FcεRI-Mediated Induction of TNF-α Gene Expression in the RBL-2H3 Mast Cell Line: Regulation by a Novel NF-κB-Like Nuclear Binding Complex

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FcεRI-Mediated Induction of TNF-α Gene Expression in the RBL-2H3 Mast Cell Line: Regulation by a Novel NF-κB-Like Nuclear Binding Complex

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Using rat basophilic leukemia (RBL-2H3) cells as a model, we investigated how aggregation of the high affinity receptor for IgE (FcεRI) regulates TNF-α gene expression. Antigenic stimulation of RBL-2H3 cells led to an increase in newly synthesized TNF-α mRNA that was dependent on continuous receptor aggregation and did not require de novo protein synthesis. Kinetic analysis showed that maximal levels were achieved at 60 min and waned by 180 min of stimulation. Concomitant with the transcriptional activation of the TNF-α gene, the rapid appearance and disappearance of a previously uncharacterized nuclear NF-κB DNA binding activity, comprised of two distinct protein complexes, were observed. These protein complexes bound to NF-κB sites within the TNF-α gene and contained novel biologic functions (2). Mast cell-derived TNF-α has a protective role in acute septic peritonitis and is involved in the recruitment of neutrophils for clearance at sites of bacterial infection (3, 4). It is also clear that the production of TNF-α has a detrimental role in allergy and asthma as a mediator of late phase inflammatory reactions (5). In mast cells, TNF-α has been found prestored in cytoplasmic granules and is released with histamine and other preformed mediators within minutes following antigenic stimulation, suggesting a potential role in early inflammation as well (6). In addition, there is a prolonged secretion of TNF-α, since it is newly synthesized and secreted via a regulated Golgi secretory pathway that requires continuous aggregation of receptors (7).

Due to its potent and pleiotropic actions as an inflammatory mediator and the delicate balance of beneficial and toxic effects, the production of TNF-α is tightly regulated in many cell types, including mast cells (8–10). Regulation involves both transcriptional and post-transcriptional mechanisms (10–12). Transcriptional activation of the TNF-α gene expression is induced by diverse stimuli, some relatively nonspecific such as LPS and PMA, and others more specific such as TNF-α, IFN-γ, and Ag activation of FcR and TCR (10). Multiple consensus sequences for transcription factors have been identified within the TNF-α gene loci (9, 13–15). The transcription factors implicated are differentially regulated and appear to depend, in addition to the stimulus, on the cell type and the species studied. For example, both murine and human promoters contain several binding sites for the transcription factor NF-kB. In murine macrophages, LPS induction of TNF-α gene expression was mediated by the binding of NF-kB to its target DNA sequence (16, 17), while, in contrast, analysis of the human promoter sequence suggested that NF-kB consensus binding sequences are neither required nor sufficient for LPS- or virus-induced TNF-α gene expression (18). Regulation in murine macrophages and rat astrocytes also seems to involve a κB element downstream of the TNF-α polyadenylation site (13, 15). Recently, a NF-AT binding site in the human TNF-α promoter was shown to confer a stimulation-dependent induction of this gene in lymphocytes and mast cells (19, 20). In T lymphocytes the NF-AT binding element was found to function coordinately with an upstream localized cAMP-responsive element that bound activating transcription factor 2 and Jun family proteins (21, 22). In addition to NF-κB and NF-AT other transcription factors, such as AP-1 (23), ets (24), and C/EBPβ (25), also appear to be involved in TNF-α gene expression.

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3 Abbreviations used in this paper: FcεRI, high affinity IgE receptor; NF-AT, nuclear factor of activated T cells; AP-1, activator protein-1; RBL-2H3, rat basophilic leukemia 2H3 cell line; PDTC, pyrrolidine dithiocarbamate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TPCK, Nα-αoxyl-Phe-chloromethyl ketone; EMSA, electrophoretic mobility shift assay; BrdUrd, bromodeoxyuridine; CAT, chloramphenicol acetyltransferase.
In this study we focused on the role of NF-κB binding elements in activating transcription of the TNF-α gene in RBL-2H3 cells stimulated via FceRI. We provide evidence for a mechanism implicating the transient activation of the DNA binding activity of a previously uncharacterized NF-κB-related binding complex in the nucleus as important to transient activation of the TNF-α gene in response to aggregation of FceRI.

