# Transcriptional and Post-Transcriptional Alterations of IκBα in Active Minimal-Change Nephrotic Syndrome

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*Abstract.* Minimal-change nephrotic syndrome (MCNS) is a renal disease characterized by heavy glomerular proteinuria and increased production of cytokines by immune cells. Because of the central role of nuclear factor-κB (NF-κB) in the regulation of cytokine expression, its activity during the relapse and remission phases of steroid-sensitive MCNS was analyzed. During relapse, nuclear extracts from peripheral blood mononuclear cells displayed high levels of NF-κB DNA-binding activity, consisting primarily of p50/RelA (p65) complexes. NF-κB p65 and IκBα proteins were barely detected or not detected in cytosolic fractions during relapse, in contrast to remission. The lack of expression of IκBα protein was associated with downregulation of IκBα mRNA and increases in the levels of the mRNA encoding the proteasome α2 subunit proteolytic pathway. In addition, inhibition of proteasome activity induced cytosolic accumulation of phosphorylated IκBα and significant reductions in the NF-κB binding activity in nuclear extracts from peripheral blood mononuclear cells from patients experiencing relapses. These results suggest that alterations in the NF-κB/IκBα regulatory feedback loop may contribute to the immunologic abnormalities that occur in steroid-sensitive MCNS.

Minimal-change nephrotic syndrome (MCNS) is a kidney disease defined by selective proteinuria and hypoalbuminemia occurring in the absence of cellular glomerular infiltrates or Ig deposits. Electron microscopy reveals changes focused on glomerular epithelial cells, in the form of foot process effacement (1). Most children with primary nephrotic syndrome respond to steroid therapy, but the disease is often characterized by a relapsing course. For such patients, prolonged remission can be obtained with the addition of cyclosporin A or cyclophosphamide. However, a minority of patients fail to respond to this treatment and may develop chronic renal failure (2). Because the relapses occur concurrently with immune alterations, it has been suggested that the proteinuria is a consequence of a putative circulating factor produced by immune cells. Indirect evidence is derived from experiments demonstrating that systemic infusion of supernatants of cultured peripheral blood mononuclear cells (PBMC) from patients with MCNS relapses induced proteinuria in rats (3–5).

Recent studies of T cell compartments in MCNS demonstrated expansion of CD4+ and CD8+ T cell populations, including those with the CD45RO memory phenotype (6,7). T cell expansion was often associated with increased synthesis of several cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-13 (IL-13) (8,9). Clinical observations and experimental data also suggest that T cells with Th2-like phenotypes are involved in the pathophysiologic processes of MCNS. First, the production of IgE is often increased during relapses, independent of previous atopic manifestations. Second, both cell-mediated immunity and delayed hypersensitivity, which are characteristic of Th1 cell function, seem to be defective in relapses (10). Immune dysfunction seems not to be restricted to T cells, because levels of cytokines produced primarily by monocytes, such as IL-1 and IL-8, were increased in relapses. In contrast, remissions were characterized by downregulation of these cytokines (11). These findings suggest that molecular events upstream from cytokine production may be impaired in MCNS.

Nuclear factor-κB (NF-κB) plays a key role in the regulation of cytokine expression, through association with other transcription factors and protein-protein interactions with coactivator proteins (12). Because most cytokines whose levels are increased during relapses and downregulated during remissions are partly or predominantly regulated by NF-κB, we postulated possible involvement of this regulatory pathway in MCNS.

The human NF-κB/Rel family includes five members, *i.e.*, NF-κB1 (p50), NF-κB2 (p52), RelA (p65), cRel, and RelB, which form various homo- and heterodimers. Their activity is

regulated by IκB proteins (IκBα, IκBβ, and Bcl-3) (12). IκBα and IκBβ specifically interact with RelA (p65), whereas Bcl-3 binds p50 homodimers. In unstimulated immune mononuclear cells, NF-κB, which is primarily composed of p50 and p65, is inactivated in the cytoplasm through reversible association with IκB proteins (12,13). After activation, cytosolic IκBα is phosphorylated by the IκB kinase complex and degraded by the proteasome system (12). Free NF-κB complexes can subsequently move to the nucleus, where they regulate NF-κB-dependent gene expression. After activation of NF-κB, IκBα is rapidly resynthesized and sequestrates NF-κB in the cytoplasm, thus switching off the NF-κB activity (12). This negative feedback occurs despite continuous activation by cytokines or protein kinase activators and results from transcriptional activation of the IκBα gene by NF-κB (14–16).

We report for the first time that, during MCNS relapses, strong persistent stimulation of NF-κB activity was observed in PBMC, whereas the expression of IκBα was hardly or not detected. Downregulation of IκBα protein was associated with low levels of IκBα mRNA and increases in the levels of proteasome α2 subunit (α2P) mRNA. The inhibition of proteasome activity in PBMC during relapse concomitantly induced significant reduction of NF-κB binding activity and accumulation of phosphorylated IκBα. In contrast, remission was associated with downregulation of NF-κB and stabilization of IκBα protein. These new data suggest that subtle alterations of the NF-κB pathway might contribute to immune abnormalities underlying the pathophysiologic processes of MCNS.

## Materials and Methods
### Patients
Most patients included in this study were monitored by their respective physicians. For children, the criteria of the International Study of Nephrosis were used for the diagnosis of MCNS, determination of responses to steroids, criteria for kidney histopathologic features, and application of treatment protocols (17). For adult patients, the diagnosis of MCNS or membranous nephropathy (MN) was confirmed by renal biopsy before inclusion. Blood sampling was performed before the initiation of treatment. For three children, relapses occurred during low-dose steroid treatment, without other immunosuppressive therapy. Control subjects were normal children studied while undergoing routine examinations and normal adult volunteers.

All patients (children and adults) exhibited proteinuria of >3 g/24 h and severe hypoproteinemia. Serum albumin levels were not available for all patients at the time of blood sampling.

Relapse was defined by a sudden onset of the nephrotic syndrome (proteinuria with at least 3+ protein levels, as assessed by urine dipsticks, for 3 d consecutively) in a patient previously free of proteinuria, regardless of therapy. In all cases, the diagnosis of nephrotic relapse was established at the time of blood sampling. Remission was defined by the disappearance of the nephrotic syndrome, with proteinuria of <0.5 g/24 h for 13 patients and 0.6 g/24 h for one.

The clinical and laboratory characteristics of the patients with MCNS are summarized in Table 1. Informed consent was obtained from the parents and whenever possible from the pediatric patients, as well as from adult patients and normal volunteers.

### Purification of PBMC and T Cell Subsets
PBMC were purified through a Ficoll/Hypaque density gradient (Eurobio, France). A CD4+ T cell-enriched population was collected by immunomagnetic negative selection, using a cocktail of hapten-conjugated CD8-, CD11b-, CD16-, CD19-, CD36-, and CD56-specific antibodies and magnetic cell-sorting microbeads coupled to an anti-hapten monoclonal antibody (Miltenyi Biotech, Auburn, CA). The purity of the preparation was 90 to 95%, as assessed by flow cytometric analysis using FITC-conjugated CD2-, CD4-, CD19-, and CD8-specific antibodies.

### Electromobility Shift Assays
Cytosolic and nuclear fractions were prepared essentially as described previously (18). Protein concentrations were assayed using the Bio-Rad dye reagent (Bio-Rad, Richmond, CA), following the instructions provided by the manufacturer.

