhibition of NF-κB activity has been shown to result in apoptosis in several cell types (43, 44). One important role of constitutive NF-κB activity in TT cells may be to continuously deliver antiapoptotic signals. To test the effect of NF-κB withdrawal in TT cells, proteasome-mediated IκBα degradation was blocked by incubation with Lactacystin. This treatment resulted in 50% apoptotic cell death as determined by TUNEL staining, although no fluorescence could be detected in untreated controls (Fig. 11, *A* and *B*).

Inhibition of NF-κB Elicits a Cell Death Response in TT Cells. To confirm an antiapoptotic function in TT cells, specifically linked to NF-κB activity, we performed cell viability assays using β-Gal expression. Cotransfection of the dominant-negative mutants of IKKα, IKKβ, and MEKK1Δ, as well as IκBα used in our previous experiments (Figs. 3*B* and 10), together with pcDNA3*LacZ*, led to a significant decrease in numbers of β-Gal-positive cells (Fig. 11*C*). Except for IKKα, which was less effective, all inhibiting mutants led to an ~50% reduction of viable cells as compared with the control trans-

fected with vector alone. The tight correlation between the effects on NF-κB-dependent transcription and cell viability exerted by these mutants suggests that constitutive NF-κB activity represents an important survival factor for TT cells.

NF-κB Inhibition Blocks Focus Formation of NIH 3T3 Cells Induced by Oncogenic RET. To determine whether NF-κB is required for RET-induced cellular transformation, we asked whether the inhibition of NF-κB would affect the ability of RET C634R or RET M918T to cause morphologically transformed foci in cultured NIH 3T3 fibroblasts. Coexpression of oncogenic RET with the super repressor form of IκBα blocked RET-induced focus formation by >50% (Fig. 11*D*), indicating that cellular transformation induced by oncogenic RET is dependent on NF-κB activity.

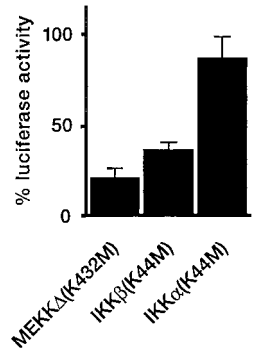

Fig. 10. Effect of dominant-negative MEKK1Δ, IKKβ, or IKKα on NF-κB activity in TT cells. TT cells were transiently cotransfected with 3 μg of 3xκBIFNβLuc along with the indicated expression constructs (4 μg each). Values relative to pcDNA3 represent means of three independent transfections performed in triplicate; *bars*, SE.

DISCUSSION

In this study, we have identified NF-κB as a downstream target of a signaling pathway initiated by oncogenic RET. This assumption is based on the following findings: (*a*) nuclear localization of RelA(p65) is found in tissue specimens of MEN 2-associated and sporadic C-cell carcinomas but not in adjacent normal thyroid tissue; (*b*) oncogenic RET (MEN 2A- and MEN 2B-type) activates a NF-κB-dependent reporter construct in HeLa cells; (*c*) TT, a human MTC-derived cell line expressing MEN 2A-type RET, displays constitutive NF-κB binding and transcriptional activity; (*d*) Overexpression of RET MEN 2A enhances IKKβ-mediated phosphorylation of IκBα.

The analysis of 16 human MTC specimens revealed a strong correlation between expression of a mutated RET receptor and nuclear staining for p65, suggesting that NF-κB is a target of RET signaling in these cells. Surprisingly, NF-κB activation was observed in both MEN 2A cases as well as in MEN 2B cases. This was not expected because MEN 2A-type and MEN 2B-type mutations differ in their mechanism of activation. Although MEN 2A mutations induce the formation of disulfide-linked receptor dimers, through unpaired cysteines that otherwise partner intramolecularly with the deleted residues (45), the MEN 2B mutation changes the RET substrate specificity because of an altered conformation of the kinase domain (12). Thus, NF-κB represents a common downstream target for both oncogenic RET isoforms, unless the differences in intracellular signal transmission. In line with this, transient transfection of the expression vectors RET C634R (MEN 2A), RET M918T (MEN 2B), and wild-type RET in HeLa cells revealed that the oncogenic isoforms but not the wild-type construct were able to strongly activate a NF-κB-dependent reporter gene.