Materials and Methods

Reagents

Supernatants from serum-free hollow-fiber cultures of the hybridoma Hi-DNP-e-26.82 (26) were used as a source of anti-DNP-specific IgE at a dilution of 1/200 for all sensitization experiments. Rabbit serum to TNF-α was purchased from Genzyme (Cambridge, MA). Abs to NF-κB complex proteins, p50 (N and C terminus), p65 (Rel-A, N and C terminus), Rel-B, Rel-C, and p52 and a pan specific Abs to NF-AT complex proteins were purchased as supershift reagents from Santa Cruz Biotechnologies (Santa Cruz, CA). Additional Abs to the p50 and p65 constituent of NF-κB were provided by Drs. A. Israel (Institut Pasteur, Paris, France) and U. Siebenlist (National Institutes of Health, Bethesda, MD). DNP-BSA was a gift from Drs. A. Prouvost-Danon (Institut Pasteur). PMA, EDTA, EGTA, pyrrolidine-dithiocarbamate (PDTC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DNP-lisine, and cycloheximide were purchased from Sigma (St. Louis, MO). Actinomycin D and 4′,6-diamidino-2-phenylchloromethyl ketone (TPCK) were obtained from Boehringer Mannheim (Mannheim, Germany).

Cell activation

RBL-2H3 cells were maintained as previously described (27). For RNA preparations, one 75-cm² flask (≈2 × 10⁷ cells) was used per experiment. Cells were sensitized for 1 to 2 h in 20 ml of culture medium supplemented with 20 mM HEPES and DNP-specific IgE as described above. After two washes with PBS, cells were stimulated with DNP-BSA (50 or 100 ng/ml) in culture medium prewarmed to 37°C. At each time point, samples were immediately processed for RNA extractions (see below). For analysis of nuclear extracts, cell nuclei were isolated and resuspended at 2 × 10⁶ cells/ml in complete culture medium containing 20 mM HEPES and DNP-specific IgE. After 1 h, suspension cells were washed and stimulated with DNP-BSA (50 or 100 ng/ml) at 37°C. Reactions were stopped by adding ice-cold PBS before the preparation of nuclear protein extracts (see below). For measurement of TNF-α and β-hexosaminidase secretion, adherent cells (5 × 10⁵ cells in 0.5 ml, 24-well plates) were sensitized for 1 to 2 h with DNP-specific IgE in medium supplemented with 20 mM HEPES (37°C). Cells were subsequently washed with PBS and stimulated with DNP-BSA in complete medium at 37°C for the indicated times. A sample of the medium (450 μl) was collected from the wells, briefly centrifuged, aliquoted, and stored at −20°C before testing.

RNA isolation and RNase protection assay

RNA isolation and RNase protection assays were conducted as previously described (27). Rat TNF-α cDNA (28) was obtained from Dr. K. Decker (Biochemisches Institut, Universität Freiburg, Freiburg, Germany). To develop antisense riboprobes of TNF-α and control β-actin genes, nucleotides 199 to 433 of the TNF-α-coding sequence covering exons 2 to 4 of the rat TNF-α gene and nucleotides 2309 to 2460 in exon 3 of the rat β-actin gene were amplified by PCR using plasmid DNA as a template and RT-PCR, respectively. The TNF-α probe yielded a protected fragment of 234 bp; the β-actin probe yielded a protected fragment of 151 bp. Hybridizations were conducted using 20 to 30 μg of total RNA, 500,000 cpm of the 32P-labeled anti-sense rat TNF-α riboprobe, and 50,000 cpm of the 32P-labeled antisense rat β-actin riboprobe.

Nuclear extracts

All procedures were performed at 4°C. Nuclear extracts were prepared according to the method of Dignam et al. (29) with minor modifications. Briefly, 5 × 10⁶ cells were washed in PBS and resuspended in HNB solution (15 mM Tris, pH 7.5, containing 0.5 M sucrose, 60 mM KCl, 0.25 mM EDTA, and 0.125 mM EGTA); then Nonidet P-40 was added to a final concentration of 0.2%. Cell lysis and integrity of nuclei were examined by light microscopy after addition of trypan blue. The cell homogenate was centrifuged for 10 min at 1,200 × g to pellet nuclei. The latter were resuspended in nuclear extract buffer (20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM MgCl₂, 0.25 mM EDTA, and 0.37 M NaCl), and the released chromatin was pelleted by centrifugation at 50,000 × g for 30 min. Ammonium sulfate was added to a final concentration of 0.3 g/ml, and the precipitated proteins were centrifuged for 1 h at 100,000 × g. The protein pellet was resuspended in 100 μl of 20 mM HEPES, pH 7.9, containing 60 mM KCl, 20% glycerol, 0.25 mM EDTA, 0.125 mM EGTA, and protease inhibitors as previously described (29). The samples were dialyzed three times in 1 h each time in 300 ml of the same buffer. Insoluble material was removed by centrifuging for 3 min at 14,000 × g in a microcentrifuge. Soluble proteins were stored at −80°C in 30-μ1 aliquots; a 2-μl sample was reserved for determination of protein concentration by the Bradford assay (30).