*Table 1.* Clinical parameters for patients with MCNS

| | Relapse[a] | Remission[a] | Adult Patients with MCNS with Relapses[b] |
|---|---|---|---|
| No. of patients | 19 | 14 | 4 |
| Age (yr) | 8.2[c] (5 to 15)[d] | 9 (2 to 14) | 29 (21 to 45) |
| Male/female | 9/7 | 6/8 | 4/0 |
| Proteinuria (g/d) | 10 (3 to 30) | 0.3 (0 to 0.6) | 12 (8 to 12) |
| Proteinemia (g/L) (normal, 65 to 78 g/L) | 43 (39 to 53) | 68 (64 to 77) | 50 (47 to 54) |
| Serum C-reactive protein concentration (mg/L) | 20 (15 to 30) | <15 | <15 |
| Serum creatinine concentration ($\mu$M) | 60 (50 to 80) | 45 (30 to 50) | 110 (80 to 130) |
| Steroid therapy[e] | 16, none; 3, steroids[f] | Prednisone[g] | None |

[a] Pediatric patients.
[b] The diagnosis of minimal-change nephrotic syndrome (MCNS) was established by renal biopsy.
[c] Mean values are indicated.
[d] Ranges are indicated in parentheses.
[e] At the time of blood sampling.
[f] Prednisone doses were 7, 10, and 15 mg/alternate day.
[g] The mean prednisone dose was 6 mg/m² on alternate days.

The double-stranded oligonucleotide probes (100 ng), with the consensus (sc-2505) and mutant (sc-2511) NF-κB sequences (Santa Cruz Biotechnology, Santa Cruz, CA), were labeled with [γ-$^{32}$P]ATP (3000 Ci/mmol) and purified on Chromaspin 30 columns (Clontech, Palo Alto, CA). Binding assays were performed for 30 min at 4°C with gentle shaking, using 10 to 20 μg of nuclear extracts, 4 μg of poly(dI-dC) in 20 μl of binding buffer [10 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.2 mM ethylenediaminetetraacetate, 12% glycerol, 1 mM levels of protease inhibitors], 0.1% Nonidet P-40, and 50,000 cpm of NFκB probe. The samples were loaded onto a native 5% acrylamide gel in 0.5× TBE buffer (90 mM Tris-HCl, pH 8.3, 90 mM borate, 4 mM ethylenediaminetetraacetate). Migration of the samples was performed at 4°C for 2 h. The subunit composition of DNA-protein complexes containing NF-κB was determined by preincubation of nuclear extracts with 2 μg of polyclonal antibodies raised against p50 (sc-7178X), p65 (RelA) (sc-7151X), cRel (sc-278X), RelB (sc-226x), or p52 (sc-298; Santa Cruz Biotechnology), for 1 h before addition of the probe. Gels were dried and analyzed, after overnight exposure, using a PhosphorImager (Storm 840; Molecular Dynamics, Sunnyvale, CA). Band shifts were quantified using ImageQuant analysis software (version 1.11). Similar electromobility shift assays were performed using the interferon-γ (IFN-γ)-activated consensus site (GAS) and the IFN-α-stimulated response element (ISRE) consensus binding sites (Santa Cruz Biotechnology).

## Western Blotting

Polyclonal antibodies raised against human NF-κB p65 (sc-109), Sp1 (sc-420), and IκBα (sc-209 or sc-371) were obtained from Santa Cruz Biotechnology. Polyclonal anti-actin antibody (A 2066) was purchased from Sigma Chemical Co. (St. Louis, MO). Cytoplasmic extracts (50 μg of protein) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. Immunoblotting and detection (ECL system; Amersham, Buckinghamshire, UK) were performed according to the instructions provided by the manufacturer.

## Semiquantitative Reverse Transcription-PCR

Total RNA was isolated from PBMC using an RNeasy kit (Qiagen, Chatsworth, CA). The primer sequences and main PCR characteristics are indicated in Table 2. The forward primer of each pair was labeled with the 6-Fam dye, except for the TNF-α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Reverse transcription-PCR (RT-PCR) was performed in a 9600 Perkin Elmer apparatus, using a RT-PCR access kit (Promega, Madison, WI). The amplified products were detected in an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems, Foster City, CA) and quantified using the GeneScan program. For five patients who were studied in both relapse and remission, the expression of IκBα and α2P mRNA was analyzed by semiquantitative RT-PCR, using 2 μg of total RNA (19). After Southern blotting, PCR products were detected with specific internal oligonucleotides. The expression of GAPDH mRNA was analyzed in parallel, as a control.

## Quantification of TNF-α mRNA by Quantitative RT-PCR

The level of TNF-α mRNA expression was analyzed by RT as described above. Quantitative PCR was performed using a LightCycler (Roche Molecular Biochemicals, Welwyn Garden City, UK). The samples (2 μl of the RT reaction mixture, corresponding to 20 ng of total RNA) were amplified in a 20-μl reaction mixture containing 0.5 mM levels of each primer and 1× LightCycler DNA master SYBR green buffer (Roche Molecular Biochemicals). Carryover was prevented by using dUTP instead of dTTP, with heat-labile uracil DNA glycosylase. A standard curve was prepared by using dilutions of RNA prepared from the pUc9-TNF-α plasmid. The TNF-α primers amplified a 391-bp sequence (Table 2). The PCR was initiated by denaturation at 95°C for 2 min, followed by 40 three-step cycles (95°C for 1 s, 60°C for 10 s, and 72°C for 24 s). The relative value for each sample was calculated using LightCycler analysis software. All PCR findings were normalized to GAPDH expression, to control for variations in the RT reactions.

## Incubation of PBMC with the Proteasome Inhibitor MG132

PBMC were suspended in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μg/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator containing

*Table 2.* Sets of primers used for semiquantitative and quantitative RT-PCR[a]

| Primer | Oligonucleotides | Accession No. | Expected Size (bp) | Annealing Temperature | No. of PCR Cycles |
|---|---|---|---|---|---|
| β2-Microglobulin | 5′-(6-F)GAGATGTCTCGCTCCGTGGCCTTAGC-3′; 5′-CCATGATGCTGCTTACATGTCTCTGATCC-3′ | NM004048 | 374 | 58°C | 21 |
| CD3 | 5′-(6-F)TCTATAGGTATCTTGAAGGGGCTC-3′; 5′-CCAGGCTGATAGTTCGGTGA-3′ | X03934 | 135 | 58°C | 25 |
| IκBα | 5′-(6-F)TATTTCTCCCTACCAGCTCAC-3′; 5′-CCATGATGCTGCTTACATGTCTCTGATCC-3′ | M69043 | 569 | 58°C | 23 |
| Proteasome α2 subunit | 5′-(6-F)CTCAAGGAAAGTCTTCCCATTCAC-3′; 5′-TATGCTTTGGCTGCTGTAGCTGGA-3′ | D00760 | 455 | 58°C | 27 |
| GAPDH | 5′-ACCACAGTCCATGCCATCAC-3′; 5′-TCCACCACCCTGTTGCTGTA-3′ | AF261085 | 451 | 60°C | 26 |
| TNF-α | 5′-ATGGCGTGGAGCTGAGAGATA-3′; 5′-GGGGAGGCGTTTGGGAAGGTT-3′ | AF043342 | 391 | 60°C | 40 |

[a] The oligonucleotides were selected from sequences for which the accession numbers are indicated. The forward primer of each set for semiquantitative reverse transcription (RT)-PCR was labeled with the 6-Fam (6-F) dye, except for the tumor necrosis factor-α (TNF-α) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. The size of each amplified sequence, the annealing temperature, and the number of PCR cycles are indicated.