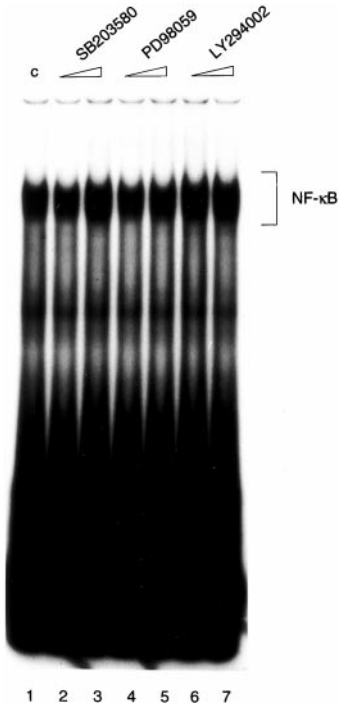

Fig. 9. Treatment of TT cells with SB203580, PD98059, or LY294002 does not interfere with constitutive NF-κB DNA binding. TT cells were incubated with specific inhibitors, SB203580 (5 and 10 μM), PD98059, or LY294002 (10 and 20 μM each). After 1 h of incubation, nuclear extracts were prepared and assayed for NF-κB DNA binding activity by EMSA.

To further characterize RET-mediated NF-κB activation, we exploited TT cells that stably express C-cell differentiation markers and therefore represent a suitable *in vitro* model reflecting cellular transformation induced by oncogenic RET (41, 42). Despite a strong nuclear p65 signal, we noted low steady-state IκBα protein amounts in TT cells. High nuclear p50/p65 levels normally are expected to cause a strong transcriptional up-regulation of the *iκbα* gene, sequestering excess free RelA(p65). This has been demonstrated in transgenic animals, where overexpressed RelA(p65) is masked efficiently by up-regulated IκBα and kept in the cytoplasm (46). At least two explanations can account for the constellation described here. TT cells could have acquired either loss of IκBα function because of *iκbα* mutations, as it is known for Hodgkin's disease tumor cells, or alterations in signal-induced IκBα degradation (47, 48). Incubating TT cells with a proteasome inhibitor resulted in the stabilization of IκBα protein, paralleled by a decrease in NF-κB DNA binding activity. This suggests that up-regulated degradation rather than structural alterations of IκBα result in constitutive NF-κB activity in TT cells.

IκBα degradation *in vivo* is initiated through specific phosphorylation by the multisubunit IKK complex (20). Because TT cells display constitutive IKK activity as judged by GST-IκBα phosphorylation, we examined the role of IKKα and IKKβ for RET-mediated NF-κB activation using dominant-negative mutants. Whereas kinase-deficient IKKα(K44M) exerted only a weak inhibitory effect, IKKβ(K44M) strongly inhibited RET-induced NF-κB-dependent transcription. This is consistent with our observation that RET C643R is able to increase the catalytic activity of IKKβ but not IKKα and suggests that IKKβ represents the downstream target of RET.

Kinases known to be involved in IKK activation include MEKK1, NIK, and NAK (20, 24). Transfection of kinase-dead versions of NIK as well as NAK failed to demonstrate an inhibitory effect on RET-induced NF-κB luciferase activity in NIH 3T3 cells.[4] In contrast,

[4] Unpublished observation.