Electrophoretic mobility shift assay (EMSA) and UV cross-linking

For oligonucleotide mobility probes the following double-stranded oligonucleotides were synthesized and used as labeled and nonlabeled consensus probes in noncompetitive and competitive binding experiments (only the sequence of one strand is shown, and consensus binding sites are underlined; complementary probes were synthesized to give a 1-bp overhang): 5′-GACAG AGGGAGTTCCGAGA-3′ corresponding to the NF-κB binding site in the Ig κ-chain enhancer, 5′-GCCTCTTGATGAGTCAGCGGAGA-3′ corresponding to a 1-bp site in the rat β-actin gene, 5′-GAGGAGAAGATCTCT-3′ corresponding to the distal NF-AT recognition site of the human IL-2 promoter. Double-stranded oligonucleotides corresponding to κB elements in the TNF-α gene (see also Fig. 6A) were the following: κB1, 5′-GACTCCCTGAACTTCCAGGAG-3′; κB2, 5′-GACCAACAGGGGATTCCTGGCCT-3′; κB3, 5′-CGAGGAGGAGATCTCTGACGTCCTG-3′; κB4, κB5-κB6, 5′-TGGATCAGTGGTTTCCACCTGGCT-3′; κB7, 5′-CCCTGCTGTTGAGAAATCTGGCT-3′; and κB2, 5′-GCTCTGGGGTGCAGCTTGGCCTTG-3′. Oligonucleotide AP-1 and NF-κB binding reactions were performed in a 20-μl volume containing 10 μg of the nuclear extract and 1 μg of poly(dI-dC) in binding buffer (4% Ficoll, 20 mM HEPES (pH 7.9), 70 mM NaCl, 2 mM DTT, 0.01% BSA, and 1 mM EDTA). The mixtures containing competitor oligonucleotide (100×) or Abs were preincubated for 10 min at room temperature. One nanogram of double-stranded oligonucleotides, which were end labeled with [γ-32P]dATP and T4 polynucleotide kinase, were added to the samples (sp. act., ~100,000 cpm/ng) and allowed to bind for 20 min at room temperature. Samples were loaded onto a 5% nondenaturing polyacrylamide gel in 0.5 × Tris/borate/EDTA buffer and allowed to migrate for 90 min at 200 V. Samples were UV-irradiated on a transilluminator (306 nm) for 15 min at room temperature and exposed under a PhosphorImager screen for 15 min for localization of the shifted complexes. The complexes were excised from the gel equilibrated in SDS sample buffer, resolved on a 6 to 12% gradient SDS polyacrylamide gel, and visualized as described above. The wet gel was UV-irradiated on a transilluminator (306 nm) for 15 min at room temperature and exposed under a PhosphorImager screen for 15 min for localization of the shifted complexes. The complexes were excised from the gel equilibrated in SDS sample buffer, resolved on a 6 to 12% gradient SDS polyacrylamide gel, and visualized as described above.

CAT assays

A NF-κB reporter construct carrying the tandem NF-κB site of HIV-1 as well as a control construct (31) were provided by Dr. M. Körner. The rat TNF-α promoter construct (15) was gifts from Dr. E. Beneniste. For transfection, 4 × 10⁶ cells resuspended in complete medium were incubated with 10 μg of plasmid DNA and electroporated using a gene pulser apparatus (Eurogentec, Brussels, Belgium) at settings of 250 V and 1500 μF and were plated in 100-mm culture dishes. After 30 h IgE-sensitized cells were stimulated for a period of 16 to 18 h with DNP-BSA (100 ng/ml). Cells were scraped from dishes and lysed by freeze-thawing in 0.25 M Tris, pH 8.0. CAT assays were conducted in duplicate as previously described (15). The amount of CAT activity was normalized to the sample protein, and values are expressed as the fold induction relative to that in an unstimulated control.
Assay of β-hexosaminidase and TNF-α secretion