5% $CO_2$, at a concentration of $2 \times 10^6$ cells/ml. Cells were divided into two equal fractions and incubated overnight at 37°C in the absence (control) or the presence of 10 $\mu$M levels of the proteasome inhibitor MG132 (carbobenzoxyl-leucinyl-leucinyl-leucinal-H; Calbiochem, San Diego, CA). After incubation, cells were pelleted by centrifugation at $1200 \times g$ for 10 min and washed three times with ice-cold phosphate-buffered saline, and protein extracts were prepared.

### Incubation with Serum

To assess the effects of serum on NF-$\kappa$B activation, PBMC were incubated with autologous serum from patients experiencing MCNS relapses. Normal autologous human serum was used as a control. Nuclear and cytoplasmic extracts were prepared as described above.

### Incubation with IFN-$\alpha$

Normal PBMC were cocultured with IFN-$\alpha$ (Schering Plough, Kenilworth, NJ) at a concentration of 1000 IU/ml and were then processed as described above.

### Statistical Analyses

Results were analyzed using ANOVA. Statistical significance was determined using the nonparametric Mann-Whitney $U$ test.

## Results

### High NF-$\kappa$B DNA-Binding Activity in Nuclear Extracts and Loss of Cytoplasmic I$\kappa$B$\alpha$ Protein in PBMC from Patients Experiencing MCNS Relapses

We assayed in parallel the NF-$\kappa$B DNA-binding activity in nuclear extracts and the expression of I$\kappa$B$\alpha$ in cytoplasmic preparations derived from the same PBMC samples (11 relapses and eight remissions) and four normal control samples. All PBMC nuclear extracts from patients with nephrotic relapses who were not receiving steroids exhibited high levels of NF-$\kappa$B DNA-binding activity (Figure 1A), whereas the expression of I$\kappa$B$\alpha$ protein was barely detectable in the corresponding cytosolic extracts (Figure 1B). In contrast, the NF-$\kappa$B DNA-binding activity was barely or not detectable in nuclear
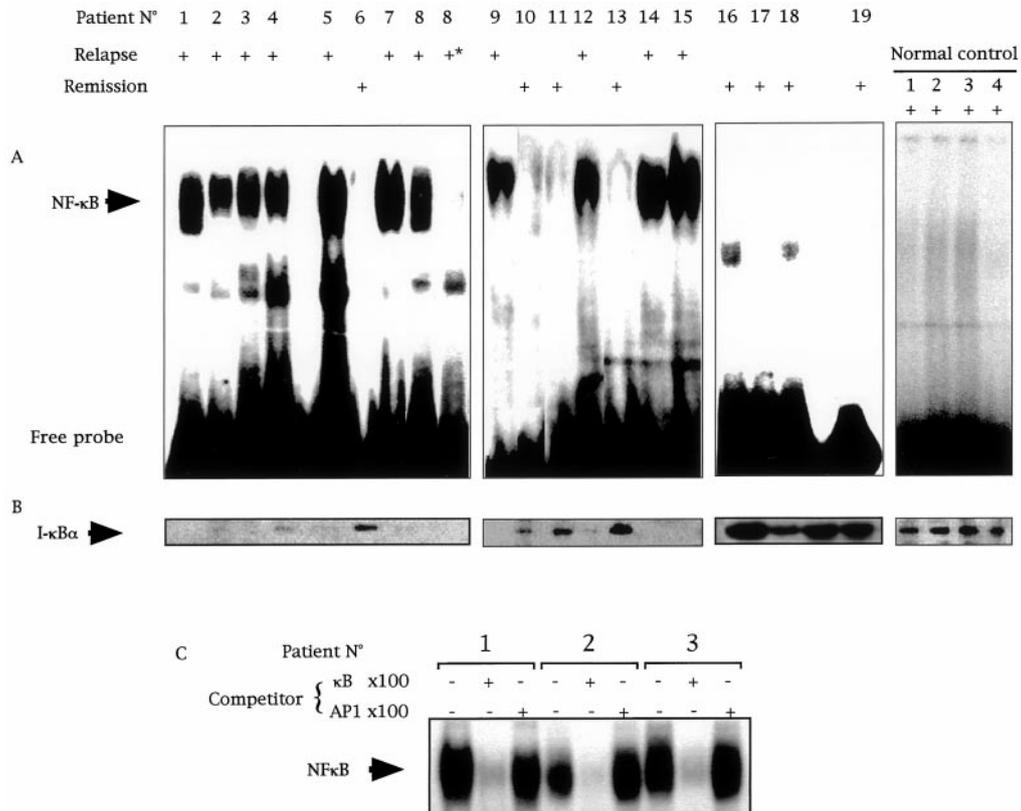


*Figure 1.* Nuclear factor-$\kappa$B (NF-$\kappa$B) activation and downregulation of I$\kappa$B$\alpha$ protein in minimal-change nephrotic syndrome (MCNS) relapses, observed in the absence of exogenous stimulation. Nuclear (A and C) and corresponding cytoplasmic (B) extracts were prepared from peripheral blood mononuclear cells (PBMC) as described in the Materials and Methods section. (A) NF-$\kappa$B DNA-binding activity of 20 $\mu$g of nuclear protein. Nuclear extracts were incubated with the wild-type NF-$\kappa$B oligonucleotide, except one lane (*) in which the mutant-type NF-$\kappa$B oligonucleotide was used as a control. (B) Expression of I$\kappa$B$\alpha$. Immunoblots were performed with 50 $\mu$g of protein, using antibodies raised against I$\kappa$B$\alpha$. The patients experiencing nephrotic relapses were not receiving steroids, whereas those experiencing remissions were receiving steroids, except for patients 6 and 16. Patients 10, 11, and 13 were receiving cyclosporin A and steroid therapy. The panels represent independent electromobility shift assays and Western blotting experiments. (C) Specificity of DNA binding. Nuclear extracts (20 $\mu$g) from three patients experiencing relapses were analyzed for the NF-$\kappa$B site in electromobility shift assays, in the presence or absence of competitor. The specificity of the band shift was demonstrated by the loss of this band in the presence of a 100-fold excess of unlabeled NF-$\kappa$B but not activator protein-1 (AP1) oligonucleotide.

extracts from patients experiencing remission, and I$\kappa$B$\alpha$ protein was easily identified. The specificity of the NF-$\kappa$B binding was demonstrated by loss of the band shifts in the presence of an excess of unlabeled or mutant NF-$\kappa$B probe. Moreover, no displacement of NF-$\kappa$B complexes was obtained in the presence of an unlabeled activator protein-1-specific oligonucleotide (Figure 1C). Immunoblotting of nuclear extracts demonstrated no reactivity with anti-actin antibodies, excluding the possibility of cross-contamination by cytoplasmic fractions (data not shown).