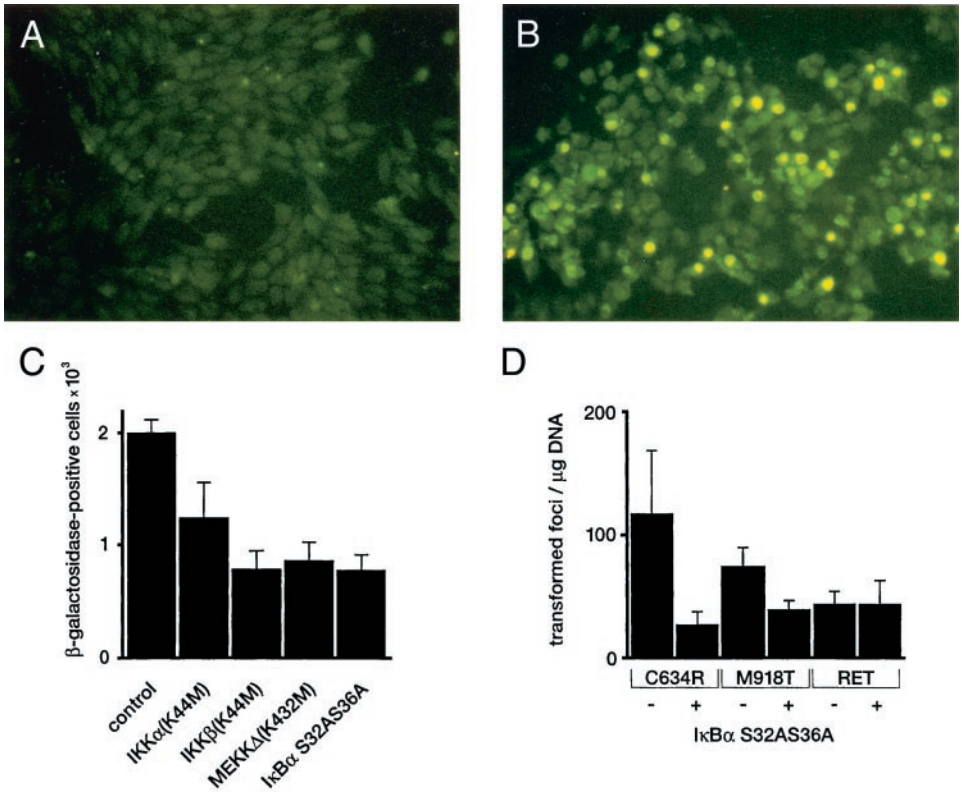

Fig. 11. NF-κB inhibition affects viability of TT cells and blocks RET-induced transformation of NIH 3T3 cells. Inhibition of proteasome-mediated IκBα degradation induces apoptosis in TT cells. TT cells incubated equivalent to the cells described in Fig. 4*B* with Lactacystin were assessed for apoptotic cell death using TUNEL assay. *A*, untreated control. *B*, strong nuclear fluorescence indicative of apoptotic cell death in ~50% of cells (×200). *C*, inhibition of NF-κB induces cell death of TT cells. TT cells were transiently transfected with expression vectors of IKKβ(K44M), IKKα(K44M), MEKK1Δ(K432M), or IκBα S32AS36A together with pcDNA3*LacZ* (3 μg/35-mm well each). Cotransfection of empty pcDNA3 with pcDNA3*LacZ* served as control. Seventy-two h after transfection, cells were fixed, stained with X-Gal, and counted in each well. Values for β-Gal-positive cells represent means of at least three independent transfections; *bars*, SE. *D*, NF-κB is required for cellular transformation induced by oncogenic RET. NIH 3T3 cells were transfected with 1 μg of pcDNA-RET C634R, pcDNA-RET M918T, or pcDNA-RET together with empty pcDNA3 or pcDNA-IκBα S32AS36 and genomic DNA as carrier. Transformed foci were scored 14–21 days after transfection. Values represent means of at least three independent transfections; *bars*, SE.

dominant-negative MEKK1 was able to efficiently inhibit NF-κB activity in TT cells. Although we were not able to confirm a direct interaction between MEKK1 and IKK in TT cells by coprecipitation experiments,[4] this suggests that RET-induced IKKβ activity critically depends on MEKK1. Consistent with this, MEKK1 has been reported to preferentially stimulate the kinase activity of IKKβ (22, 49).

Transfection experiments using chimeric epidermal growth factor/RET receptors have demonstrated clearly that Ras represents a downstream target of RET in NIH 3T3 and in neuroectodermal tumor cells (15, 16). RET-induced Ras activity described thus far was linked to RET signal transduction via the classical mitogenic kinase cascade involving MEK1 and ERK1/2 proteins (15). In addition to that, we suppose that Ras is indispensable for the RET-mediated NF-κB activation because expression of a dominant-negative N17 Ras inhibited constitutive NF-κB transcriptional activity in TT cells as well as RET C634R-induced NF-κB-dependent transcription in NIH 3T3 cells. Immunocomplex kinase assays revealed that N17 Ras was able to almost completely block RET C634R-induced IKKβ activity, indicating that RET-mediated NF-κB activation via IKK is Ras dependent.