The release of mast cell mediators by exocytosis was monitored using the β-hexosaminidase assay as described previously (27). For the TNF-α bioactivity assay, based on the lysis of the target cells (32), WEHI 164 clone 13 cells (3 × 10^5) in 50 μl of complete RPMI 1640 medium were plated in 96-well plates and incubated at 37°C for 24 h. RBL-2H3 test supernatants (50 μl of 3-fold serial dilutions in complete RPMI containing 2 μg/ml actinomycin D) were added. After an overnight incubation, viable cells were quantitated by use of the colorimetric MTT assay (33). Briefly, 25 μl of MTT (5 mg/ml) was added to the wells and incubated for 1 h. A 50-μl aliquot of the supernatant was discarded, and 100 μl of solubilization solution was added. After overnight incubation OD was measured at 570 nm. One unit of TNF-α was defined as the dilution of supernatant required to lyse 50% of WEHI 164 clone 13 target cells. As a positive control for each experiment, serial dilutions of known concentrations of recombinant murine TNF-α (Genzyme, Cambridge, MA) were used. To test the specificity of the cytotoxic effect, test supernatants were preincubated with a 100-fold dilution of serum specific to mouse TNF-α (Genzyme) before incubation with the target cells. Quantitation of secreted TNF-α was also performed using an ELISA kit specific for rat TNF-α according to the manufacturer’s instruction (BioSource, Camarillo, CA).

Statistical analysis of the data

A minimum of three experiments using independent cell cultures were performed for all reported data except when stated otherwise. The experiments shown are representative of each series of experiments or are a summary of the data from at least three experiments and are presented as the mean ± SEM. Statistical significance of the data was determined using Student’s paired t test.

Results

TNF-α mRNA transcription in RBL-2H3 cells is independent of de novo protein synthesis but requires continuous stimulation through FcεRI

IgE-sensitized RBL-2H3 cells were stimulated with various concentrations of Ag, and TNF-α-mediated cytotoxicity toward the TNF-α-sensitive WEHI 164 target cells was measured. Our results shown in Figure 1A are consistent with prior studies showing that TNF-α is secreted by RBL-2H3 cells in response to antigenic stimulation (7, 34, 35). Ag concentrations in the range of 50 to 100 ng/ml of DNP-BSA appeared to induce a maximal response. Unstimulated cells did not secrete TNF-α, since the values obtained were not significantly different from those with medium alone. The cytotoxic effect of TNF-α on the WEHI 164 cells was completely inhibited by preincubation of the stimulated cell supernatants with an Ab specific for TNF-α (data not shown). In kinetic experiments (Fig. 1B) the secretion of TNF-α appeared as early as 30 min following stimulation; maximal levels were achieved between 2 and 6 h and slowly declined thereafter.

We developed a quantitative RNase protection assay based on the simultaneous hybridization of RNA with control β-actin and experimental TNF-α antisense riboprobes to measure TNF-α steady state mRNA levels in FcεRI-stimulated RBL-2H3 cells. Figure 2A shows a representative kinetic experiment. Unstimulated cells failed to induce TNF-α mRNA levels above the background control of transfer RNA. However, aggregation of FcεRI induced a rapid and easily detectable increase in TNF-α mRNA within 20 min. Maximum levels were achieved at approximately 60 min of stimulation followed by a rapid decrease to the background level of unstimulated cells within 180 min of stimulus addition (Fig. 2, A and B). Quantitative analysis showed that stimulation of the FcεRI increased the steady state mRNA levels by at least 15-fold (Fig. 2B).

To test whether the induction of TNF-α transcription required continuous receptor aggregation, IgE α-DNP-sensitized RBL-2H3 cells were stimulated with DNP-BSA, and further aggregation of FcεRI was terminated at various time points by the addition of an excess of hapten DNP-lysine. After incubation of cells for a total of 30 min, the TNF-α relative mRNA levels were determined by RNase protection assay. As shown in Figure 3 TNF-α mRNA steady state levels were diminished in samples that had received hapten compared with that in the sample that had been stimulated continuously for 30 min. The decrease was more pronounced the earlier the hapten had been added, clearly indicating that the induction of TNF-α mRNA expression can be promptly interrupted and requires ongoing receptor stimulation.

Since TNF-α production can be regulated at multiple levels, including at post-transcriptional and translational levels, we examined whether the increase in steady state TNF-α mRNA was due to de novo transcription. Actinomycin D treatment at 2 μg/ml inhibited the FcεRI-mediated increase in TNF-α mRNA by 92.9 ± 8.2% without any significant effect on secretion of granule contents (4.0 ± 0.5%). These findings demonstrate that in RBL-2H3 cells, FcεRI-mediated TNF-α secretion is largely dependent on the synthesis of new TNF-α mRNA. Treatment with cycloheximide (10 μg/ml), a potent inhibitor of protein synthesis, diminished TNF-α secretion by 97.0 ± 3.0%. Although the secretion of TNF-α was effectively inhibited, cycloheximide treatment did not abrogate the synthesis of TNF-α mRNA but instead increased the steady state levels of this mRNA. Within 60 min TNF-α mRNA expression reached more than twice the level (253 ± 47.6%) in the absence of cycloheximide, suggesting that synthesis of proteins is necessary for the rapid down-regulation of TNF-α gene expression. These results also demonstrate that TNF-α mRNA expression
in RBL-2H3 cells is independent of de novo protein synthesis and follows the rules of an immediate early gene. Transcription factors described to mediate immediate early gene transcription include NF-κB and NF-AT. Consensus sites for both these factors have been shown to be present in the TNF-α gene (see Fig. 6A), and a role for NF-AT in TNF-α mRNA synthesis of mast cells has been proposed recently (19, 36). We therefore focused on the examination of whether NF-κB also plays a role in the activation of TNF-α gene transcription.