### Lack of DNA-Binding Activities Induced by IFN-$\alpha$ or -$\gamma$ in Nuclear Extracts

Viral infections elicit high levels of the endogenous cytokines IFN-$\alpha$ and IFN-$\gamma$. Both IFN forms induce signal transduction cascades and transactivation of target genes bearing specific response elements, such as GAS and ISRE. To determine whether the NF-$\kappa$B activation in MCNS might result from viral infection, nuclear extracts were analyzed for GAS- and ISRE-binding activity. No DNA-protein complexes were detected by mobility gel shift assays in PBMC nuclear extracts from MCNS relapses, whereas control PBMC cocultured with IFN-$\alpha$ displayed clear binding activity with the GAS/ISRE consensus oligonucleotides (Figure 2). These results suggest that NF-$\kappa$B activation did not result from viral infection.

### Evidence that NF-$\kappa$B Complexes Activated in MCNS Consist Mainly of p50 and p65 Subunits

The subunit composition of the NF-$\kappa$B-DNA complexes was analyzed by preincubation of nuclear extracts with antibodies raised against p50, p52, p65 (RelA), cRel, RelB, or Bcl-3,

before addition of the NF-$\kappa$B probe. The complexes were supershifted with both p50- and p65-specific antibodies but not with those for cRel, p52, RelB, or Bcl-3, suggesting that, in active MCNS, the NF-$\kappa$B complexes were predominantly composed of p50-p65 (RelA) heterodimers or $p50_2$ or $p65_2$ homodimers (Figure 3A). Supershifting was incomplete, as reported by others (20), possibly because of steric hindrance. In a control experiment, we analyzed the NF-$\kappa$B activity for five adult patients with nephrotic syndrome related to MN. NF-$\kappa$B DNA-binding activity was barely detected in nuclear PBMC from these patients (Figure 3B). For patient 5, the very low level of NF-$\kappa$B binding likely corresponded to basal activity (13). However, no supershift could be identified. Quantification of NF-$\kappa$B band shifts demonstrated that the activation level for patients with MCNS was consistently several orders of magnitude higher than that observed for patients with MN (Figure 3C).

### Virtual Absence of I$\kappa$B$\alpha$ and NF-$\kappa$B p65 Proteins from the Cytoplasm of PBMC during Relapse

We further compared the cytoplasmic expression of I$\kappa$B$\alpha$ and NF-$\kappa$B p65 proteins in PBMC from normal and patient samples. I$\kappa$B$\alpha$ was expressed at similar levels by two healthy control subjects and two patients experiencing remission, whereas it was not detected in PBMC from five patients with MCNS relapses (Figure 4). All samples displayed similar patterns for cytoplasmic NF-$\kappa$B p65 and I$\kappa$B$\alpha$ proteins. These results suggest that the NF-$\kappa$B binding activity in cells from patients with nephrotic relapses (Figures 1A and 3B) results from the disappearance of I$\kappa$B$\alpha$ and the translocation of p65 in the nucleus. Conversely, the cytoplasmic levels of NF-$\kappa$B p65 and I$\kappa$B$\alpha$ were normalized in remission.

### Contributions of Both CD4$^+$ and Non-CD4$^+$ T Cells to NF-$\kappa$B Activity during Relapse

To address whether NF-$\kappa$B activity was selectively increased in the CD4$^+$ T cell subset, we performed immunomagnetic negative selection of CD4$^+$ T cells from PBMC from four patients with MCNS relapses who were not receiving steroids. Nuclear extracts were prepared and assayed for NF-$\kappa$B activity. The highest level of NF-$\kappa$B binding was observed in non-CD4$^+$ T cell populations (Figure 5).

### Similar Levels of NF-$\kappa$B Activation in Relapses with and without Steroid Treatment

To assess whether patients experiencing MCNS relapses while receiving steroids and those experiencing relapses while not receiving steroids exhibit differences in NF-$\kappa$B activation, we further studied three patients who experienced relapses while receiving steroids and five patients who experienced relapses while no longer receiving this treatment. The patients in the two groups exhibited similar high levels of NF-$\kappa$B activity, which had been reduced to basal levels during remission (Figure 6). These results suggest that MCNS relapses were associated with increased NF-$\kappa$B activity, regardless of steroid treatment at the time of relapse.
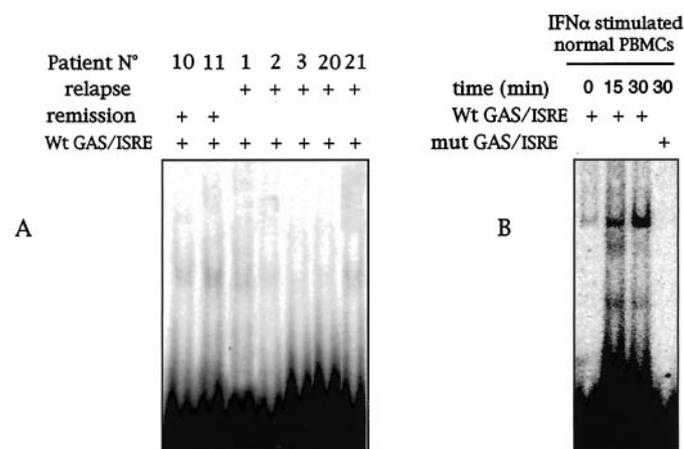