Possible downstream effectors of Ras include PI3K, which has been shown to be involved in growth factor-mediated and cytokine-induced NF-κB activation (31, 50). Although PI3K/Akt signal cascades are activated in TT cells, the specific PI3K inhibitor LY294002 did not influence NF-κB DNA binding activity in these cells. Furthermore, neither LY294002 treatment nor transfection of a kinase-dead Akt construct (not shown) led to an inhibition of NF-κB-dependent transcription in TT cells. This argues against a role for PI3K/Akt in RET C634R-mediated NF-κB activation and contrasts with the observation that GDNF-initiated RET signaling, which is not supposed to be oncogenic, activates NF-κB via PI3K (51).

Oncogenic H-Ras(V12) as well as Akt have been demonstrated to activate NF-κB by stimulating the transactivation capacity of the p65 subunit (27). These signals are mediated at least in part by PI3K/Akt and IKK (28). We were able to show that oncogenic RET induces the transactivation potential of p65 in a Gal4 reporter assay.[5] This pathway, however, does not involve PI3K/Akt, because LY294002 failed to inhibit NF-κB transcriptional activity as measured with the 3xκBIFNβLuc reporter (not shown). Therefore, a contribution of a PI3K/Akt-mediated transactivation pathway to the NF-κB activation in TT cells seems unlikely.

Ras has been shown previously to activate NF-κB in a p38 mitogen-activated protein kinase-dependent manner (30, 52, 53). Although p38 is activated in TT cells, specific inhibition by treatment with SB203580 exhibited no effect on constitutive NF-κB activity in these cells. Thus, p38 signaling presumably does not contribute to NF-κB activation in TT cells. Because NF-κB activation induced by oncogenic RET was clearly dependent on Ras, we investigated the role of the Ras effector Raf-1. A dominant-negative Raf-1 mutant partially inhibited IKKβ activity in TT cells as well as the RET C634R-associated NF-κB-luciferase activity in NIH 3T3 cells. Although Raf effects are predominantly mediated by phosphorylation of the downstream targets MEK1 and ERK1/2, inhibition of MEK1 did not decrease NF-κB activation in our experiments. This led to the speculation that MEKK1 acts downstream of Ras/Raf to mediate IKK activation in TT cells. In line with this, constitutively activated Raf has been shown recently to stimulate the activity of IKKβ (40). In that case, NF-κB activation induced by oncogenic Raf was dependent on MEKK1 but independent of the classical mitogenic signal cascade.

Oncogenic transformation is supposed to require mitogenic as well as antiapoptotic signals. The capacity of NF-κB to up-regulate an antiapoptotic gene expression program has been studied extensively (18, 43, 44). More recently, a growth-promoting function has been ascribed to NF-κB by controlling the cyclin D1 promoter (54, 55). In this context, it is intriguing that NF-κB activation induced by onco-

[5] L. Ludwig and R. M. Schmid, unpublished data.

genic RET could potentially provide both a mitogenic as well as an antiapoptotic signal. Inhibition of NF-κB using the proteasome inhibitor Lactacystin induced apoptosis in TT cells. Similarly, dominant-negative versions of MEKK1, IKKα, and IKKβ or IκBα were able to initiate a cell death response in these cells, while on the other hand, NF-κB was essential for RET-mediated transformation. This is reminiscent of the situation observed with oncogenic H-Ras(V12), which stimulates NF-κB-dependent transcription to overcome transformation-induced apoptosis.

In summary, our data suggest that RET-mediated transformation is dependent on NF-κB-delivered antiapoptotic as well as mitogenic signals. Because NF-κB activation induced by oncogenic RET isoforms seems to be a prerequisite for RET-induced C-cell carcinogenesis, we suppose that strategies to inhibit NF-κB in these tumors bear important therapeutic potential.

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