Induction of nuclear NF-κB binding activity following FcεRI cross-linking

To examine whether antigenic stimulation of FcεRI led to the induction of nuclear NF-κB DNA binding activity we employed a probe corresponding to the consensus binding site for NF-κB from the Ig light chain κ enhancer. As a positive control we analyzed the induction of nuclear AP-1 DNA binding activity that was recently reported to occur in mast cells following antigenic stimulation (37). Figure 4 shows that NF-κB binding activity was detected in nuclear extracts following stimulation of FcεRI. The protein-probe complex appeared as a broad band that could be resolved to a doublet (upper complex and lower complex) in most exposures of the gels.

FIGURE 2. RNase protection assay of steady state TNF-α mRNA levels in Ag-stimulated RBL-2H3 cells. A, Total RNA (30 μg) from unstimulated cells or cells stimulated with DNP-BSA (100 ng/ml) for various time points and control transfer RNA (30 μg) were hybridized simultaneously with antisense TNF-α and β-actin riboprobes. The ribonuclease-resistant product from a representative experiment is shown, as analyzed by electrophoresis. The migrations of protected fragments corresponding to TNF-α and β-actin mRNA are indicated. Molecular size markers correspond to 32P-labeled fragments from a pBR322 MspI digest. B, Corresponding quantitative PhosphorImager analysis of the experiment shown in A. For each time point the ODs of TNF-α mRNA levels were normalized to β-actin mRNA levels. The 0 h point was arbitrarily normalized to 1 (dashed line).

FIGURE 3. Addition of excess hapten halts FcεRI-mediated TNF-α mRNA synthesis. RBL-2H3 cells were stimulated with 100 ng/ml DNP-BSA or were left unstimulated (NS). For certain batches of cells further aggregation of FcεRI was prevented by adding hapten (50 μM DNP-lysine) at various times (5, 10, 15, and 20 min) followed by incubation for a total of 30 min at 37°C. Relative amounts of TNF-α mRNA were determined in duplicate by RNase protection as described in Figure 2. For the purpose of this comparison the relative amounts of TNF-α mRNA obtained from cells continuously stimulated for 30 min were arbitrarily defined as 100%. The data are representative of two similar experiments.

FIGURE 4. Induction of NF-κB (A) and AP-1 (B) nuclear binding activity in Ag-stimulated cells. Nuclear extracts were prepared from IgE-sensitized RBL-2H3 cells stimulated or not with Ag (DNP-BSA, 100 ng/ml) for 1 h. Nuclear extracts were incubated with 32P-labeled oligonucleotides corresponding to NF-κB (A) and AP-1 (B) consensus motifs in the absence or the presence of a 100-fold excess of unlabeled oligonucleotides and were analyzed by EMSA. NF-κB- and AP-1-specific complexes are indicated. The arrows correspond to the position of the uncomplexed DNA probe.
Kinetics of NF-κB and AP-1 nuclear binding activity in Ag-stimulated cells and analysis of the requirement for continuous stimulation. A, IgE-sensitized RBL-2H3 cells were stimulated with DNP-BSA (100 ng/ml) for the indicated time before preparation of nuclear extracts. Data are from a representative EMSA using NF-κB- and AP-1-specific 32P-labeled oligonucleotides. B, Corresponding quantitative PhosphorImager analysis of NF-κB and AP-1 binding activities. Values for unstimulated cells (dashed line) were arbitrarily normalized to 1, and the relative ODs for the various times were determined. C, Three batches of IgE-sensitized RBL-2H3 cells were stimulated with 100 ng/ml of DNP-BSA. At various time points (10 and 20 min) further aggregation of receptors was prevented by addition of hapten, and incubation was continued for a total of 45 min (see Materials and Methods). The third sample was incubated with DNP-BSA for 45 min and did not receive hapten. Nuclear extracts were prepared and analyzed for nuclear NF-κB and AP-1 binding activities by EMSA.