Figure 2. Lack of detection of DNA binding to the interferon-$\gamma$ (IFN-$\gamma$)-activated consensus site (GAS)/IFN-$\alpha$-stimulated response element (ISRE). (A) Nuclear extracts (20 $\mu$g) were analyzed for DNA-binding activity with wild-type (Wt) GAS/ISRE oligonucleotide. Patients with MCNS who were experiencing relapses were receiving (patients 20 and 21) or were not receiving (patients 1, 2, and 3) steroids. Patients 10 and 11 were experiencing remission. (B) As a positive control, nuclear extracts (20 $\mu$g) from normal PBMC were incubated with IFN-$\alpha$ (1000 IU/ml) for the indicated times. The specificity of the binding was assessed using mutant (mut) GAS/ISRE.
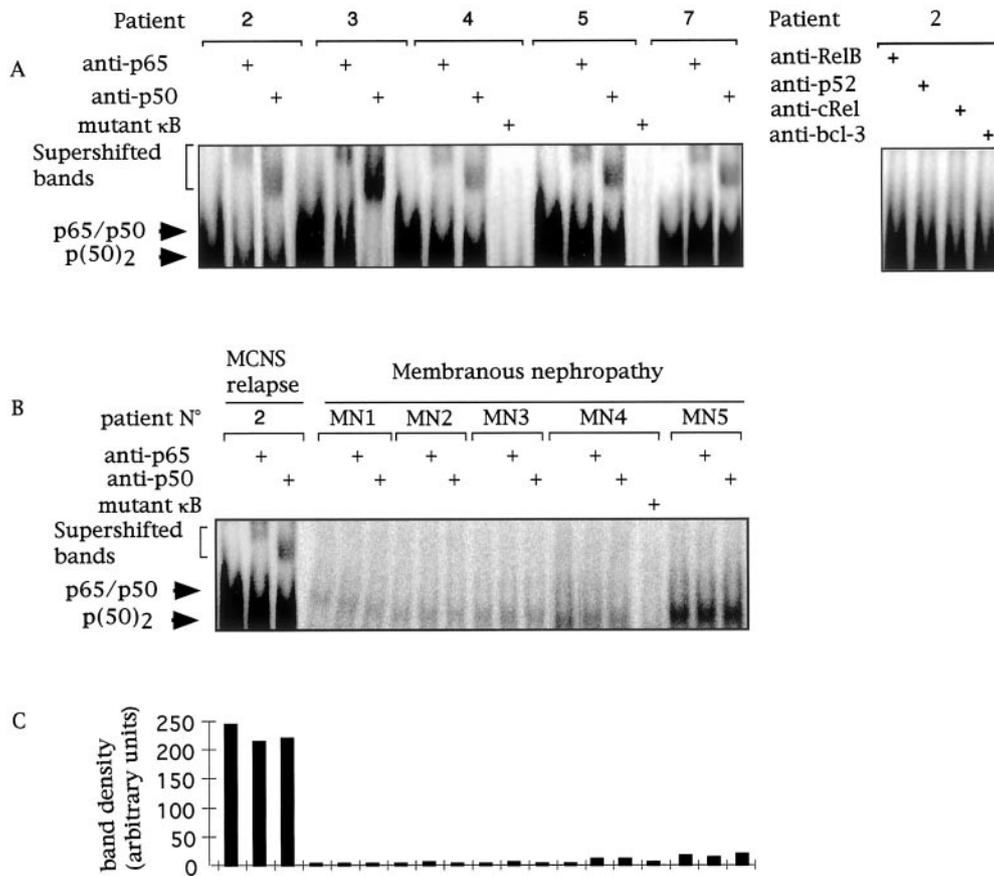
*Figure 3.* NF-κB DNA-binding activity in MCNS. (A) Identification of DNA-protein complexes. Nuclear extracts (20 μg) isolated from PBMC from five patients with MCNS relapses were analyzed for the NF-κB site in electromobility shift assays, in the absence or presence (+) of antibodies directed against members of the Rel family. Supershifted bands (indicated by brackets) were present when anti-p50 or anti-p65 antibody was incubated with extracts before probe addition. Antibodies to other members of the Rel family (anti-p52, anti-cRel, anti-RelB, and anti-Bcl-3) failed to alter the NF-κB binding pattern. The specificity of the NF-κB interaction was monitored by using the mutant NF-κB oligonucleotide (patients 4 and 5). Arrows indicate the composition of the NF-κB complexes. (B) Lack of detectable NF-κB activation in cells from patients with nephrotic membranous nephropathy (MN). Nuclear extracts (20 μg) isolated from PBMC from five patients with MN were analyzed for the NF-κB site in electromobility shift assays, in the absence or presence (+) of anti-p50 or anti-p65 (RelA) antibodies. The specificity of the NF-κB interaction was monitored by using the mutant NF-κB oligonucleotide (patient 4). For comparison, an equal amount of nuclear extract from patient 2, with an MCNS relapse, is shown in the left lane. Arrows indicate the composition of the NF-κB complexes. (C) Band densities quantified by using ImageQuant analysis software (version 1.11).
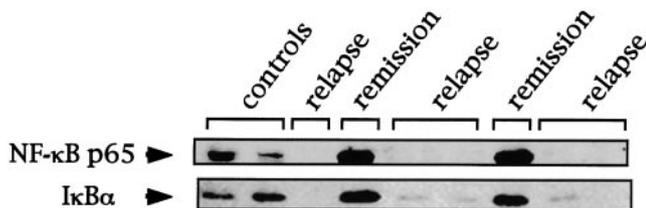


*Figure 4.* Immunodetection of cytosolic IκBα and NF-κB p65 proteins in relapse and remission of MCNS. Proteins (50 μg) from cytosolic extracts were analyzed by Western blotting, using antibodies raised against IκBα and NF-κB p65.

### Effects of MCNS Relapse Serum on NF-κB Activity

To determine whether the serum of patients with MCNS relapses was involved in the upregulation of NF-κB activity, PBMC were incubated overnight with control or MCNS sera.

Normal control PBMC incubated with MCNS sera did not display significant NF-κB DNA-binding activity (Figure 7). Surprisingly, when PBMC from patients with active MCNS were incubated with sera from patients with nephrotic relapses or normal sera, we observed that the NF-κB activity was downregulated in the presence of sera from patients with MCNS relapses but not normal sera. These results suggest that NF-κB activation precedes mononuclear cell activation and cytokine production.

### Lack of Correlation between NF-κB Activation and TNF-α mRNA Levels

To investigate whether the NF-κB activation in MCNS relapses was induced by cytokines such as TNF-α (a potent inducer of NF-κB activity), we analyzed TNF-α mRNA expression levels, under nonstimulated conditions, by quantitative RT-PCR. Paired data were obtained for five patients who
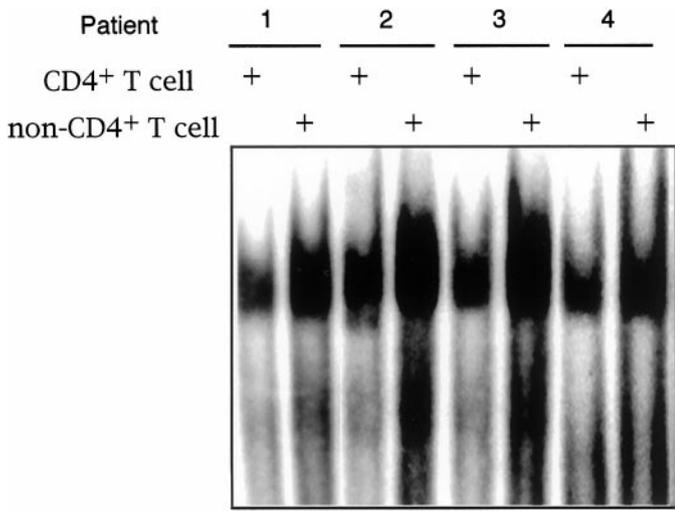
*Figure 5.* Activation of NF-κB in CD4⁺ and non-CD4⁺ T cells in MCNS relapse. PBMC from four adult patients experiencing MCNS relapses were isolated, and CD4⁺ T cell-enriched populations were collected by immunomagnetic negative selection. Nuclear extracts (10 $\mu$g) were prepared from CD4⁺ and non-CD4⁺ T cells and analyzed by electromobility shift assays.

exhibited high levels of NF-κB activity. The TNF-α mRNA levels were higher in relapse than in remission for two patients, whereas no significant difference was detected between the relapse and remission phases for three patients (Figure 8). These results suggest that the production of TNF-α does not fully account for the NF-κB activation detected in these patients.
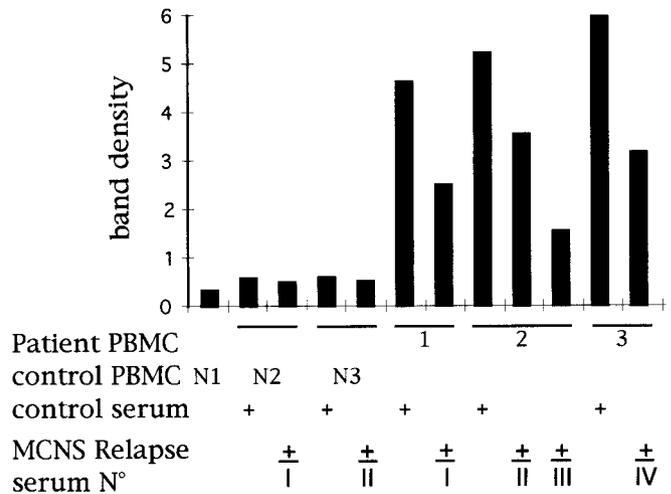


*Figure 7.* Effect of serum on NF-κB activity. PBMC from three patients experiencing MCNS relapses and two normal control subjects were incubated overnight with normal serum or sera from four different MCNS relapses (I, II, III, and IV). Nuclear extracts (20 $\mu$g) were isolated and analyzed by electromobility shift assays. The band shifts were quantified by using ImageQuant analysis software (version 1.11).