(see also Fig. 6, B and C). Both bands of the doublet were equally competed by an excess of unlabeled oligonucleotides (Fig. 4A), whereas oligonucleotides corresponding to the binding site for AP-1 did not compete for binding. The AP-1 activity was detectable in unstimulated cells (Fig. 4B), and antigenic stimulation led to increased AP-1 binding activity, which was inhibited with excess unlabeled AP-1 consensus binding site oligonucleotides, but not with oligonucleotides corresponding to the NF-κB site (Fig. 4B).

Kinetic experiments were performed to quantitate the induction of NF-κB activity following antigenic stimulation. As shown in Figure 5A no significant NF-κB binding activity was detected in unstimulated cells. Following exposure to Ag, NF-κB binding activity could be detected as early as 15 min (data not shown) and reached maximal activity at approximately 60 min of stimulation (Fig. 5A). Continued stimulation for 150 min resulted in decreased NF-κB binding activity to levels similar to those found in unstimulated cells. Quantitative analysis showed a maximal induction of 6- to 16-fold for NF-κB binding activity in the nucleus in five independent experiments (Fig. 5B). In comparison, the AP-1 binding activity present in unstimulated cells also increased, with a maximal response at approximately 60 min of stimulation (Fig. 5A). However, unlike NF-κB, AP-1 binding activity was stable for a prolonged period of time, with elevated levels maintained at 150 min of stimulation. Quantitative analysis of the induced levels of AP-1 binding activity showed an increase of 3- to 4-fold (Fig. 5B). The requirement for ongoing receptor stimulation was also examined. Figure 5C shows that when receptor aggregation was terminated by adding an excess of hapten at early time points AP-1 binding activity was not altered, suggesting that a short stimulus was sufficient to induce maximal binding activity even in the absence of stimulation (Fig. 5C). In contrast, NF-κB binding activity depends, like TNF-α mRNA production, on the continuous stimulation through FcεRI.

Implication of NF-κB consensus binding sites in transcriptional induction of the TNF-α gene

To investigate whether the induced NF-κB binding activity could play a role in TNF-α gene transcription we examined whether the various κB sites present in the TNF-α gene could also bind the induced NF-κB binding complexes. As a first approach we tested the different individual sites present within the TNF-α gene (Fig. 6A) for their capacity to inhibit formation of the protein-probe complex. The results given in Figure 6B show that besides the consensus site double-stranded oligonucleotide, which was the most powerful inhibitor, all other oligonucleotides inhibited complex formation to some degree. Differences in the capacity to inhibit the upper and lower complexes were noted. The oligonucleotides corresponding to the κB4/NF-AT and κB3’ sites substantially inhibited both lower and upper complexes, whereas the other κB as well as κB pseudo site CK2 oligonucleotides preferentially inhibited the upper complex. This was further confirmed when the oligonucleotide probes were directly tested for their binding capacities (Fig. 6C). Interestingly, in these experiments we could not detect significant binding with oligonucleotide probes corresponding to κB1 and κB3 sites for the upper complex and κB4/NF-AT for the lower complex, suggesting that the interaction with these sites may be of low affinity.

To functionally test the role of the inducible NF-κB binding activity we used NF-κB and TNF-α promoter constructs encoding CAT as a reporter gene. In the first set of experiments we tested a promoter construct containing the tandem NF-κB site of the HIV-1 promoter as well as a control construct where both these sites have been deleted (see Fig. 7A). As shown in Figure 7B stimulation of RBL-2H3 cells transfected with this construct readily led to an induction of CAT activity. No activity was induced with the control promoter construct. These results indicate that stimulation of FcεRI in RBL-2H3 cells can induce NF-κB-dependent transcription of a heterologous promoter. We next examined the effect of stimulation on a TNF-α promoter construct containing all 5’ κB sites as well as a promoter construct containing only the most proximal κB4/NF-AT site. The latter site was shown to bind the transcription factor NF-AT and to play a role in the induction of TNF-α in a variety of cells (21, 36). A construct in which the entire promoter sequence was inserted in a reverse orientation was used as a control. As shown in Figure 7B, following stimulation of
transfected RBL-2H3 cells CAT activity could be induced in constructs containing the full promoter. No significant induction was detectable in constructs in which three \( \kappa B \) sites, but not the proximal \( \kappa B4/NF-AT \) site, were deleted.