### Low Levels of IκBα mRNA in PBMC from Patients with Active MCNS

Because IκBα protein was barely detected or not detected for patients with MCNS relapses, IκBα mRNA expression levels were analyzed by RT-PCR. The IκBα PCR primers
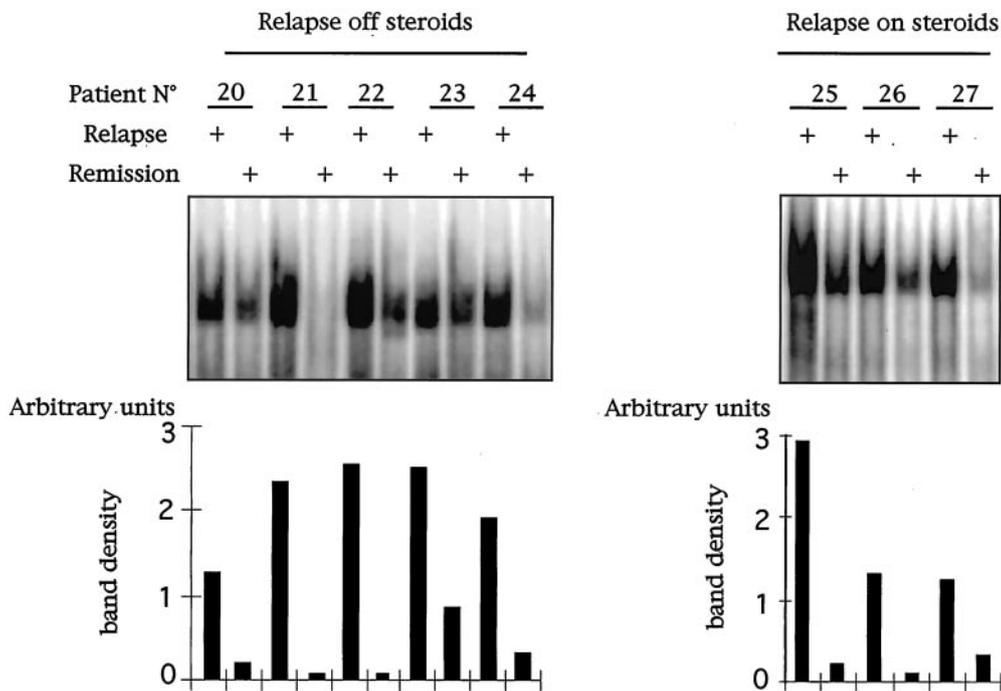


*Figure 6.* Activation of NF-κB among patients experiencing MCNS relapses while receiving ($n = 3$) or not receiving ($n = 5$) steroids. (Upper) Electromobility shift assays of 20-$\mu$g nuclear extracts from patients receiving or not receiving steroids. (Lower) Band densities quantified by ImageQuant analysis software (version 1.11).
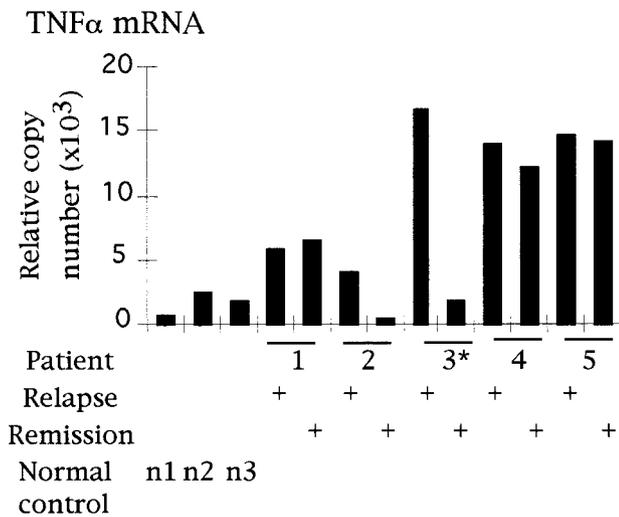
## TNFα mRNA



*Figure 8.* Lack of correlation between tumor necrosis factor-α (TNF-α) mRNA levels and NF-κB activation. Total RNA was isolated from PBMC from patients experiencing MCNS relapses (patients 1 to 5) and was then subjected to quantitative reverse transcription (RT)-PCR analysis of TNF-α mRNA expression, as described in Materials and Methods. Results are expressed as the TNF-α copy number per $10^6$ copies of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The asterisk indicates a patient receiving steroids.

amplified a PCR product of 569 bp, corresponding to mature mRNA, in all samples (19). IκBα mRNA levels were reduced for most patients (>50%) experiencing relapses, compared with normal control subjects ($P < 0.01$) and patients experiencing remissions ($P < 0.001$) (Figure 9). In contrast, IκBα mRNA levels were increased severalfold for patients experiencing remission, compared with normal control subjects ($P < 0.005$). For all samples, IκBα primers did not amplify a 921-bp product, corresponding to an unspliced form of IκBα mRNA, suggesting normal maturation of this transcript during relapse (19).

### Upregulation of α2P mRNA during Active MCNS

Although IκBα mRNA levels were reduced during relapse, these results might not entirely account for the virtual lack of IκBα protein expression. Previous studies demonstrated that stimulation of the proteasome proteolytic pathway was associated with an increase in the mRNA-encoding components of this system, such as α2P (21). Therefore, we determined α2P mRNA levels during the relapse and remission phases of MCNS. As demonstrated in Figure 10A, α2P mRNA levels were significantly increased during relapse, compared with those measured during remission and those for normal groups ($P < 0.01$). These results demonstrated that the downregulation of IκBα was associated with the upregulation of α2P mRNA during relapse.

In addition, the expression of IκBα and α2P was analyzed for five patients in both relapse and remission, by using RT-PCR followed by Southern blotting. The IκBα mRNA levels were lower, whereas the α2P mRNA levels was higher, in
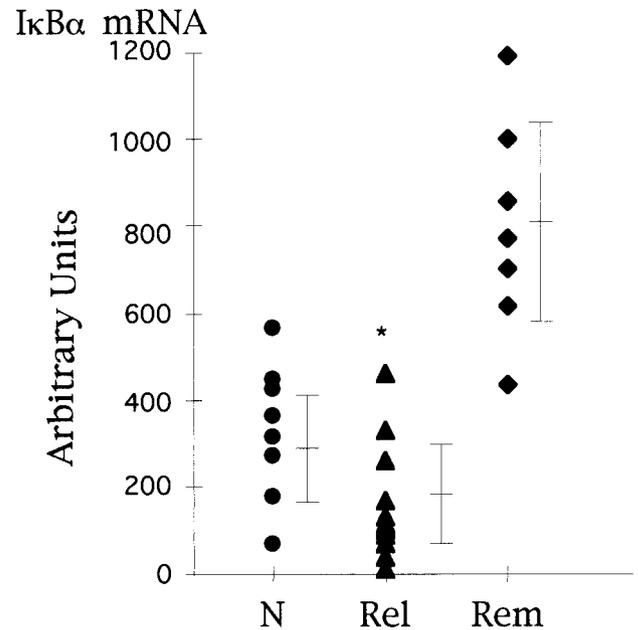
## IκBα mRNA



*Figure 9.* Downregulation of IκBα mRNA in MCNS. The relative expression of IκBα mRNA in samples obtained from normal control subjects (N) ($n = 8$), patients experiencing relapses while not receiving steroids (Rel) ($n = 11$), and patients experiencing remissions while receiving steroids (Rem) ($n = 7$) was measured. Semiquantitative RT-PCR was performed with 5 ng of total RNA, as described in the Materials and Methods section. A dot plot of the relative fluorescence values (expressed in arbitrary units), after normalization with respect to the corresponding β2-microglobulin mRNA values, is presented. The mean IκBα mRNA level is shown as a horizontal bar. *, $P < 0.001$, relapses *versus* remissions.

relapse (Figure 10B). Together, the increase in α2P mRNA levels and the decrease in IκBα mRNA levels led to sharp reduction of the IκBα/α2P ratio in relapse, compared with remission (Figure 10C). This ratio was significantly increased in remission, compared with the control group.

### Effects of Proteasome Inhibition on NF-κB Activity and on IκBα Protein Expression in Active MCNS

To further investigate whether the inhibition of proteasome activity could affect NF-κB activity, PBMC from four patients who experienced MCNS relapses while not receiving steroids were incubated with the proteasome inhibitor MG132. MG132 clearly inhibited NF-κB binding activity, and no supershift could be detected (Figure 11A). IκBα was concomitantly stabilized, as suggested by the accumulation of its phosphorylated form. In contrast, no NF-κB activity was detected in supershift experiments with nuclear extracts from cells from patients experiencing remissions while receiving steroids. Furthermore, preincubation with MG132 did not induce accumulation of phosphorylated IκBα (Figure 11B), further indicating that IκBα is stabilized during remission. Together, these results strongly suggest that both transcriptional and post-transcriptional mechanisms are involved in the downregulation of IκBα in MCNS.
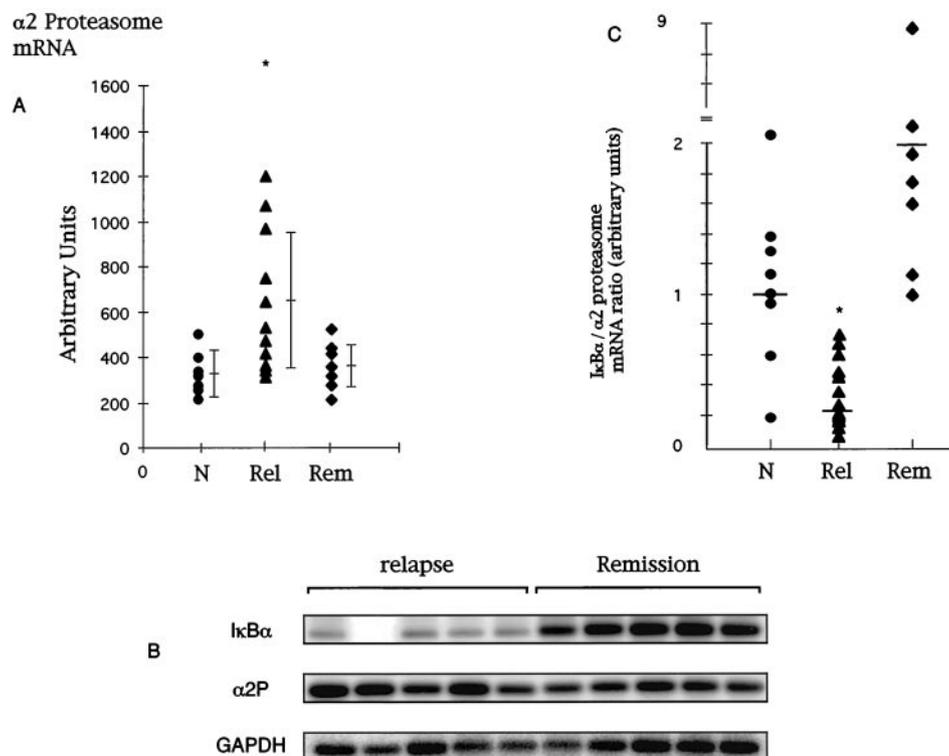
*Figure 10.* Regulation of proteasome $\alpha2$ subunit ($\alpha2P$) mRNA in MCNS. (A) Relative expression of $\alpha2P$ mRNA in samples obtained from normal control subjects (N) ($n = 7$), patients experiencing relapses while not receiving steroids (Rel) ($n = 11$), and patients experiencing remissions while receiving steroids (Rem) ($n = 7$). Semiquantitative RT-PCR was performed with 5 ng of total RNA, as described in the Material and Methods section. A dot plot of the relative fluorescence values (expressed in arbitrary units), after normalization with respect to the corresponding $\beta2$-microglobulin mRNA values, is presented. *, $P < 0.01$, normal values *versus* relapses. (B) Southern blots of PCR products for $I\kappa B\alpha$ and $\alpha2P$. Semiquantitative RT-PCR was performed with 2 $\mu$g of total RNA from PBMC from five patients studied during relapses and remissions. After Southern blotting, the PCR products were detected with specific probes. (C) Comparison of $I\kappa B\alpha/\alpha2P$ mRNA ratios. *, $P < 0.0007$.

## Discussion

In this work, we demonstrated that PBMC from patients with MCNS who were experiencing nephrotic relapses exhibited increased NF-$\kappa$B binding activity in nuclear extracts. In contrast, the binding activity returned to basal levels among patients experiencing remission. We also demonstrated that no DNA-binding activity for the IFN-$\gamma$- or -$\alpha$-responsive elements could be detected in the same nuclear extracts, suggesting that viral infection was not responsible for the upregulation of NF-$\kappa$B in MCNS. Moreover, the typical absence of cellular infiltrates and Ig deposits in the kidney argues against an inflammatory process, in contrast to conditions prevailing in other glomerular diseases (22).

We observed that NF-$\kappa$B activation was not induced by sera from patients with active disease, which unexpectedly seemed to downregulate this activity. Moreover, the upregulation of NF-$\kappa$B activity was not closely related to increased production of TNF-$\alpha$, a known inducer of NF-$\kappa$B activity. Therefore, it seems that the increase in NF-$\kappa$B activity results primarily from molecular events that occur at early stages in the pathophysiologic processes of MCNS. Nevertheless, we cannot exclude, at least for some patients, the possibility that increased release of TNF-$\alpha$ and/or other cytokines contributes to NF-$\kappa$B activation.