To further confirm the role of NF-\( \kappa B \) in FceRI-dependent transcription we used an alternative approach based on the use of pharmacologic inhibitors. This allowed the study of FceRI-dependent transcription of TNF-\( \alpha \) mRNA at the level of the gene in its native chromatin structure. In the first series of experiments we used a potent inhibitor of the nuclear localization of NF-\( \kappa B \)-related factors, the antioxidant PDTC (38–40). RBL-2H3 cells pretreated or not with PDTC were subsequently stimulated with Ag, and the effects on TNF-\( \alpha \) mRNA expression and nuclear localization of several transcription factors were examined. For the latter we particularly focused on the nuclear DNA binding activities of NF-\( \kappa B \), AP-1, and NF-AT. As revealed in Figure 8 TNF-\( \alpha \) mRNA expression (Fig. 8B) was dramatically inhibited at concentrations where NF-\( \kappa B \) binding activity was also substantially inhibited (Fig. 8A). No significant effect was observed on the binding activities of AP-1 and NF-AT in the presence of PDTC (Fig. 8A). The latter binding activity appeared as several bands that were inhibited with
an excess of unlabeled oligonucleotides (data not shown). In addition, we examined the effect of the protease inhibitor TPCK, which has also been shown to be a powerful inhibitor of NF-κB (41). As shown in Figure 8A, in RBL-2H3 cells this compound inhibited TNF-α mRNA steady state levels in a dose-dependent manner as well as the activation of NF-κB nuclear binding activity. The binding activity of the transcription factor AP-1 was not significantly affected, while that of NF-AT was enhanced in the presence of TPCK (Fig. 8A).

**Molecular characterization of NF-κB binding complexes**

To determine the composition of the related NF-κB complexes we initially tested, using supershift analysis, whether the p50 (NF-κB1) and p65 (Rel-A) proteins might be present in the binding complexes, since these are the most abundant NF-κB protein components in various cells (42). Whereas both Abs to p50 and p65 readily led to a supershift of NF-κB binding proteins present in nuclear extracts of LPS-stimulated rat spleen cells (Fig. 9A), no supershift was observed with the complex present in nuclear extracts from RBL-2H3 cells. Two other Abs to p50 and p65 directed toward epitopes that differed from the above Abs also did not induce a supershift (data not shown). In addition, as shown in Figure 9A, the shifted complexes in RBL-2H3 mast cells migrated with slower mobility than the classical NF-κB complex present in LPS-stimulated rat splenocytes. Therefore, we tested Abs directed to related NF-κB proteins such as Rel-B, Rel-C, p52, and an Ab directed to a conserved region in NF-AT proteins that has been shown to bind to NF-κB elements under certain conditions (43). However, none of these Abs had an effect on the relative mobility of the original complex (data not shown). These results suggested that the induced NF-κB binding activity is not likely to be comprised of the classical NF-κB binding proteins. This was further investigated by assessing the molecular mass of the protein adducts using UV cross-linking experiments with a BrdUrd-modified NF-κB consensus oligonucleotide probe. In preliminary experiments we verified that the presence of BrdUrd in the probes did not affect the migration of the complexes. Cross-linking was performed by UV irradiating the native acrylamide gel. Due to the close migration of the UV cross-linked upper and lower complexes in acrylamide gels we excised both complexes together and resolved the components by SDS-PAGE. As shown in Figure 9B, cross-linking revealed the presence of three species with molecular masses of 90, 100, and 110 kDa. The same pattern was obtained with the 3′ flanking region of the TNF-α gene. Cross-linked samples from RBL-2H3 cells were preincubated with DNAseI and resolved by 6 to 12% gradient SDS-PAGE and analyzed by a PhosphorImager.
mass of previously described protein adducts of p50, p65 (Rel-A), or Rel-C (44).

Discussion

TNF-α gene expression has been shown to be tightly regulated and to involve an array of different transcription factors whose activity depends largely on the stimulus as well as on the cell type. Regulation of TNF-α gene expression in mast cells is of particular interest, since in these cells, TNF-α is released as a preformed component of cytoplasmic granules but is also newly synthesized upon cell activation. The newly synthesized TNF-α accounts for the sustained release of the cytokine. Our study with RBL-2H3 cells as well as a previous study (2) show that the new synthesis of TNF-α gene transcripts follows the rules of an immediate early gene and does not require de novo synthesis. Transcription factors such as NF-AT and NF-κB are likely candidates for the regulation of immediate early genes, and consensus binding sites for both these transcription factors are present in the TNF-α gene (16, 21). Recent evidence implicates the transcription factor NF-AT in the expression of newly synthesized TNF-α in PMA/ionophore- or FcεRI-stimulated CPII mast cells (19, 36). The results of these studies are consistent with NF-AT regulation of the TNF-α gene observed in human B and T lymphocytes, where the most proximal human κB promoter element was reported to bind NF-AT and drive reporter-dependent gene expression following stimulation (20, 21). However, species-specific regulation of the TNF-α gene is suggested by the fact that this binding site is not completely conserved among the mouse, rat, and human (14, 21). In addition, differences in the differentiation stage and tissue origin of the cell may influence how the genes are regulated. For example, in CPII cells induction of NF-κB binding activity was not observed, although it was detected in MC/9 mast cells by NF-κB-dependent reporter assays (45) and in Cl. MC/C57.1 mast cells by EMSAs (46).