We demonstrated concomitant downregulation of $I\kappa B\alpha$ protein for these patients, which seemed to be responsible for the sustained NF-$\kappa$B activation. The downregulation of $I\kappa B\alpha$ occurred at the mRNA and protein levels. The low $I\kappa B\alpha$ mRNA levels during relapse were not expected, because the activation of NF-$\kappa$B is known to stimulate transcription of the $I\kappa B\alpha$ gene under physiologic conditions (15,16). Two mechanisms may account for this observation, *i.e.*, lack of induction of $I\kappa B\alpha$ gene transcription or increased $I\kappa B\alpha$ mRNA decay. The first mechanism has been described in the experimental model of monocyte adherence, where, in contrast to several cytokine genes, the basal rates of $I\kappa B\alpha$ gene transcription are not changed after NF-$\kappa$B activation (19). Therefore, the nuclear processing of $I\kappa B\alpha$ mRNA, independent of the transcription rate, was suggested to be a determinant in the regulation of $I\kappa B\alpha$ protein turnover (19). The low $I\kappa B\alpha$ mRNA levels during relapse may also be attributable to increased destabilization, the second mechanism. Like that of many short-lived mRNA, including those encoding cytokines and proto-oncogenes, the 3'-untranslated region of $I\kappa B\alpha$ mRNA contains multiple AU-rich destabilizing sequence elements (ARE), which are involved in rapid mRNA decay (19,23). This rapid decay of ARE-containing mRNA is mediated by a 20S protein complex (24), which was recently partly identified (25). It
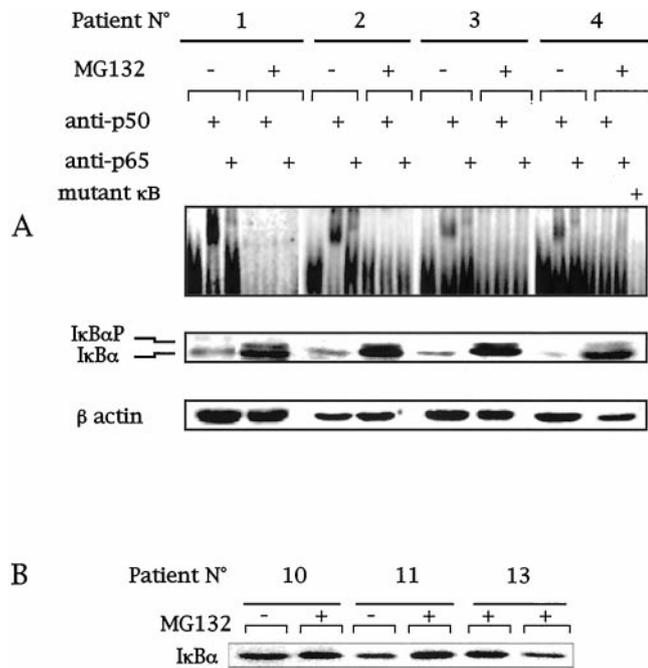
*Figure 11.* Suppression of NF-κB activation and induction of accumulation of phosphorylated IκBα with the inhibition of proteasome activity in cells from patients experiencing nephrotic relapses. PBMC from four patients experiencing relapses while not receiving steroids were incubated for 15 h in the absence (−) or presence (+) of 10 μM levels of the proteasome inhibitor MG132. (A) NF-κB activity. (Upper) Nuclear extracts (20 μg) were analyzed for the NF-κB site in electromobility shift assays, in the absence or presence (+) of anti-p50 and anti-p65 (RelA) antibodies. The specificity of the NF-κB interaction was monitored using the mutant NF-κB oligonucleotide. (Lower) The cytosolic expression of IκBα was analyzed by immunoblotting, using antibodies raised against amino-terminal (sc-209) and carboxy-terminal (sc-371) epitopes of IκBα. The positions of IκBα (lower band of the doublet) and the phosphorylated form of IκBα (IκBαP) (upper band of the doublet) are indicated. The blot was subsequently stripped and reprobed with anti-actin antibodies. (B) Stabilization of IκBα in remission and lack of effect of the proteasome inhibitor MG132 on IκBα levels. PBMC from patients experiencing remissions (n = 3) were incubated for 15 h in the absence (−) or presence (+) of 10 μM MG132. The cytosolic expression of IκBα was analyzed by immunoblotting, using antibodies raised against amino-terminal (sc-209) and carboxy-terminal (sc-371) epitopes of IκBα. The position of IκBα is indicated. The phosphorylated form of IκBα could not be detected in the presence of the proteasome inhibitor MG132.

includes the ARE-binding protein AUF1, heat shock protein 70 (hsp70), the translation initiation factor elf4G, and poly(A)-binding protein. Ubiquitin-proteasome controls the activity of this protein complex and thus IκBα mRNA decay.

Lower IκBα mRNA levels alone do not fully account for the lack of detection of IκBα protein for most patients with MCNS who are experiencing nephrotic relapses. In fact, it has been demonstrated that the degradation of IκBα protein is dependent on the proteasome complex (12). Our data suggest that the increased proteasome activity in these patients might also be involved in IκBα protein downregulation during relapses. The

latter hypothesis is supported by the fact that the degradation of IκBα was blocked in the presence of the proteasome inhibitor MG132, as suggested by accumulation of the IκBα phosphorylated form.

The mechanisms by which glucocorticoids (the first-line therapy for MCNS) induce remission are not understood. Most cytokine genes that are upregulated in MCNS do not carry a glucocorticoid-responsive element, but their promoter exhibits binding sites for NF-κB (26). Therefore, glucocorticoid-induced inhibition of NF-κB may explain the downregulation of many cytokines during remission. This effect relies on a combination of mechanisms. Similar to NF-κB, the glucocorticoid receptor is maintained in an inactive state in the cytoplasm, complexed to hsp90, which acts as a "protein chaperon" and prevents the targeting of the glucocorticoid receptor to the nucleus (27). After binding to glucocorticoids, the glucocorticoid receptor dissociates from hsp90 and moves into the nucleus, where it downregulates NF-κB activity essentially by preventing NF-κB from binding to DNA (28,29) and/or by increasing transcription of the IκBα gene (30). The high levels of IκBα mRNA in remission support the latter hypothesis.

There was a striking correlation between the activation of NF-κB and the course of the disease. This was not the case for MN, which is characterized by a nephrotic syndrome similar to MCNS but for which no NF-κB activation was demonstrated in our cases.

Dysregulation of NF-κB activity was previously reported for some immune-mediated diseases, such as systemic sclerosis (31). In contrast to MCNS, patients with active systemic lupus erythematosus exhibit decreased NF-κB activity associated with a lack of p65 subunit protein expression in nuclear and whole-lymphocyte extracts (32). This activity remains weak with time and is independent of the disease index activity and steroid therapy. The regulation of IκBα has not yet been reported.

The restoration of IκBα protein and the associated inhibition of NF-κB in remission argue against a constitutive defect of IκBα in MCNS, as reported for Hodgkin's disease (20,33). In Hodgkin cells, the IκBα protein is either truncated or functionally defective or displays a very short half-life because of excessive phosphorylation, whereas the IκBα mRNA levels remain normal. In addition, constitutive activation of NF-κB in some Hodgkin cell lines results from the release of a soluble factor (33).

In conclusion, this study supports an important role for the NF-κB pathway in the pathogenesis of MCNS. The downregulation of IκBα mRNA and protein levels during relapse involves both transcriptional and post-transcriptional mechanisms and suggests alteration of the NF-κB/IκBα autoregulatory feedback loop.

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