To reexamine the relationship among FcεRI stimulation, TNF-α gene expression, and nuclear localization of transcription factor complexes we used RBL-2H3 cells as a model system. Our findings reveal a novel and previously uncharacterized NF-κB-like binding activity as important to transient activation of the TNF-α gene in response to aggregation of FcεRI. These results provide a candidate activity that may explain the apparent discrepancies in the above-mentioned studies on different mast cell lines. Several independent approaches established the relationship between TNF-α mRNA expression and induction of this novel nuclear NF-κB binding activity in RBL-2H3 cells: 1) the analysis of κB elements present in the TNF-α gene loci showed the presence of several elements that have the capacity to inhibit binding and/or bind directly the nuclear complex; 2) both TNF-α mRNA expression and nuclear NF-κB binding activity were transiently induced with similar kinetics, and both required continuous receptor stimulation; 3) analysis of AP-1, NF-AT, and NF-κB nuclear binding activities in the presence of pharmacologic agents that inhibit nuclear activation of the described NF-κB binding activity revealed that the specific loss of NF-κB binding activity also resulted in a loss of TNF-α mRNA expression; and 4) deletion of 5′ κB elements present in the TNF-α promoter (except the one most proximal to the initiation site) abolished all measurable FcεRI-dependent induction in TNF-α promoter-reporter assays.

An interesting feature of the induced NF-κB binding activity in RBL-2H3 cells was the rapid and transient nature of the response, which was remarkably similar to the kinetics of TNF-α mRNA induction. In general, it is thought that NF-κB binding activity is relatively stable (42); however, a similar rapid and transient response has been shown in murine pre B (70Z/3) and lymphoma (EL4) cell lines when stimulated via the type I IL-1R (47). We also found that interruption of receptor aggregation with hapten resulted in a rapid reversal of both nuclear NF-κB binding activity and TNF-α gene expression, suggesting that both processes can be rapidly modulated and are dependent on continuous stimulation. This is consistent with a previous report (48) of the sustained NF-κB binding activity as being dependent on the continuous presence of the external stimulus. In contrast, we demonstrated that AP-1 activity is not reversed by aborting the formation of new receptor aggregates. In a similar manner, mRNA expression of the immediate early gene c-fos after a short stimulus could not be terminated or reversed by the interruption of receptor aggregate formation (49).

NF-κB-dependent expression of TNF-α has been reported in a number of systems. These include LPS-stimulated macrophages and monocytes, where increased TNF-α production was found to correlate with increased NF-κB binding activity (16, 17, 38). NF-κB-mediated induction in murine macrophages and rat astrocytes has also been defined to function through κB elements found in the 3′-flanking region of the TNF-α gene downstream of the polyadenylation site (13, 15). In our study the oligonucleotide corresponding to the 3′-flanking region of the TNF-α gene strongly inhibited formation of both upper and lower nuclear complexes observed in RBL-2H3 cells and could itself induce a shift of these complexes. Thus, the absence of the 3′ κB elements from the TNF-α promoter-CAT construct probably explains the lower transcriptional activity observed with this reporter compared with that with the NF-κB reporter construct. However, a direct test of this hypothesis would require mutational analysis of the 3′ κB elements in the native gene.

Molecular analysis of the induced NF-κB complexes suggests that they are comprised of novel and of as yet uncharacterized proteins by several criteria: 1) the observed complexes migrated with a lower electrophoretic mobility than the classical NF-κB binding activity of LPS-stimulated rat splenocytes, which were shown to contain both p50 and p65; 2) using a series of Abs we did not detect the presence of p50, p65, Rel-B, Rel-C, or p52 in the complex; and 3) molecular size analysis using UV cross-linking experiments revealed proteins with molecular masses of 90 to 110 kDa. These proteins were not detected with the NF-κB complex from LPS-stimulated rat splenocytes. These results suggest that in addition to the classical NF-κB binding complexes implicated in TNF-α gene expression we have identified novel NF-κB binding proteins that also regulate the induction of TNF-α mRNA. Novel NF-κB-like complexes have been reported in other systems and include a p50-related molecule of 55 kDa and a complex of proteins, with a molecular mass of 110 to 115 kDa, that are expressed in the brain during development (50, 51). The molecular mass of the latter is similar to that observed in the RBL mast cell model. Whether these proteins are identical with those we describe awaits verification.

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